Molecular Modeling and 3D-QSAR Studies on the Interaction Mechanism of Tripeptidyl Thrombin Inhibitors with Human α-Thrombin†

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The mechanism of inhibition of peptidyl inhibitors with thrombin was studied using molecular modeling, molecular mechanics, and CoMFA statistical analysis. A new procedure for the elucidation of binding conformations, BCSPL, is described and was employed to obtain the binding conformers of a series of 18 tripeptidyl thrombin inhibitors. Energetic studies and QSAR analysis of the BCSPL-derived conformers indicated a modest correlation between the calculated binding energies of the title compounds and their inhibitory activities to human α-thrombin. CoMFA analysis of the BCSPL alignment resulted in a satisfactory model of the thrombin active site.

Introduction

The discovery of novel and highly potent therapeutic agents with high potency for the treatment of cardiovascular diseases is a stimulating and exciting challenge.[1,2] Among the many potential targets for therapeutics, one important serine protease, thrombin, is central to both haemostasis and thrombosis.[3] In the coagulation cascade, thrombin is the final key enzyme, which proteolytically cleaves fibrinogen, releasing fibrinopeptides A and B to generate fibrin, which then polymerize to form a haemostatic plug. In addition to fibrinogen cleavage, thrombin catalyzes its own production by activating coagulation factor V and VII. It also activates factor XIII, which cross-links and stabilizes the fibrin polymer. Thrombin also has been shown to acts in many other cells, such as tumor, endothelial, and leukocytes cells, in addition to its role in coagulation.[4]

Such factors make thrombin an attractive target in the design of new drugs for the treatment of cardiovascular and other diseases.[1,2,4] Since Bode and co-workers solved the X-ray crystal structure of human α-thrombin,[5] a number of structures of thrombin−inhibitor complexes have appeared in the literature.[6−13] From these structures, insights into binding modes for both substrates and inhibitors have led to the elucidation of structure-activity relationship of inhibition for many of the serine proteases.[4] Knowledge of the three-dimensional structures for thrombin−inhibitor complexes clearly is useful for the structure-based drug design (SBDD) of novel thrombin inhibitors.

We have performed a theoretical study on the mechanism of interaction of a series of tripeptidyl thrombin inhibitors[14] which are analogs of PPACK.[5] We first determined the binding conformers of these inhibitors using a newly developed method and then performed both traditional and 3D-QSAR analysis (CoMFA)[15] against known activity measurements. Our results indicate that this new paradigm, which we have called BCSPL (binding conformation search program for ligand),[16] provides a reliable and useful alignment mechanism for such methods as predictive models for activity were obtained. In addition, analysis of the resulting molecular fields indicated good agreement with existing features of the thrombin active site. Finally, these results provided some clues for designing more potent inhibitors.

Table 1. Structural Formulae and *in Vitro* Enzyme Inhibitory Activities of R-Pro-Arg-H in This Study

compd	R	IC_{50} (μM)	−log IC_{50}
1	Boc-D-Chg	0.012	7.92
2	Boc-D-Phg	0.016	7.80
3	D-MePhg	0.018	7.74
4	D-Phg	0.018	7.74
5	Bov-D-2-Nag	0.018	7.74
6	D-1-Tiq	0.019	7.72
7	Boc-D-1-Nag	0.023	7.64
8	D-3-Tiq	0.025	7.60
9	Boc-D-Phg(3-CF_3)	0.027	7.57
10	Boc-D-Phg(F)	0.030	7.52
11	Boc-D-Phe	0.045	7.35
12	Boc-1-Nag	0.055	7.26
13	Boc-1-Nag	0.085	7.07
14	Boc-D-Phg(OH)	0.089	7.05
15	(*R*)-(+)-MeCH(Ph)CO	0.094	7.03
16	(*R*)-(+)-EtCH(Ph)CO	0.130	6.89
17	(*R*)-(+)-MeOCH(Ph)CO	0.250	6.60
18	(*S*)-(+)-MeCH(Ph)CO	5.100	5.29

Computational Methods

A series of 18 analogs of PPACK[14] were employed in this work (Table 1). The X-ray structure of the human α-thrombin−PPACK complex was kindly provided by professor A. Tulinsky (structure presently available as 1ppb from the Brookhaven Protein Data Bank). Sybyl 6.2[17] was employed for all calculation, program development, and visualization. All calculations were performed on Silicon Graphics Indigo XZ R4000 workstations.

