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The crystal structures of human α-thrombin complexed with active site-directed diamino benzo[*b*]thiophene derivatives: A binding mode for a structurally novel class of inhibitors

N.Y. CHIRGADZE, D.J. SALL, S.L. BRIGGS, D.K. CLAWSON, M. ZHANG, G.F. SMITH, AND R.W. SCHEVITZ

Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285

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Abstract

The crystal structures of four active site-directed thrombin inhibitors, 1–4, in a complex with human α-thrombin have been determined and refined at up to 2.0 Å resolution using X-ray crystallography. These compounds belong to a structurally novel family of inhibitors based on a 2,3-disubstituted benzo[*b*]thiophene structure. Compared to traditional active-site directed inhibitors, the X-ray crystal structures of these complexes reveal a novel binding mode. Unexpectedly, the lipophilic benzo[*b*]thiophene nucleus of the inhibitor appears to bind in the S_1 specificity pocket. At the same time, the basic amine of the C-3 side chain of the inhibitor interacts with the mostly hydrophobic proximal, S_2, and distal, S_3, binding sites. The second, basic amine side chain at C-2 was found to point away from the active site, occupying a location between the S_1 and S_1' sites. Together, the aromatic rings of the C-2 and C-3 side chains sandwich the indole ring of Trp60D contained in the thrombin S_2 insertion loop defined by the sequence "Tyr-Pro-Pro-Trp." [The thrombin residue numbering used in this study is equivalent to that reported for chymotrypsinogen (Hartley BS, Shotton DM, 1971, *The enzymes*, vol. 3. New York: Academic Press. pp 323–373).] In contrast to the binding mode of more classical thrombin inhibitors (D-Phe-Pro-Arg-H, NAPAP, Argatroban), this novel class of benzo[*b*]thiophene derivatives does not engage in hydrogen bond formation with Gly216 of the thrombin active site. A detailed analysis of the three-dimensional structures not only provides a clearer understanding of the interaction of these agents with thrombin, but forms a foundation for rational structure-based drug design. The use of the data from this study has led to the design of derivatives that are up to 2,900-fold more potent than the screening hit 1.

Keywords: benzo[*b*]thiophene; human α-thrombin; nonpeptidyl inhibitor; structure-based drug design; X-ray crystallography

α-Thrombin (E.C.3.4.21.5) belongs to the trypsin-like serine protease family and plays a central regulatory role in blood coagulation (Machovich, 1984; Hirsh et al., 1987; Barnett, 1991; Berliner, 1992; Colman et al., 1994a, 1994b). It is activated by factor X_a (in the presence of factor V_a, lipids, and Ca^{2++} ions) and subsequently converts polymeric fibrinogen to fibrin monomers, which aggregate to form a soft clot. Thrombin simultaneously activates Factor XIII, which, in turn, cross-links the fibrin monomers, thereby stabilizing the soft clot. Under normal physiological conditions, blood coagulation is controlled through an intricate system of feedback mechanisms (Machovich, 1984; Hirsh et al., 1987; Barnett, 1991; Berliner, 1992; Colman et al., 1994a; 1994b). However, small perturbations in this complicated system can lead to aberrant coagulation and result in a variety of thrombotic disorders. In fact, thromboembolic diseases resulting from uncontrolled coagulation are a leading cause of mortality and morbidity in developed societies. Accordingly, the ability to control the blood coagulation process, in particular through thrombin inhibition, has been a major focus of the pharmaceutical industry.

In recent years, a number of very potent and selective active-site directed thrombin inhibitors have been designed using arginine or benzamidine as an active site recognition element (Edmunds & Rapundalo, 1996; Wiley & Fisher, 1997; Kaiser, 1998; Menear, 1998; Sanderson & Naylor-Olsen, 1998; Uzan, 1998; Want et al., 1998; Kimball, 1999). Crystal structures of the complexes between thrombin and a number of these inhibitors, including the arginal tripeptide D-Phe-Pro-Arg-H and its related analogs, NAPAP, Argatroban (MD-805), and cyclotheonamide A, have been determined (Bode et al., 1989, 1992; Banner & Hadvary, 1991; Chirgadze et al., 1992; Maryanoff et al., 1993). All have a similar binding

Reprint requests to: N.Y. Chirgadze, Lilly Research Labs., Lilly Corporate Center, Indianapolis, Indiana 46285; e-mail: nyc@lilly.com.

orientation, namely an antiparallel beta-sheet arrangement between the inhibitor and enzyme that is stabilized by hydrogen bonds to Gly216. A second type of binding mode ("retro") has also been published in which a parallel beta-sheet arrangement between enzyme and inhibitor was observed (Iwanowicz et al., 1994). This binding orientation is also reinforced through hydrogen bonding with Gly216. More recently, we reported the crystal structure of thrombin complexed with a nonpeptidyl series of amidinoindoles (Chirgadze et al., 1997).

