Design of Highly Potent Noncovalent Thrombin Inhibitors That Utilize a Novel Lipophilic Binding Pocket in the Thrombin Active Site

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Thrombin is a trypsin-like serine protease that plays a critical role in the blood coagulation cascade. Thrombin is responsible for the final conversion of fibrinogen to fibrin and is the most potent known stimulator of platelet aggregation. We have targeted this enzyme in our efforts to develop novel, safe, selective, and orally bioavailable antithrombotic agents. Our early lead structure compound **1** (Figure 1)[1] which contained the *trans*-4-(aminomethyl)cyclohexylamine P_1 group proved to be a very potent thrombin inhibitor. We chose, however, to focus our efforts on compounds that did not contain activated carbonyl serine traps due to the potential synthetic and metabolic liabilities associated with these functionalities. Simple removal of the keto amide functionality from **1** provided the second generation lead compound **2** (Figure 1),[2] which retained a good level of inhibitory potency. In an effort to develop analogs with increased potency and oral bioavailability, we began a synthetic program of incorporating lipophilic groups onto compound **2**. Strategically, the P_3 region of the molecule appeared to be the most logical area in which to add lipophilic groups given the known interactions of this region of the molecule with the lipophilic S_3 thrombin binding pocket.[3]

In this paper, we describe our progress and report the discovery of a novel lipophilic binding pocket in the thrombin active site.[4]

In the early 1990s, researchers at the Thrombosis Research Institute reported a series of thrombin inhibitors that used the unique amino acid D-diphenylalanine as a P_3 ligand.[5] The compounds exhibited an ∼3−10 fold potency enhancement versus the corresponding D-phenylalanine analogs. Incorporation of this amino acid into the P_3 position of our inhibitors using standard synthetic approaches[6] provided compound **3** (Figure 1), which exhibited a 30-fold increase in inhibitory potency-(K_i thrombin = 0.10 nM). Simultaneously, we became interested in employing lipophilic groups on the N-terminus of our lead structure **2**. We envisioned the use of an N-terminal alkyl sulfonamide[7] as an additional way of adding lipophilicity to the P_3 region of these

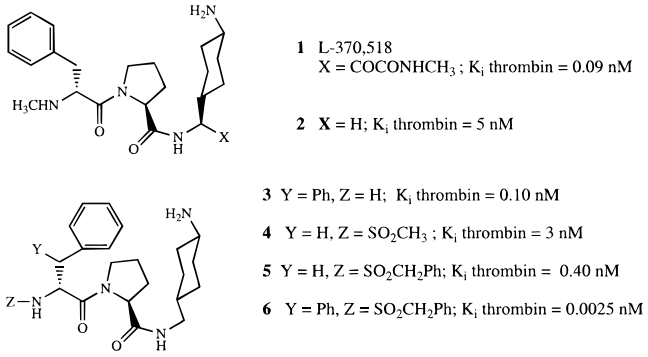

Figure 1.