Conformational Search of the Inhibitors. Conformational searching of the inhibitors was performed using the Systematic Search option of Sybyl with a limit of 500 000 conformations per molecule. The conforma-

† All amino acids are in the L configuration unless otherwise noted. The abbreviations used are as follows: PPACK, D-Phe-Pro-Arg chloromethyl ketone; Boc, *tert*-butyloxycarbonyl; Chg, cyclohexylglycine; Phg, phenylglycine; 1-Tiq, 1-carboxy-1,2,3,4-tetrahydroisoquinoline; 3-Tiq, 3-carboxy-1,2,3,4-tetrahydroisoquinoline; Nag, naphthylglycine; Phg(OH), (4-hydroxyphenyl)glycine; Phg(F), (4-fluorophenyl)glycine; Phg(CF_3), (4-(trifluoromethyl)phenyl)glycine; Me, methyl; Ph, phenyl; Me_3Si, trimethylsilyl; Mpg, (methoxypropyl)glycine; $C_{10}H_{16}$, pinanediol; Z, benzyloxycarbonyl; Dpa, β,β-diphenylalanine; Mpg^P, (α-amino-δ-methoxybutyl)phosphonyl.

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tion of the common Pro-Arg backbone was set and initially restricted to the crystallographic coordinates of PPACK, and only the bonds in the N-terminal amino acid and side chain were subjected to the search. Following this search, a second systematic search was performed on the C-terminal Arg for each inhibitor. As the N-terminal amino acid residue and the C-terminal Arg residue are located in the S3 and S1 pockets of thrombin, respectively, the conformations of these two parts actually do not influence each other once the inhibitor binds to thrombin. Therefore, the systematic search for the two "ends" of the tripeptides was carried out separately to save computational time.

Binding Conformation Search Method (BCSPL). By combining the power of systematic search protocols with a molecular docking approach,[18] we have developed a new method to find the binding conformer of a ligand based on the three-dimensional structure of a receptor site. Using Sybyl Programming Language (SPL),[19] we created a computer program named BCSPL (binding conformation search program for ligands)[16] which automates this methodology. The algorithm of BCSPL is as follows:

(1) Perform a systematic conformational search for an isolated ligand, saving the sampled conformers into a molecular spreadsheet (MSS). Other conformational search methods such as random search or molecular dynamics could also be used to sample the conformations of the ligand.

(2) Merge or "dock" the individual conformers from the MSS into the active site of the receptor, calculating the total energies of each ligand−receptor complex ($E_{complex}$).

(3) Calculate the binding energy (E_{bind}) of each conformer according to the formula

$$E_{bind} = E_{complex} - E_{ligand} - E_{receptor} \qquad (1)$$

where E_{ligand} is the energy of the ligand corresponding to the overall minimum energy for the conformational search, and $E_{receptor}$ is the energy of the receptor. All energies are calculated using Tripos force field and the routine MAXIMIN2 encoded in Sybyl.[17]

(4) Save the binding energies, E_{bind}, back into the MSS table, and use the statistical tools of MSS to choose the conformer having the lowest binding energy, which is the probable binding conformer of the ligand.

