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TEMPLATES FOR DESIGN OF INHIBITORS FOR SERINE PROTEASES: APPLICATION OF THE PROGRAM DOCK TO THE DISCOVERY OF NOVEL INHIBITORS FOR THROMBIN.

Irina Massova,[a] Philip Martin,[b] Alexey Bulychev,[a] Remek Kocz,[a] Maureen Doyle,[b] Brian F. P. Edwards,[b] and Shahriar Mobashery[a,*]

[a]*Department of Chemistry, and of* [b]*Biochemistry and Molecular Biology, Wayne State University, Detroit, MI 48202-3489*

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Abstract: The program DOCK was used to search for novel inhibitors for α-thrombin. Four among the top twelve best scoring compounds from the Cambridge Structural Data Base inhibited this enzyme, and three of them inhibited α-thrombin in a competitive mode. These molecules are expected to serve as general templates for structural elaboration in targeting diverse serine proteases for selective inhibition.

Serine proteases constitute a large family of proteolytic enzymes with a diverse set of functions spanning various metabolic and pathologic processes.[1] Despite their disparate roles in biological systems, they all have the same general protein fold with certain important active-site elements being entirely conserved. A key feature is the catalytic triad, comprised of amino-acid residues aspartate, histidine and serine, of which serine undergoes acylation and deacylation during turnover of substrates. All serine proteases possess a pocket at the S_1 site.[2] The substrate specificity of these enzymes is largely determined by the residue at P_1, which is driven by interaction of the P_1 side chain of the substrate with the S_1 pocket of the enzyme. The binding of typical substrates to the enzyme can be further characterized by interactions with the S_2 site. Some of the serine proteases are known to have more extended binding sites for substrates, such as the "fibrinogen binding" site of thrombin.[3]

Because of the critical roles that these enzymes play in biological systems, new types of inhibitors for them are highly sought. We have used the program DOCK,[4] which has successfully found bioactive lead compounds in other systems,[5–9] to search for novel templates for the design of inhibitors for serine proteases. DOCK describes molecular surface in terms of a solvent accessible area, usually the Connolly surface. The program matches potential small ligands to various sites on this surface, which is described in terms of a negative image of the surface produced by DOCK. A simple scoring procedure is used to eliminate molecules with excessive van der Waals overlap and those with weak interactions with the potential binding sites on the surface. We searched 76,500 structures in the Cambridge Structural Data Base for molecules that could complement the topology of the active site of α-thrombin, an important serine protease involved in fibrinolysis, thrombosis and hemostasis.[10] Nature has developed an elaborate process for α-thrombin regulation. Mostly, this is through the action of high-molecular-weight complexes, such as the heparin–antithrombin complex,[11] and others. Nevertheless, native mechanisms of α-thrombin regulation frequently fail, and low-molecular-weight synthetic drugs are used as a treatment in such cases. Many of these antithrombotic drugs were developed over the past thirty years.[12] However, along with high potency, they often have critical side effects and shortcomings such as toxicity, release of the plasminogen activator and prostacyclin, vasoconstriction, smooth muscle growth and propagation of experimental metastasis.[13] Oral administration is impossible in most cases, as these drugs have a very short life-time which requires continuous administration.[13] Therefore, development of novel classes of α-thrombin inhibitors is highly desirable. The structure of this enzyme has been available from our

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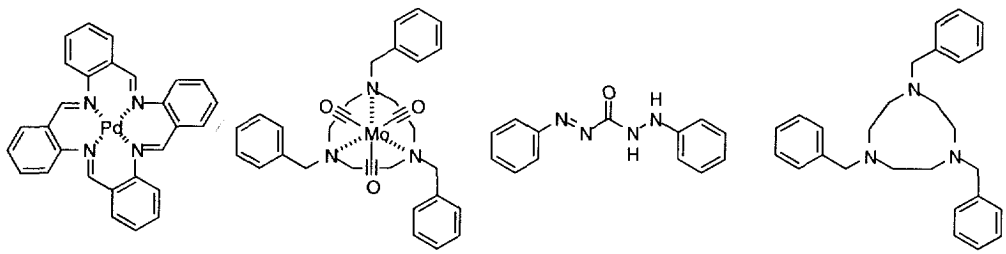
laboratories, which we have used in the present study.[14]

We synthesized 12 of the molecules with high score in the search protocol. The study of inhibition kinetics for some of these compounds was difficult because of their low solubility or presence of strong interfering chromophores in the molecules. Compounds **1**, **2**, and **3** were three of the compounds identified by DOCK. Palladium was important for the stability of compound **1**. However, molybdenum was not necessary for stability of **2**; hence it was left out of the structure, and the parent structure **4** was studied. Compounds **1**, **3**, and **4** were investigated further and they were found to be competitive inhibitors of α-thrombin.[15] The dissociation constants (K_i) for compounds **1**, **3**, and **4** were 170 ± 50 μM, 1.7 ± 0.2 mM, and 410 ± 40 μM, respectively.[16]