In an effort to identify structurally novel, nonpeptidyl thrombin inhibitors that might have a more favorable pharmacokinetic profile than their arginine or benzamidine derived counterparts, we undertook a broad screening approach. Recently, we reported the identification and characterization of a series of diamino benzo[*b*]-thiophene derivatives as a structurally novel class of orally active thrombin inhibitors (Sall et al., 1997). While the series demonstrated competitive inhibition kinetics, suggesting the compounds interact in the active site of the enzyme, their unique structure limited speculation about their binding mode. Accordingly, X-ray crystallographic studies were undertaken to better understand the interaction of these inhibitors with thrombin. Using the crystallographic information, structure activity relationship (SAR) studies surrounding the initial lead 1 led to the more potent derivatives 2, 3, and 4 (Fig. 1). Here, we report the structures of cocrystals formed between human α-thrombin and diamino benzo[*b*]thiophene compounds 1–4. The usefulness of these data in explaining SAR results and its application to the structure-based design of novel, more potent derivatives will also be discussed.

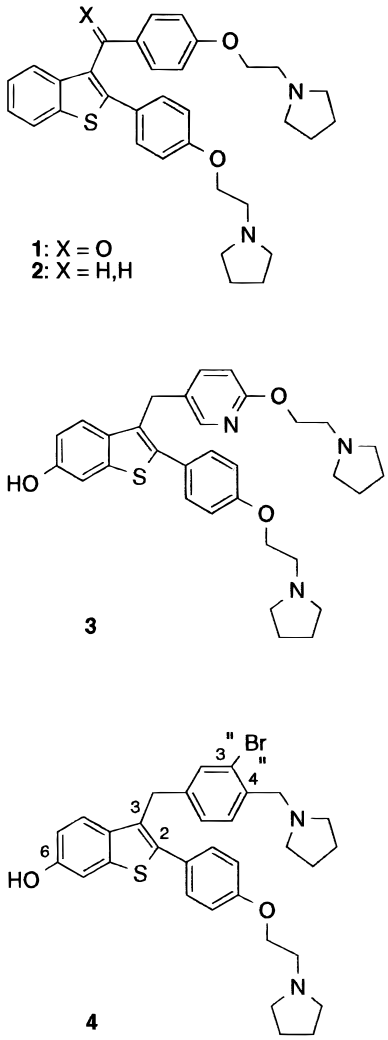


Fig. 1. Structures of diamino benzo[*b*]thiophene-derived thrombin inhibitors 1–4.

Results

The crystal structure of the thrombin/inhibitor 1 complex solved here (Fig. 2A) has provided the first structural information about the interactions of these agents with thrombin. Consistent with the competitive inhibition kinetics observed for this series, this agent binds in the active site of the enzyme (Sall et al., 1997). The binding mode of benzo[*b*]thiophene based inhibitors is unique in regard to thrombin inhibitors known to date.

The binding of inhibitors from this class is characterized primarily by hydrophobic interactions. The crystal structures of the thrombin/inhibitor complexes 1–4 (Fig. 2) reveal three principal regions of the thrombin active site at which the diamino benzo[*b*]-thiophene inhibitors bind.

The first region

The first site of interaction is at the specificity pocket (S_1) with Asp189 at its base. In proteases of the trypsin-like family, this binding pocket is specific for basic amino acids. Thrombin, in particular, has a preference for arginine. Despite its lipophilic nature and lack of a positive charge, the bulky benzo[*b*]thiophene nucleus binds with good complementarily in the S_1 specificity pocket. One might have expected that either the C-2 or C-3 side chains (Fig. 1, Table 1), both containing basic amines, could have fit in this pocket and interacted with the carboxyl of Asp189. The electron density corresponding to the inhibitor also indicates that the C-3 side chain induces a noticeable twist/bend in the benzo[*b*]-thiophene nucleus, forcing the C-3 carbon atom from the benzo[*b*]-thiophene ring plane by about 0.5 Å (Fig. 2). This structural distortion likely introduces a degree of strain and may result in reduced inhibitory activity.