Energy Calculation and Structure Optimization. Employing the BCSPL method described above, we generated initial structures of the inhibitor−thrombin complexes. These structures were then successively refined using both the Dock functionality and minimization options of Sybyl. Docking of the flexible ligands was first performed while holding the receptor site fixed. The entire complex was then minimized using 200 steps of steepest descent, followed by conjugate gradient minimization to a root-mean-square (rms) energy gradient of 0.10 kcal/mol Å^2. Gasteiger−Hückel charges were employed throughout. Solvation energies were not explicitly considered; however calculations were performed with a dielectric constant of 5 to simulate the solvation effect of the inhibitor in the protein environment.

Interaction Mechanism

Figure 1 illustrates the binding conformations for the 18 thrombin inhibitors predicted from the BCSPL program[16] and minimization techniques described above. Table 2 provides some of the geometric data for these conformers, and in Table 3 are compiled the BCSPL binding data of the inhibitor−thrombin complexes.

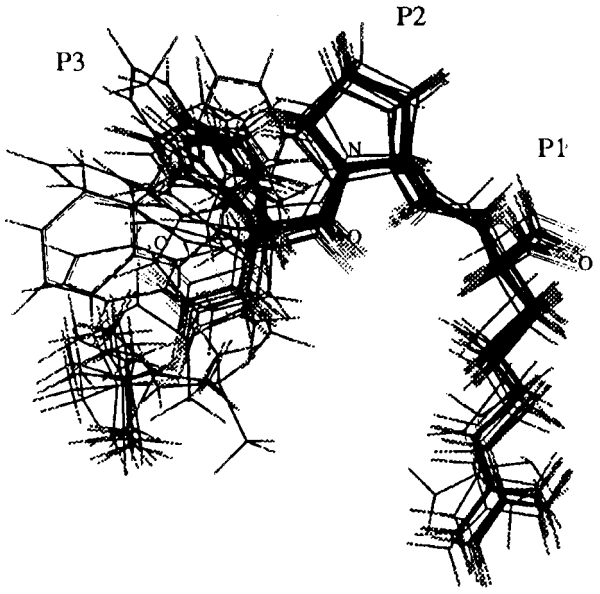


Figure 1. The alignment of binding conformers for the title compounds.

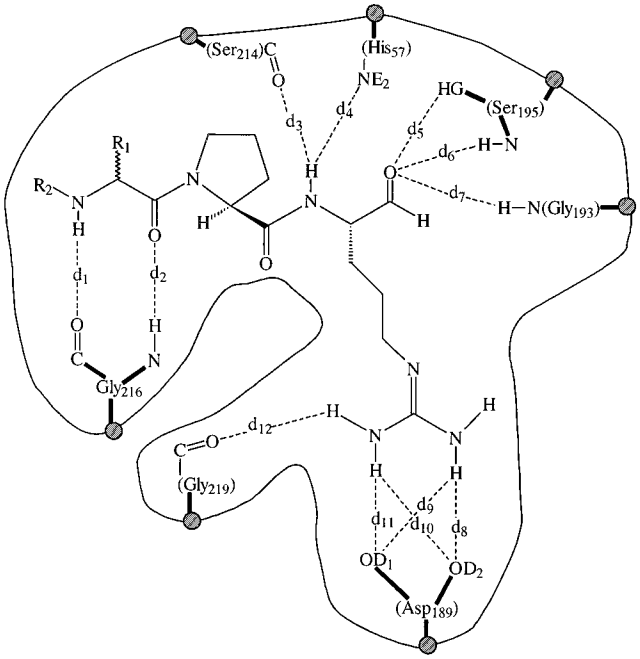
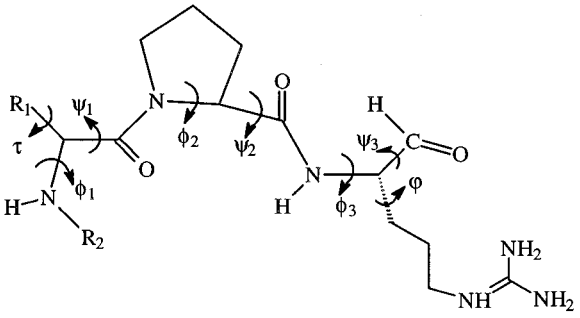


Figure 2. Schematic presentation of the hydrogen-bonding interaction between title inhibitors and thrombin.