We attempted to generate crystals for the complexes of these inhibitors with α-thrombin. We were able to



1 **2** **3** **4**

grow crystals of the complex with **3** that were amenable to X-ray analysis.[17] The crystallographic data revealed that **3** was bound in the active site (Fig. 1A), as predicted by DOCK. The positioning of compound **3** predicted by DOCK is highly similar to that found in the crystal structure. The inhibitor has one phenyl ring in a hydrophobic pocket formed by Leu-99, Ile-174, and Trp-215. This site is known to be the S_2-binding site. The other ring of the inhibitor is in contact with the main-chain atoms of Gly-219 and Ser-193, respectively. Interestingly, the side chains of the catalytic residues His-57, Asp-102, and Ser-195 do not interact with the inhibitor. We can also see that the two conformations of the inhibitor are due to the presence of the double bonded nitrogen in **3**. In one conformation the double bond is found closer to the phenyl ring in the S_2 pocket and in the other conformer, the single bonded nitrogen takes this position since the inhibitor is nearly symmetric about the carbonyl carbon.

The structure of compound **1** was energy-minimized in the active site of α-thrombin by using the AMBER force-field according to the procedure reported previously.[18] The mode of binding of compound **1** remained essentially unchanged after energy-minimization, providing the same critical interactions to the active-site, as predicted by DOCK. Figure 1B shows the energy-minimized complex of **1** in the active site of α-thrombin superimposed on the crystal structure of the bound known α-thrombin inhibitor PPACK.[19] Compound **1** appears to fit well in the active site, occupying the known "aryl binding site" and the S_2 hydrophobic sites.[20] The various aromatic rings of the inhibitor are involved in hydrophobic interactions, aromatic stacking, or edge-to-face interactions with various subsites in the active site of the enzyme. One aromatic ring shows hydrophobic interactions with Ile-174, and has stacking interactions with residues Trp-215 and Phe-227 near the "aryl binding site". This site is usually occupied by the phenyl ring of Phe-8' of fibrinogen Aα-chain or that of D-Phe of PPACK. The overall conformation of the bound **1** in the active site of α-thrombin repeats the profile of the inhibitor PPACK (Fig. 1B).

The binding mode for inhibitors in the energy-minimized complex of **1**, the predicted complex of **2** by DOCK (not shown), and the crystal structure of **3** in α-thrombin are all very similar to the binding of the potent thrombin inhibitors NAPAP, MQPA, and 4-TAPAP.[21] These three potent α-thrombin inhibitors possess several

hydrophobic groups which interact with the enzyme analogously to inhibitors **1**, **3**, and **4**.

The crystallized complex of α-thrombin with **3** and inhibition of α-thrombin by **1**, **3**, and **4** demonstrate how small and relatively hydrophobic compounds can bind to the active site of α-thrombin entirely independently of the S_1-binding site. Compounds **1**, **3** and **4** are novel templates for development of effective inhibitors for α-thrombin and other serine proteases. We anticipate that incorporation of different moieties in places of substituents in compounds **1**, **3**, and **4** or their analogues, would yield highly effective inhibitors for various serine proteases (including α-thrombin) in light of the overal shared topology by this family of enzymes.