The second region

The C-3 side chain of inhibitor 1 is oriented perpendicular to the plane of the benzo[*b*]thiophene nucleus and extends past the S_2 pocket and into the S_3 site. The C-3 phenyl ring lies at the entrance of this hydrophobic S_2 pocket formed by residues Leu99, Tyr60A, Trp60D, and His57. The lipophilic portion of the pyrrolidine ring binds in the shallow hydrophobic S_3 binding site that is partially exposed to the solvent and is adjacent to the S_2 pocket. The S_3 subsite is formed by residues Leu99, Ile174, and Trp215. Like the benzo[*b*]thiophene core the C-3 side chain of these analogs occupies a binding site region shared by the majority of other active-site directed thrombin inhibitors (Bode et al., 1989, 1992; Banner & Hadvary, 1991; Chirgadze et al., 1992; Maryanoff et al., 1993). In contrast to classical thrombin inhibitors, however (i.e., D-Phe-Pro-Arg-H, NAPAP, Argatroban), no specific interactions with the backbone of residues Ser214–Gly216 were observed. Despite the lack of this commonly observed interaction, members of the benzo[*b*]-thiophene series can still exhibit very potent thrombin inhibitory activity.

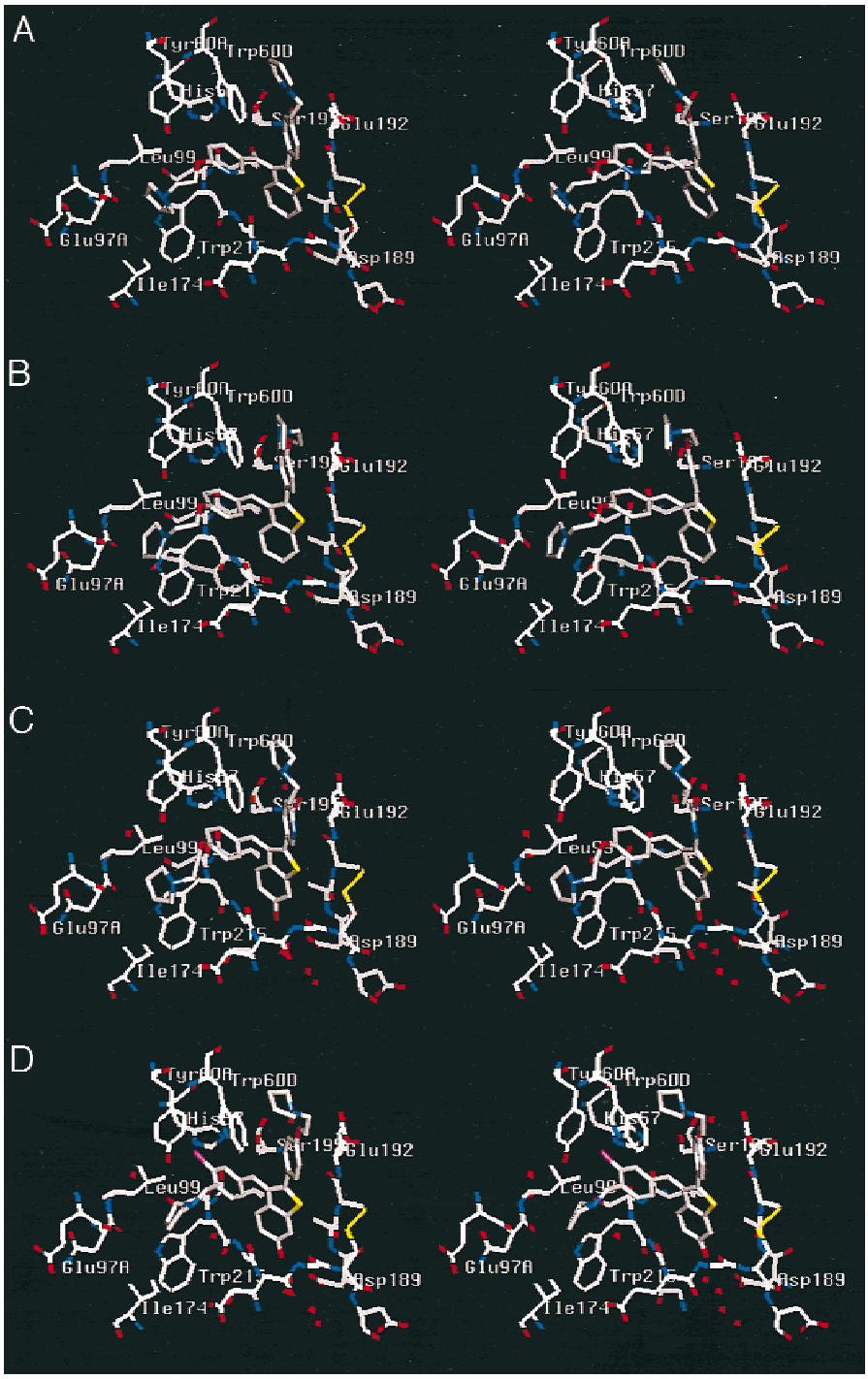
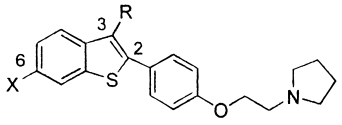
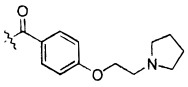
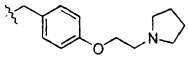
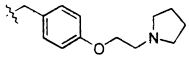
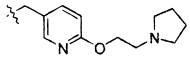
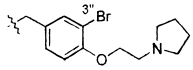
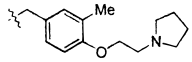
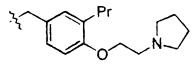
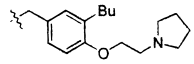
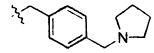
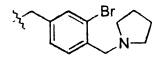