We also examined the hydrogen bonding patterns for the BCSPL-predicted conformers and compared them to that seen for PPACK. Figure 2 illustrates the predicted hydrogen-bonding pattern.

The hydrogen-bonding modes for these compounds compare favorably to those observed with PPACK[5] and phosphonopeptides.[16] In general, the Arg-H unit for all of the inhibitors is bound in an extended conformation to the S1 subsite of thrombin, allowing the guanidinium moiety to form multiple hydrogen bonds to OD1 and OD2 atoms of Asp189 and also to Gly219 (d8−d12 in Figure 2). The agreement between the structures of PPACK, phosphonopeptides, and the inhibitors in this

Table 2. Dihedral Angles of Some of the Backbone Bonds for the Binding Conformers of the Title Inhibitors



compd	ϕ_1	ψ_1	ϕ_2	ψ_2	ϕ_3	ψ_3	τ	φ
1	157.3	−156.9	−68.8	136.3	−72.4	59.1	−84.3	−89.8
2	148.1	−159.6	−60.9	139.6	−78.4	48.7	−130.6	−83.2
3	68.4	−157.0	−63.3	132.0	−73.0	57.4	−129.1	−85.0
4	102.0	−161.8	−64.3	129.6	−74.1	54.4	−129.1	−85.0
5	129.2	−127.3	−64.2	−175.5	−127.8	37.8	141.8	−81.3
6	−171.7	−160.5	−65.5	129.0	−73.4	56.0	176.2	−85.9
7	168.0	−159.0	−63.6	134.4	−76.4	48.8	−116.7	−83.8
8	166.7	−135.5	−61.1	129.5	−78.4	45.2	−176.5	−89.3
9	170.5	−163.3	−58.8	161.3	−108.9	42.3	−123.9	−79.4
10	180.0	−160.0	−58.4	140.7	−87.4	44.8	−149.1	−78.3
11	163.5	−133.0	−63.9	−173.4	−136.3	45.3	−90.4	−85.0
12	−160.0	−160.0	−57.1	155.2	−108.6	38.5	−43.7	−79.1
13	−160.0	−160.0	−55.6	150.0	−104.2	38.3	−63.6	−78.5
14	180.0	−160.0	−59.4	143.2	−89.2	44.1	−118.3	−78.7
15	-	−67.7	−68.3	133.6	−80.8	47.0	−142.5	−84.0
16	160.0	−166.7	−60.8	147.8	−96.8	42.9	−150.0	−81.4
17	70.0	−166.5	−60.5	146.6	−93.2	43.1	−150.0	−80.3
18	-	−168.1	−61.4	155.5	−104.6	41.0	−170.0	−81.8

Table 3. Binding Energies (kcal/mol) for the Title Inhibitors Interacting with Thrombin[a]

compd	E_{total}	E_{steric}	E_{elec}
1	−210.703	−130.211	−80.492
2	−191.999	−127.828	−64.171
3	−190.725	−121.880	−68.880
4	−184.575	−117.163	−67.412
5	−188.203	−134.227	−53.977
6	−186.541	−119.608	−66.932
7	−200.080	−136.447	−63.633
8	−174.416	−107.793	−66.623
9	−178.631	−129.218	−49.143
10	−178.051	−123.644	−54.408
11	−183.060	−132.542	−50.518
12	−173.029	−116.853	−56.175
13	−172.481	−116.386	−56.095
14	−183.553	−127.643	−55.910
15	−174.985	−116.026	−58.958
16	−165.476	−114.106	−51.370
17	−166.304	−112.908	−55.396
18	−165.086	−111.453	−53.633