inhibitors. A preliminary molecular modeling study[8] with the methanesulfonamide compound **4** suggested that one of the sulfonamide oxygens might be capable of hydrogen bonding with the N-H of Gly 219 on the thrombin β-sheet. Compound **4** was synthesized using standard synthetic approaches;[6] however, no enhancement in potency (K_i thrombin = 3 nM) versus the lead compound **2** was observed. Despite this unsuccessful result, the use of an N-terminal sulfonamide as a scaffold for adding an additional aromatic group to the P_3 region of this series of inhibitors remained of interest. It appeared that a flexible aralkyl sulfonamide such as a benzylsulfonamide might be an alternative to D-diphenylalanine as a means of introducing a second aromatic ring into the S_3 binding pocket of thrombin. Incorporation of the benzylsulfonamide onto the N-terminus of **2** using standard synthetic approaches[6] provided the novel inhibitor compound **5** (Figure 1), which showed a 10-fold potency enhancement (K_i thrombin = 0.40 nM) versus **2**. To understand the binding of these compounds to the thrombin active site, both X-ray crystallographic and molecular modeling studies[8] were initiated in an effort to provide guidance for further synthetic work. Modeling of compound **3** into the thrombin active site suggested that one of the phenyl rings of the D-diphenylalanine moiety might bind in the normal D-phenylalanine mode. The second phenyl ring could be in the front of the S_3 pocket near the solvent interface, making interactions with Ile 174 and the side chain carbon atoms of Glu 217.[9] Similar modeling studies[8] with **5** suggested two potential binding modes for the benzylsulfonamide group. In one model, the group curled into the front of the S_3 pocket, occupying a position near that predicted for the second phenyl ring of compound **3**, able to stack with the P_3 phenyl group of the inhibitor as well as with the Tyr 60A and Trp 60D aromatic rings of the thrombin insertion loop. The second model had the benzylsulfonamide group rotated ∼120° toward the solvent interface, pointing upward toward the insertion loop and stacking with the aromatic ring of Trp 60D. Both models suggested that the SO_2 of the sulfonamide might be capable of hydrogen bonding with the N-H of Gly 219 on the thrombin β-sheet. The second model implied that it might be possible to merge the features of compounds **3** and **5** into a single compound of extremely high potency. Therefore, the synthesis of the hybrid molecule **6** (Figure 1) was initiated using standard methods.[6]

During the synthesis of **6**, X-ray crystallographic data for compounds **3** and **5** bound with the thrombin molecule became available (Figure 2).[10] The structure

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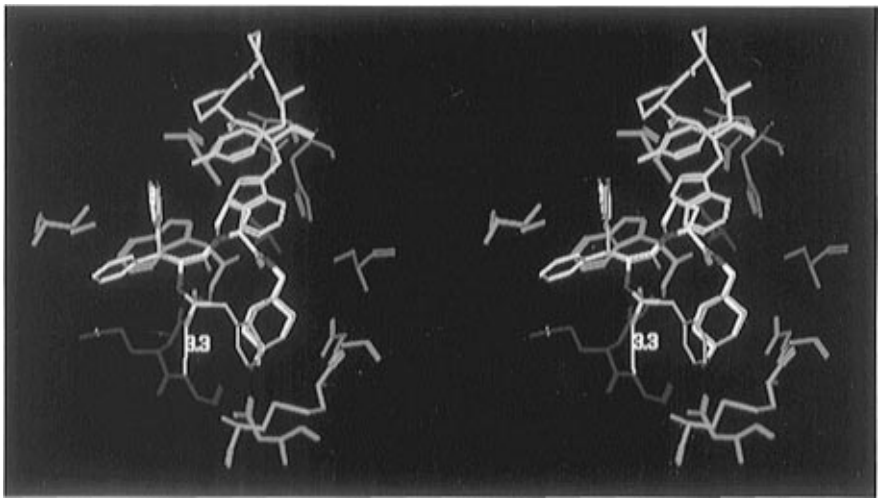

Figure 2. Stereoview of thrombin-bound inhibitors **3** (white with red oxygen and blue nitrogen atoms) and **5** (green with red oxygen, blue nitrogen, and yellow sulfur atoms) with the **5**-inhibited thrombin active site. The bound structure of **6** is essentially a superposition of the structures of **3** and **5**. The potential hydrogen-bonding interaction between the sulfonyl oxygen of **5** and the N-H of Gly 219 is indicated as a line with the distance provided in angstroms. For each structure, a single crystal was used, each diffracting to 1.8 Å resolution. Active site residues are color coded as follows: lipophilic P_3 residues (Leu 99, Ile 174, Trp 215) are colored magenta; the thrombin β-sheet is colored dark blue; the catalytic triad is colored green; the S_1 Asp 189 is colored red; the thrombin insertion loop is colored yellow; the Cys 191−Cys 220 disulfide linkage is colored orange; Glu 192 is colored light blue. Some active site residues have been omitted for clarity.