Fig. 2. The binding of the benzo[*b*]thiophene based inhibitors 1–4 in the active site of human α-thrombin. **A:** Crystal structure of the thrombin/inhibitor 1 complex reveals the binding orientation of the inhibitor in the active site. The hydrophobic benzo[*b*]thiophene nucleus binds in the S_1 specificity pocket containing Asp189. The C-3 side chain spans the proximal and distal binding sites. The C-2 side chain branches away from the active site and together with phenyl ring of the C-3 side chain, sandwiches the phenol of Trp60D. **B:** The thrombin/inhibitor 2 complex is essentially very similar to the previous structure, but the absence of the carbonyl group in the C-3 side chain makes this pivot point less rigid, allowing the inhibitor to better accommodate to the shape of the active site cavity, fitting deeper in the S_1 pocket. **C:** The thrombin/inhibitor 3 has a hydroxy group (at position 6 of the benzo[*b*]thiophene nucleus) that engages in a hydrogen bond with carboxyl group of Asp189 in the S_1 specificity pocket. Due to the similarities in the electron densities of carbon and nitrogen, it was not possible to definitively assign the nitrogen atom in the C-3 pyridyl ring. It was arbitrarily assigned so that it resided in the more hydrophilic environment. **D:** The crystal structure of the thrombin/inhibitor 4 complex. The bromine substituent (shown in purple) tightly fills proximal binding site and undergoes a π-electron/cation interaction with hydroxy group of Tyr60A. The hydroxyl of Tyr60A engages in a hydrogen bond with the nitrogen of the pyrrolidine group that occupies the distal binding site.

Table 1. *Structure–activity relationships for the diamino benzo[*b*]thiophene thrombin inhibitors*



Compound	X	R	K_{ass} (L/mol × 10^6)[a]
1[b]	H		0.43 ± 0.07
2[b]	H		3.43 ± 0.55
5	OH		20.2 ± 0.8
3[b]	OH		24.3 ± 2.0
6	H		27.5 ± 1.84
7	H		24.2 ± 3.21
8	H		1.02 ± 0.05
9	H		0.42 ± 0.09
10	H		16.7 ± 2.8
4[b]	OH		1,240 ± 249

[a]Represents the apparent association constant as measured by the methods of Smith et al. (1996).

[b]X-ray crystal structure of complex with human α-thrombin has been determined.

The third region

The C-2 side chain of benzo[*b*]thiophene 1 binds in a region of the enzyme where most classical thrombin inhibitors do not bind. This side chain protrudes out of the active site into the solvent shell. The pyrrolidine basic amine is in a favorable position for π-electron/cation interaction with the Trp60D indole ring. The C-2 side chain extends along the side chain of Gln238, having displaced it from its previously observed orientation by more than 90°. Though the pyrrolidine nitrogen is located within the vicinity of the glutamate side chain, there appears to be no hydrogen bond formation. Yet despite the absence of any obvious significant interaction, removal of the C-2 side chain results in at least an 80-fold loss in thrombin inhibition ($K_{ass} < 0.005 \times 10^6$ L/mol; 22), making this subsite an essential point of interaction. It is noteworthy that, together, the phenyl rings of the C-2 and C-3 side chains begin to sandwich the Trp60D side chain of the insertion loop, forcing the indole ring to shift toward the inhibitor by ~1 Å. Trp60D is part of the insertion loop, which is a unique structural attribute of thrombin (Bode et al., 1989). Inhibitor interaction with Trp60D may provide a basis for the high thrombin selectivity observed for this series (Sall et al., 1997).