[a] E_{total} is the total interaction energy, E_{steric} is the steric interaction energy, and E_{elec} is the electrostatic interaction energy.

study is not surprising, as all of these inhibitors belong to the same class of reaction intermediate-based inhibitors which interact with Ser195 in the active site.[4]

In addition to hydrogen bonding, thrombin presents two hydrophobic "pockets" as a potential binding site for inhibitors. The loop consisting of Try60A−Pro60B−Pro60C−Trp60D, and the residues Ser214, Trp215, Gly216, Leu99, and Ile174, form what are known as the "D-pocket" (distal to the active site serine), and the "P-pocket" (proximal to the active site serine). Similar to PPACK and phosphonopeptides, the 18 inhibitors in this study associate with the Pro residue in the P-pocket, while the substituted Gly is directed into the D-pocket via hydrophobic interactions. The inhibitors thus form an antiparallel β-sheet with Gly216.

Satisfied that the conformations predicted by BCSPL were indeed reasonable, we then performed a classical QSAR to explore whether the inhibitory activities of these compounds could be correlated to BCSPL energetic information. Employing partial least squares (PLS)[20,21] analysis with Sybyl/QSAR, we calculated regression equations for the inhibitory activities, −log IC_{50}s, using the interaction energies E_{total} (total interaction energy), E_{steris} (steric interaction energy), and E_{elec} (electrostatic interaction energy) as descriptor variables (eqs 2−5). The best correlation was found using the total interaction energy as the sole descriptor variable (Equation 2), and this relationship is shown graphically in Figure 3. Equation 5, wherein the steric and electronic components of the total energy are considered jointly as independent descriptors, is shown merely to illustrate that both steric (including hydrophobic and hydrogen bonding interactions) and electrostatic effects are necessary to fully describe the inhibitory data. The moderately predictive correlation between activity and total interaction energy, therefore, can help to establish the importance of the initial binding event relative to the rates of reaction between Ser195 and potential inhibitors. Those potential inhibitors which would exhibit stronger interaction energies using this paradigm would therefore be expected to have greater efficacy toward thrombin inhibition.

CoMFA Analysis

In order to more fully explore the specific contributions of electrostatic and steric effects in the binding of PPACK analogs to thrombin, we performed a CoMFA[15] study from the alignment predicted by BCSPL (Figure 1). For the CoMFA calculation, steric and electronic field energies were calculated using an sp^3 carbon as the steric probe atom, and a +1 charge for the electro-

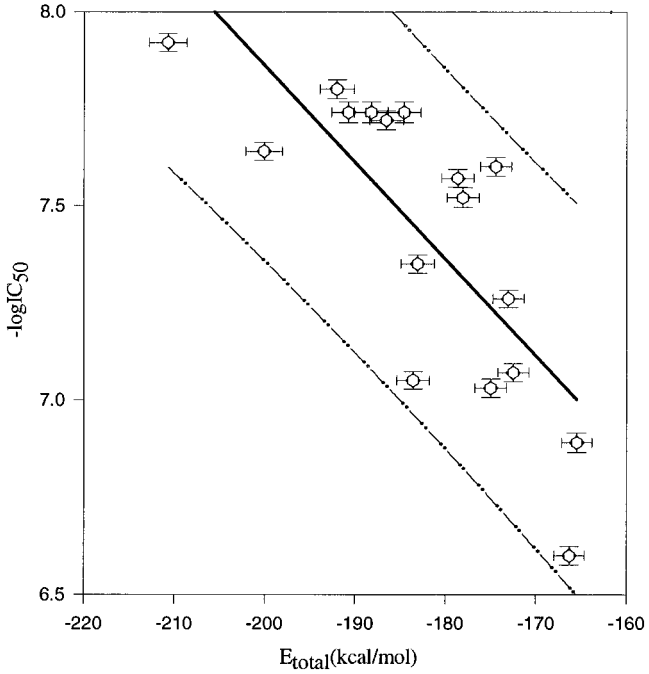


Figure 3. Correlation between total interaction energies (E_{total}) (kcal/mol) and inhibitory activities ($-\log IC_{50}$). The solid line represents the linear tendency of log IC_{50} along with the E_{total}, and the dash dot lines represent the predictive interval of $-\log IC_{50}$.