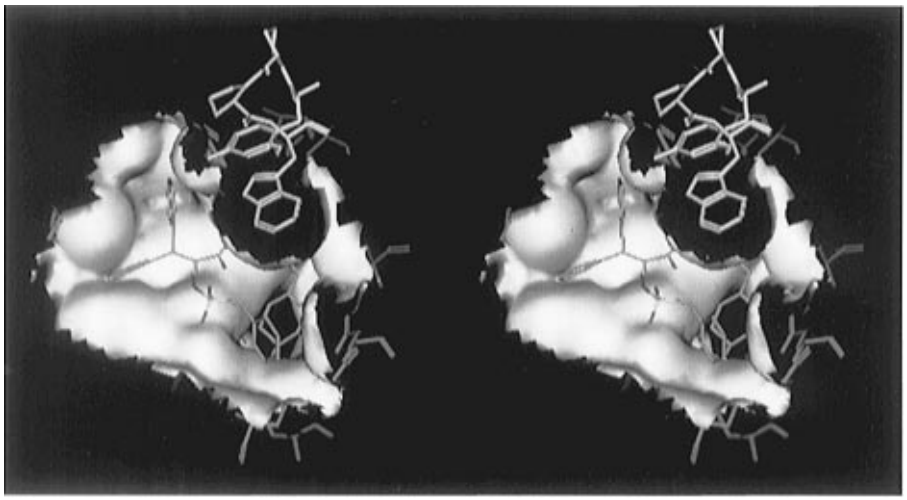

Figure 3. Van der Waals surface stereorepresentation of bound inhibitor **6** (green with red oxygen, blue nitrogens and yellow sulfur) in the **6**-inhibited active site. The view is identical to that shown in Figure 2, and the same color coding for active site residues is used.

for compound **3** (Figure 2) showed that the P_3 diphenyl rings were located essentially as suggested by molecular modeling. The structure for compound **5** (Figure 2), however, indicated a novel binding site previously unknown for thrombin. The benzylsulfonamide moiety of **5** was turned away from both the S_3 pocket and the thrombin insertion loop, lying along the solvent interface and roughly parallel to the Asn 217−Gly 219 portion of the thrombin β-sheet, with the phenyl ring lying extremely close to the P_1 cyclohexyl group. In essence, it appeared that the benzyl group had found a unique lipophilic binding site which we have labeled as the "N-terminus binding site". The crystal structure of **5** also suggested a hydrogen-bonding interaction between one of the sulfonyl oxygens and the N-H of Gly 219.

From a drug design point of view, the crystallographic results confirmed that the features of compound **3** and **5** were mutually compatible, indicating that the hybrid structure **6** should indeed possess excellent inhibitory potency. Completion of the synthesis[6] of **6** and its evaluation as a thrombin inhibitor showed this molecule to be one of the most potent and selective inhibitors of thrombin yet identified, possessing a K_i value of 2.5 ± 2 pM (n = 5) versus thrombin. The compound also demonstrates excellent selectivity versus several important human serine proteases including trypsin (K_i = 4 nM), plasmin (K_i = 490 nM), and tissue-type plasminogen activator (K_i = 6.7 mM).[6] Most remarkably, the incredible levels of potency observed with **6** were achieved without the use of an activated carbonyl serine trap, a feature present in almost all known tripeptide thrombin inhibitors achieving this level of potency. It is also noteworthy that compound **6** exhibits an extremely fast k_{on} value of 8×10^7 m^{-1} s^{-1}, approaching diffusion controlled.[6] Most highly potent thrombin inhibitors that contain serine traps are characterized as slow binding inhibitors due to the nature of their specific covalent interactions with the thrombin catalytic triad. Crystallographic studies[10] with com-