In addition to the thrombin/inhibitor 1 complex, a number of other benzo[*b*]thiophene/thrombin cocrystals have also been solved 2–4 (Fig. 2). The data from these studies have proven useful in explaining and suggesting structural modifications that have an effect on inhibitory activity. For example, it has been previously shown that reduction of the C-3 carbonyl of derivative 1, affording the corresponding methylene compound 2 enhances thrombin inhibitory activity by eightfold (Sall et al., 1997). Comparison of the complexes between thrombin and compounds 1 and 2 suggests that the C-3 ketone had unfavorable steric interactions with the C_β of the catalytic Ser195 (Fig. 2A). Removing this steric interaction, in the form of inhibitor 2, allowing the benzo[*b*]thiophene nucleus to bind more deeply in the active site pocket, results in increased hydrophobic surface of the interaction (Fig. 2B).

In addition to providing bases to explain the effects of the structural modifications on inhibitory activity, the structural information has been critical to the rational design of more potent agents (Table 1). From the orientation of the benzo[*b*]thiophene ring (1,2) in the specificity pocket, it was suggested that the presence of a hydroxyl group at position 6 would allow formation of a hydrogen bond with the carboxyl group of Asp189, mimicking the guanidino group of the arginal series. It was subsequently shown that the presence of this hydroxyl group (5) increased thrombin inhibitory activity by sixfold relative to the unsubstituted parent 2 (Sall et al., 1997). It is observed from the thrombin/inhibitor 3 complex crystal structure that this hydroxyl group indeed engages in a hydrogen bond with the carboxyl of Asp189 within the S_1 specificity pocket, thereby enhancing potency (Fig. 2C). Larger hydrogen-bond donating groups (CH_2NH_2, $CH(O)NH_2$, CH_2OH) have also been substituted at this site, but resulted only in decreased activity. It is apparent from the thrombin/inhibitor 2 crystal structure (Fig. 2B) that the benzo[*b*]thiophene nucleus binds very close to the peptide backbone of the enzyme, limiting the size of the hydrogen-bond donating group that can be employed in this region to hydroxyl.

Many of the classical active site-directed thrombin inhibitors take advantage of interactions at the S_2 and S_3 binding sites. In the benzo[*b*]thiophene series of inhibitors, the C-3 side chain spans these two regions, providing additional opportunities to enhance the activity of the series. Although we have previously demonstrated the effectiveness of increasing the interactions at the S_2 binding site with another series of thrombin inhibitors (Schacht et al., 1995; Chirgadze et al., 1996), the thrombin/inhibitor 1 complex shows that this pocket is largely unoccupied in the current benzo[*b*]thiophene series. Based upon consideration of the crystal structures that show that the orientation of the C-3 phenyl ring places the C-3″ carbon at the opening of the S_2 binding site, providing a site suitable for substituting lipophilic groups that can provide additional interactions with this pocket, SAR studies were undertaken to explore the effect of incorporating a wide variety of substituents at the C-3″ position (Sall et al., 1998). The results of these studies indicated that small lipophilic substituents such as bromo and methyl (i.e., 6 and 7, respectively) enhanced thrombin inhibitory activity by 10-fold, presumably through interaction at the proximal binding site. The activity enhancement erodes, however, as the size of the substituent increases, possibly due to un-

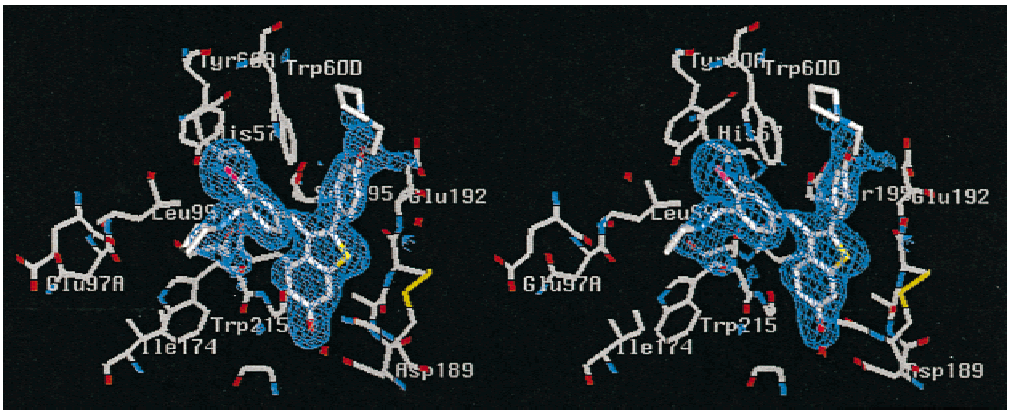


Fig. 3. Stereo view of the human α-thrombin active site region for the thrombin/inhibitor 4 complex. The electron density map corresponding to the inhibitor molecule that was used to position a ligand in the active site is overlaid on the final refined model. The map calculated with ($F_o - F_c$, α_c) Fourier coefficients was countered at 2.5 σ level at 2.0 Å resolution.