$$-\log IC_{50} = 2.882 - 0.025E_{total} \quad (2)$$

$$r^2 = 0.598, \quad F = 22.297, \quad s = 0.248$$

$$-\log IC_{50} = 4.836 - 0.021E_{steric} \quad (3)$$

$$r^2 = 0.248, \quad F = 4.166, \quad s = 0.346$$

$$-\log IC_{50} = 5.780 - 0.027E_{elec} \quad (4)$$

$$r^2 = 0.378, \quad F = 8.492, \quad s = 0.309$$

$$-\log IC_{50} = 3.220 - 0.021E_{steric} - 0.028E_{elec} \quad (5)$$

$$r^2 = 0.593, \quad F = 9.487, \quad s = 0.259$$

static probe. Atomic charges on the inhibitors were precalculated using AM1 potentials,[22] and a distance-dependent dielectric was used over all intersections of a regularly spaced (0.2 nm) grid. Excessive steric and electrostatic contributions were truncated to 30 kcal/mol for all field calculations. The statistical results of these calculations and the predicted for the series of inhibitors appear in Tables 4 and 5 and are graphically reproduced below in Figure 4.

Initial calculation of the 18 inhibitors of Table 1 resulted in a rather poor, though predictive, model (model 1, Table 4) exhibiting a maximum cross-validated r^2 (r^2_{cross}) of only 0.217 at four components. Upon detailed examination of the residuals for this calculation, it became apparent that compound 18 was at least partially responsible for the poor r^2_{cross}. Omission of compound 18 as an outlier and recalculation resulted in a much better r^2_{cross} of 0.455 (model 2) at fivecomponents. Subsequently increasing the total number of components of the CoMFA fields to six provided a final model of $r^2_{cross} = 0.505$ (model 3). As these useful predictive results were derived directly from the initial BCSPL alignment, this indicates a

Table 4. CoMFA Analysis Results

	cross-validated		conventional		
compd	r^2_{cross}	optimal component	r^2	s	F
18 (model 1)	0.217	4	0.929	0.190	42.449
17 (model 2)[a]	0.455	5	0.991	0.042	327.094
17 (model 3)[b]	0.505	6	0.996	0.027	621.683

[a] Omit compound **18**, and the initial component number is 5. [b] Omit compound **18** and the initial component number is 8.

Table 5. Experimental Activities and Predictive Activities with the CoMFA Models

		model 1		model 2		model 3	
compd	EA[a]	PA[b]	δ[c]	PA[b]	δ[c]	PA[b]	δ[c]
1	7.92	7.941	−0.021	7.958	−0.038	7.924	−0.004
2	7.80	7.681	−0.119	7.795	0.005	7.794	0.006
3	7.74	8.006	−0.266	7.791	−0.051	7.774	0.034
4	7.74	7.688	0.052	7.710	0.030	7.704	0.036
5	7.74	7.667	0.073	7.701	0.039	7.764	−0.024
6	7.72	7.628	0.092	7.723	−0.003	7.725	−0.005
7	7.64	7.770	−0.130	7.657	−0.017	7.633	0.007
8	7.60	7.539	0.061	7.573	0.027	7.580	0.020
9	7.57	7.602	−0.032	7.497	0.073	7.564	0.006
10	7.52	7.517	0.003	7.548	−0.028	7.517	0.003
11	7.35	7.501	−0.151	7.389	−0.039	7.388	−0.038
12	7.26	7.275	−0.015	7.245	0.015	7.243	0.017
13	7.07	7.095	−0.025	7.104	−0.034	7.076	−0.006
14	7.05	6.893	0.157	7.033	0.017	7.027	0.023
15	7.03	7.072	−0.042	7.006	0.024	7.019	0.011
16	6.89	6.511	0.379	6.861	0.029	6.868	0.022
17	6.60	6.482	0.118	6.651	−0.051	6.639	−0.039
18	5.29	5.662	−0.372				

[a] Experimental activities. [b] Prediction. [c] Residual values.