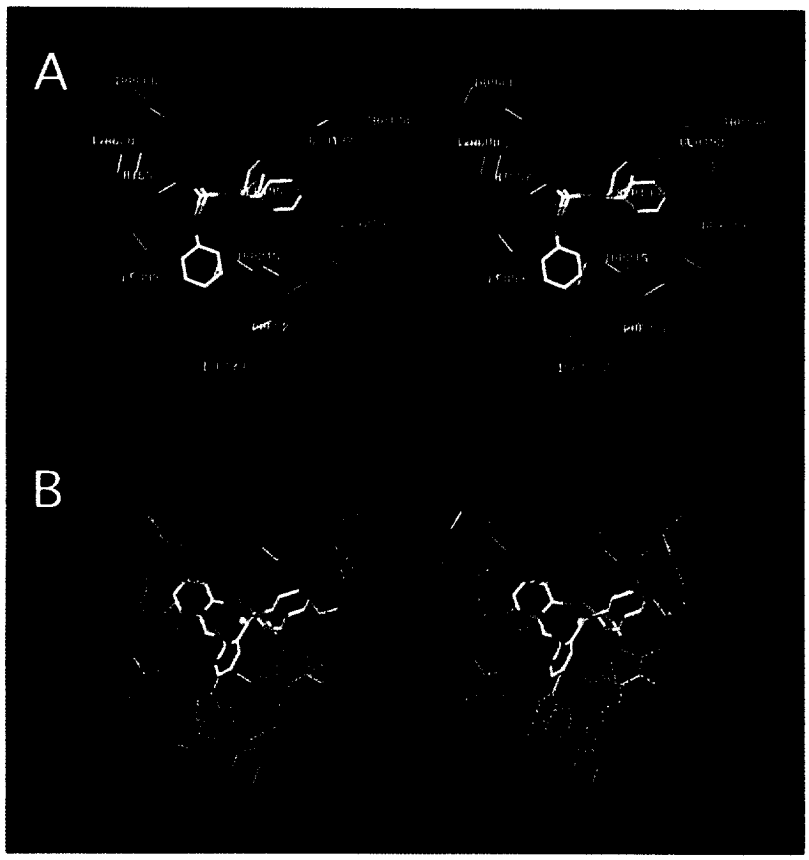


Figure 1. (A) The crystal structure of **3** bound to the active site of α-thrombin. The inhibitor is colored according to atom type (blue for nitrogens, red for oxygens and white for carbons). Enzyme residues are depicted in orange. The figure shows two conformations for the exposed phenyl ring. (B) Stereoview of the energy-minimized complex of compound **1** in the active site of α-thrombin shown in the same perspective as for the crystal structure in (A). Compound **1** is colored according to the atom types: white for carbons, blue for nitrogens and yellow sphere for palladium. The inhibitor **1** is shown superimposed on the inhibitor PPACK[19] (in red), as discerned from its crystal structure bound in the active site of the enzyme. The α-thrombin residues are shown in orange. Hydrogen atoms are not displayed. The Connolly surface of the α-thrombin active site is drawn as a green dotted surface.

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15. Compound **1** was made by the procedure of Brawner and Mertes (Brawner, S.; Mertes, K. B. *J. Inorg. Nucl. Chem.* **1979**, *41*, 764), compound **3** (phenylazoformic acid 2-phenylhydrazide) was purchased from the Aldrich Chemical Co., and compound **4** was synthesized according to the method of Beissel *et al.* (Beissel, T.; Della Vedova, B. S. P. C.; Wieghardt, K.; Boese, R. *Inorg. Chem.* **1990**, *29*, 1736).
16. All kinetic measurements were performed on a Perkin-Elmer Lambda 3B or Hewlett-Packard 8452 diode-array spectrometer. The enzyme assay and methods for determination of kinetic parameters were according to published procedures (Sonder, S. A.; Fenton, J. W., II. *Clin. Chem.* **1986**, *32*, 934). The dissociation constants (K_I) for compounds **1**, **3**, and **4** for α-thrombin were calculated by method of Dixon (Dixon, M. *Biochem. J.* **1953**, *55*, 170). A series of assay mixtures containing both Chromozym TH (Tosyl-Gly-Pro-Arg-4-nitranilide; Boehringer-Mannheim Co.) as the substrate (75 or 150 μM) and various concentrations of compounds **1**, **3** and **4** as the inhibitor (0–175 μM for **1**, 0–1250 μM for **3**, and 0-800 μM for **4**) were prepared in 50 mM Tris, 200 mM NaCl, 0.1% polyethylene glycol (PEG; Sigma), pH 7.4, at 25 °C. Organic co-solvents (10%) were used, for compound **1** (DMSO) and for **3** and **4** (methanol). A portion of the enzyme was added to give a final concentration of 5 nM in a total volume of 0.1 mL. The enzyme activity was determined immediately.
17. Crystals of the α-thrombin-inhibitor complex were grown in hanging drops prepared from equal volumes of a 12.5 mg/mL protein solution in 50 mM $NaPO_4$, 100 mM NaCl, pH 7.3, which was saturated with the inhibitor and a precipitating solution containing 25% PEG2000, 0.1 M sodium acetate, 0.2 M ammonium sulfate, 0.1% w/v NaN_3, at pH 4.6. The drops were equilibrated at 22 °C against 1 mL of the precipitating solution. The structure of the complex was solved by molecular replacement, using a high-resolution model of bovine thrombin (Martin, P. D.; Malkowski, M. G.; DiMaio, J.; Konishi, Y.; Edwards, B. F. P. *Biochemistry*, **1996**, *35*, 13030) and refined with XPLOR in successive steps of molecular dynamics (4000°), positional refinement, and temperature factor refinement. The final values for the R-factor and R_{free} were 0.19 and 0.29, respectively, for 2σ data between 7.0 and 2.7 Å.
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