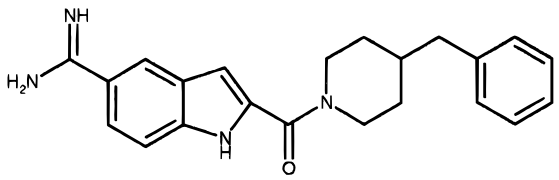
favorable steric interactions (see 8 and 9). In addition to lipophilic interactions, the bromo substituent may provide a π-electron/cation interaction with the aromatic ring of Tyr60A (Dougherty, 1996).

The thrombin/inhibitor 1–3 crystal structures also show that the lipophilic pyrrolidine ring of the C-3 side chain binds in the hydrophobic, S_3 pocket of the thrombin active site. It further shows that the linker between the phenyl and pyrrolidine groups adopts an S-shaped curved configuration to accommodate a shorter distance. SAR studies that were undertaken to vary the length of the tether between the pyrrolidine ring and the C-3 phenyl ring (Sall et al., 2000) demonstrated that contracting the length of the C-4″ side chain by two atoms (i.e., 10) provided a fivefold increase in inhibitory activity relative to compound 2. This enhancement in activity is consistent with a favorable entropy effect coming from a shorter tether with fewer degrees of freedom than its longer counterparts.

The combined impact on thrombin inhibition by all of these structural enhancements in a single molecule was determined by the preparation and evaluation of derivative 4 (Sall et al., 2000). It combines the structural modifications described in this paper with those previously reported, namely a C-6 hydroxyl, the reduced C-3 ketone, a lipophilic C-3″ bromo, and a contracted C-4″ side chain. The change in K_{ass} due to the incorporation of any single one of these structural modifications by itself is a modest factor of <10 (Table 1), yet together these modifications combine to yield a 2,900-fold improvement in inhibitory activity compared to that of screening hit 1.

Figures 2D and 3 show the crystal structure of the thrombin/inhibitor 4 complex, illustrating the optimized interaction of this agent in the thrombin active site. The C-6 phenol engages in a hydrogen bond with the carboxylate of Asp189 within the S_1 specificity pocket. The C-3″ bromo (purple) inserts into the center of the S_2 binding site as predicted by crystal structures 1–3, supporting the thought that the increased thrombin inhibition observed with this structural change is indeed due to additional interaction at this site. The crystal structure also indicates that the pyrrolidine ring of C-3 side chain has a position similar to the one in the inhibitors with the longer C-3 linker. The impact that the C-3″ bromo and the contracted C-4″ side chain have on thrombin inhibitory activity highlight the value of using the crystal structures of these studies in the structure based design of more potent derivatives.

Recently we published the X-ray crystal structure of an amidinoindole-derived inhibitor (11) bound in the active site of thrombin (Chirgadze et al., 1997; PDB accession code: 1D4P) (Fig. 4). It is interesting to compare that structure to the current thrombin/inhibitor 4 complex (Fig. 5). Despite the clear structural differences between inhibitors, both molecules still occupy common binding regions within the thrombin active site. The benzo[*b*]thiophene and the amidinoindole nuclei both occupy the S_1 specificity pocket, interacting with Asp189 through the phenol and amidine groups, respectively. The orientation of the benzo[*b*]thiophene nucleus is nearly coaxial with the S_1 pocket, which could be described as a deep cylindrical shaped cavity. Within the rest of the thrombin active site, the phenyl and pyrrolidine rings of the C-3 side chain of the benzo[*b*]thiophene occupy the same binding regions as the piperidine and phenyl rings, respectively, of amidinoindole 11. In both complexes, the protein structures are very similar, except for a few small differences. The most noticeable are the changes in the orientation and position of the side chains of Trp60D, Tyr60A, and Trp215. In the complex with inhibitor 11, Trp60D has moved away from the inhibitor by about 1 Å to provide additional space to bind the piperidine ring, thereby making the S_2 pocket wider in comparison to the inhibitor 4 structure. In general, the enzyme bound benzo[*b*]thiophene inhibitor 4 results in fewer structural changes in the protein molecule from native than does the amidinoindole



11

Fig. 4. Chemical structure of 5-amidinoindole-4-benzylpiperidine thrombin inhibitor 11.