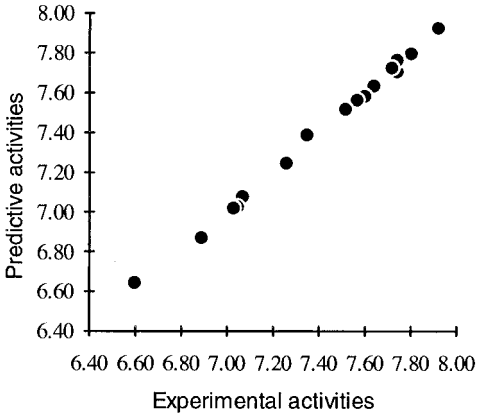


Figure 4. Experimental activities *vs* predictivies for model 3.

further application for our new binding conformation recognition approach as an appropriate alignment engine for CoMFA calculations.

The CoMFA map for model 3 is illustrated in Figure 5. Colored polyhedra in the map represent those areas in 3D space where changes in the field values for the inhibitors correlate strongly with concomitant changes in activities. Beneficial and detrimental steric interactions are displayed in green and yellow contours, respectively, while red and blue contours respectively illustrate regions of desirable negative and positive electrostatic interactions.

Several insights into the binding of thrombin inhibitors can readily be observed from the CoMFA map. First, the guanidinium group of the Arg-H side lies within a region of blue contour, indicating that a positive electrostatic field in the inhibitor is important for activity at this location. In addition to the hydrogen-