pound **6** confirmed that the compound was indeed a true hybrid of **3** and **5**, with the bound inhibitors essentially superimposable on each other. Careful examination of space-filling surface representations of the bound structure of **6** (Figure 3) indicates that the novel binding pocket is formed by the insertion loop above, the Cys 220−Cys 191 disulfide linkage below, the side chain carbons of Glu 192 on one side, and the cyclohexyl ring of the inhibitor itself to the rear. This unique binding mode appears to be an example of an inhibitor that binds in such a manner as to help create a part of its own binding site. Strongly supporting these assumptions are the close associations between the P_1 and benzylsulfonamide moieties of the inhibitor. The sulfonamide benzylic carbon and the C_2 carbon of the P_1 cyclohexyl ring are separated by only 3.4 Å, and the C_6 cyclohexyl carbon is also quite close at a distance of 3.9 Å. Similarly, the C_2 and C_6 carbons of the benzylic sulfonamide aromatic ring are both less than 4 Å away from the C_2 carbon of the cyclohexyl ring, again suggesting a specific lipophilic interaction between these groups. The observed interactions between the P_1 and the benzylsulfonamide groups may be analogous to observations previously reported with D-Phe-Pro-Arg thrombin inhibitors[11] and with the non-peptide thrombin inhibitor argatroban.[12] In each of these molecules, specific intramolecular hydrophobic interactions defined as "hydrophobic collapse" were observed.

The excellent selectivity for thrombin versus trypsin observed with compound **6** may be due to a combination of interactions present in this molecule. The P_1 cyclohexyl group is likely a key contributor to the selectivity observed with this compound. The S_1 pockets in thrombin and trypsin differ in amino acid sequence at reside 190, with this residue being an Ala in thrombin and a Ser in trypsin.[3] Therefore, compounds containing bulky lipophilic P1 groups should in theory be more selective for thrombin versus trypsin.[1] The role of the thrombin insertion loop in the formation of the novel binding site may also be important to the selectivity profile observed with compound **6**, given that the insertion loop is unique to thrombin as opposed to other similar serine proteases. The benzylsulfonamide group of **6** appears to make direct interactions with Trp 60D of the insertion loop.

In summary, we have discovered a novel lipophilic binding pocket in the thrombin active site. The novel binding pocket is formed by the insertion loop above, the Cys 220−Cys 191 disulfide linkage below, the side chain carbons of Glu 192 to the side, and the cyclohexyl ring of the inhibitor itself to the rear. By taking maximum advantage of lipophilic interactions in both the S_3 binding pocket of thrombin and the novel sulfonamide binding pocket, the extremely potent and selective noncovalent thrombin inhibitor **6** was designed and synthesized. We are continuing to explore these discoveries with the goal of developing clinically useful thrombin inhibitors and will detail our further efforts in subsequent publications.

Supporting Information Available: Summary of X-ray crystallographic studies with **3**, **5**, and **6** and experimental procedures (9 pages). Ordering information is given on any current masthead page.

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(6) Full experimental details for compounds **3**, **4**, and **5** are available as Supporting Information and are also detailed in the patent cited in ref 2b. The procedures for K_i determinations are also detailed in the patent application cited in ref 2b.

(7) A series of N-terminal sulfonamide tripeptide arginal thrombin inhibitors has been reported: Weir, L. C.; Wiley, M. R.; Borromeo, P. S.; Coffman, W. J.; Craft, T. J.; Gifford-Moore, D.; Lugor, C. W.; Schacht, A. L.; Shuman, R. T.; Smith, J. T. and Wikel J. H. MEDI 127, Book of Abstracts of 211th ACS National Meeting, New Orleans, LA, Mar 24−28, 1996.

(8) Molecular modeling was performed as follows: Models were constructed using AMF/C_View (AMF is Merck proprietary software). The ligands were energy mininized within the context of the active site using OPTIMOL with the MMFF force field. The amino group on the P_1 cyclohexyl ring was held fixed in the position corresponding to the crystal structure of **2**.

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