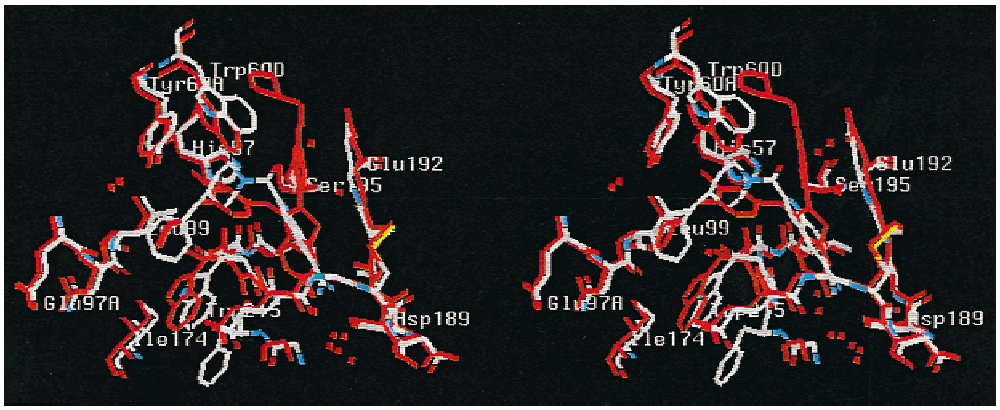


Fig. 5. Superposition of the X-ray crystal structures of the benzo[*b*]thiophene 4 (red) and the nonpeptidyl, amidinoindole derived inhibitor 11 (white) in the active site of human α-thrombin. The overlay demonstrates that the inhibitors have a common binding subsite, with different interaction points in the thrombin molecule active site (see text).

11. However, significant structural deformations of the benzo[*b*]thiophene ligand itself seem to occur upon binding. These distortions appear to provide a more complementary fit of the inhibitor to the active site of the enzyme.

Conclusions

We have reported the X-ray crystal structures of the complexes between human α-thrombin and four structurally novel, diamino benzo[*b*]thiophene-derived inhibitors (1–4). Compared to traditional active-site directed inhibitors, the X-ray crystal structures of these complexes reveal a novel binding mode. These studies have provided a detailed understanding of the interaction of these agents in the active site of the enzyme. The information gained has been used to explain the effects of specific structural modifications on inhibitory activity. More importantly, the data have aided in the rational design of additional compounds and have expedited the synthesis of more potent derivatives.

Materials and methods

The syntheses of the benzo[*b*]thiophene analogs discussed in this study is reported elsewhere (Sall et al., 1997, 2000). Thrombin inhibitory activity was determined by the methods of Smith et al. (1996, 1997) as previously described, and is reported as the apparent association constant (K_{ass}).

Human α-thrombin was obtained from Enzyme Research Laboratories, Inc. (South Bend, Indiana) as a pH 6.5 buffered solution in 50 mM sodium citrate, 0.2 M NaCl, and 0.1% PEG 8000, frozen at $-80\,^{\circ}$C. After thawing at $4\,^{\circ}$C, the enzyme preparation was concentrated to 7.5 mg/mL with simultaneous buffer exchange by use of a Centricon 10 mL stirred cell (Amicon, Beverly, Massachusetts), with a YM10 membrane, by washing five times with 25 mM sodium phosphate buffer (pH 7.5) and 375 mM NaCl. Hirugen was purchased from Bachem Biosciences, Inc. (Philadelphia, Pennsylvania). Hirugen, which consists of residues 54–65 from the C-terminus of hirudin, has the sequence Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3H)-Leu-Gln. It binds noncovalently to the basic fibrinogen recognition exosite, as has been reported for related peptides (Skrzypczak-Janjun et al., 1991). The binding of this peptide provides additional protection for α-thrombin from autocatalytic cleavage of the Arg77A–Asn78 bond that yields β-thrombin.

Crystals of the complexes suitable for X-ray analysis were grown from a solution containing 6 mg/mL human α-thrombin with three molar equivalents of both hirugen and the respective inhibitor, equilibrated against a solution of 30% PEG 3400, 100 mM sodium citrate at pH 5.6 and 200 mM ammonium acetate, using a vapor diffusion technique at $4\,^{\circ}$C temperature. Within a month, single crystals appeared that had a prism shape with dimensions of $0.2 \times 0.2 \times 0.3$ mm. These crystals belonged to the monoclinic space group C2 and contained one molecule per asymmetric unit.

X-ray diffraction data were collected using CuK_{α} radiation from a Rigaku rotating-anode generator operating at 50 kV and 90 mA with a fine focus of 0.3×3.0 mm^2. An MSC/Yale total reflection mirror focusing system was used. An R-AXIS IIc system with a Fuji imaging plate was used as a detector (Shibata, 1990). The crystal to detector distance was 100 mm. Frames with an oscillation angle of 1.0° were taken for 10 min. Each data set was collected from a single crystal at room temperature. The diffraction data were integrated and reduced using the program DENZO (Otwinowski & Minor, 1997), and the intensities were scaled with SCALEPACK (Otwinowski, 1993). A summary of the crystallographic data is shown in Table 2. A previously reported thrombin structure (Chirgadze et al., 1997) was used as the initial model (Resolution 2.1 Å, *R*-value = 21.5%, R_{free} = 23.1%). The conformation and position of the inhibitors were determined using difference electron density maps. Positional crystallographic refinement was performed using the program X-PLOR98.0 (Brünger, 1992), and the force-field parameter dictionary PARHCSDX.PRO (Engh & Huber, 1991) was used. For the high-resolution structural complexes 3 and 4, individual temperature parameters were included in the refinement. The inhibitor molecule was fit into the active site based on the electron density maps calculated with ($2F_o - F_c$, α_c) and ($F_o - F_c$, α_c) Fourier coefficients that were obtained after refinement with protein and hirugen only. The inhibitor molecule was then included in all subsequent refinements. Visual inspection and manual correction of the model between cycles of the refinement were done using QUANTA 4.1 (Molecular Simulations Inc., San Diego, California). The N-terminal residue Thr1H-Gly1D and the C-terminal residues Asp14L–Arg15 of the light chain, the C-terminal residues Gly246 and Glu247, and the loop Trp148–

Table 2. *Summary of X-ray crystallographic data*

	Thrombin/inhibitor complex (compound number)			
	1	2	3	4
Space group	C2	C2	C2	C2
Unit cell parameters				
a (Å)	71.14	71.15	71.37	71.11
b (Å)	71.85	71.73	72.01	71.89
c (Å)	72.96	73.10	73.44	73.32
β (deg)	100.50	100.56	100.36	100.60
Resolution (Å)	3.0	2.9	2.1	2.0
Number of observations	24,280	21,574	103,972	102,581
Number of unique reflections	5,740	7,999	20,979	22,097
Average redundancy	2.1	2.0	3.0	3.8
Completeness (%)				
Outer shell	81.1	99.3	96.6	82.4
Overall	79.7	98.5	98.0	95.3
Average $I/\sigma(I)$				
Outer shell	3.1	2.7	3.5	4.8
Overall	10.6	8.8	20.2	23.3
R_{merge}[a] (%)				
Outer shell	25.7	26.5	28.9	27.6
Overall	8.4	8.9	7.0	7.7

[a] $R_{merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of an individual measurement, and $\langle I\rangle$ is the mean intensity of this reflection.

Table 3. *Statistics of crystallographic refinement*

	Thrombin/inhibitor complexes (compound number)			
	1	2	3	4
PDB accession code	1D3T	1D3P	1D3Q	1D3D
Number of nonhydrogen atoms				
Overall	2,437	2,436	2,549	2,550
Protein	2,273	2,273	2,273	2,273
Hirudin peptide	108	108	108	108
Carbohydrate	15	15	15	15
Na^+ ions	2	2	2	2
Inhibitor	39	38	39	38
Water molecules	0	0	112	114
Root-mean-square deviation from target values				
Bond lengths (Å)	0.009	0.008	0.010	0.008
Bond angles (deg)	1.462	1.443	1.446	1.385
Dihedral angles (deg)	27.026	27.012	26.856	26.312
Improper angles (deg)	0.960	0.915	1.003	0.806
R_{free}[a] (%)	23.5	22.8	21.4	22.3
R-value[b] (%)	17.2	16.7	18.0	17.5
Resolution (Å)	3.0	2.9	2.1	2.0

[a] $R_{free} = \sum||F_o| - |F_c||/\sum|F_o|$ has been calculated for 7% of the reflections removed from the refinement.

[b] R-value $= \sum||F_o| - |F_c||/\sum|F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

Lys149E of the heavy chain have no visible electron density, and thus were not included in the refinement. The statistics for the final model after four iterations of 150 cycles of refinement are summarized in Table 3.

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