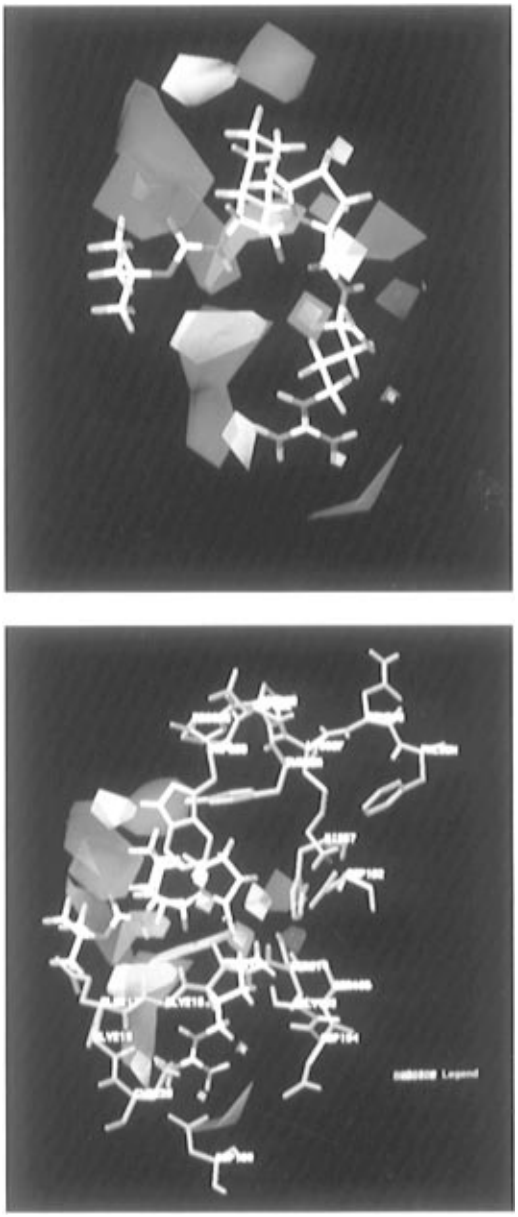


Figure 5. CoMFA contour map and its comparison with the structure of thrombin active site. (a, Top) CoMFA contour map. Sterically favored areas (contribution level of 80%) in green. Sterically unfavored areas (contribution level of 20%) in yellow. Positive potential favored areas (contribution level 80%) in blue. Positive potential unfavored areas (contribution level of 20%) in red. (b, Bottom) Comparison of CoMFA field contribution and the structure of thrombin active site (only main residues of thrombin active site are presented).

bonding noted previously, such an area of positive charge is quite complementary with the negative electrostatic field found in the thrombin active site at Asp 189 (Figure 5). This observation is in agreement with the experimental results of Tapparelli et al.,[4] Bajusz et al.,[23] Fuetani and Matsunaga,[24] and Cheng et al.[25] These groups studied the peptide inhibitors Boc-D-Me_3-SiAla-Pro-boroMpg$C_{10}H_{16}$ (SDZ219349), Z-D-Phe-Pro-boroMpg $C_{10}H_{16}$ (SDZ216744), and D-Dpa-Pro-Mpg^P-$(OPh)_2$ in which the side chain of Arg-H was substituted by a 3-methoxypropyl moiety. As these compounds also exhibited reasonable potency in thrombin inhibition, the conclusion of these studies was that the interaction of a charged guanidino group with Asp 189 through hydrogen bonding was not prerequisite for potent thrombin inhibition. Rather, substitution of Arg side chain by neutral methoxypropyl group exhibited a considerable gain in selectivity due to the increased hydrophobic interaction between the methoxypropyl group and the environment around Asp 189. This conclusion may not be complete, however. The terminus of methoxypropyl group is somewhat positively charged and may contribute to the development of a positive electrostatic field in this region. Consequently, tripeptidyl inhibitors may therefore interact with the S1 pocket not only by hydrogen-bonding interactions but also through both hydrophobic and electrostatic interactions.

Additional electrostatic field complementarity may be seen between the carbonyl group of the Arg-H and Asp102, which is part of the catalytic triad (His57⋯Asp102⋯Ser195)[5] of the serine protease, and between the Gly NH proton of the inhibitor and the side chain of Glu217 (Figure 5).

Beneficial steric effects may be observed near the Gly residue of the inhibitors. The green area indicates that bulky groups added to Gly may help to increase efficacy in the design of new inhibitors. Additionally, since many bulky groups such as *tert*-butyl, cyclohexyl, and phenyl are also hydrophobic, they may also be able to take advantage of the hydrophobic nature of the D-pocket in binding. Therefore we conclude that the CoMFA fields generated from BCSPL-aligned molecules have successfully described the structure of the active site of thrombin, which further serves to illustrate the value of this new computational paradigm for molecular design.

Conclusions

In summary, we have determined the binding conformers of a series of 18 tripeptide thrombin inhibitors employing a new alignment procedure, BCSPL. Results indicate that the binding energies of the inhibitors calculated by this method correlate well with the reported inhibitory activities against thrombin, and the calculation results provide a satisfactory explanation for the binding mechanism of the tested compounds. Additionally, we have undertaken a CoMFA study based on this alignment rule and obtained a predictive 3D-QSAR model. Finally, the resulting CoMFA field map was found to be in agreement with the structure of the active site of thrombin.

While there exist many methods to elucidate the binding conformation of a series of ligands, such as the pioneering active analog approach of marshall[26] or the more recent distance-based methodology of Martin,[27] most procedures have focused on the determination of a pharmacophoric model for the ligands rather than using a receptor site as a guide. In those cases where a three-dimensional structure of a receptor site is known, it has still been difficult to find an appropriate binding conformation for the ligands. The new BCSPL model provides a simple, rapid approach to finding such conformation based in part on the receptor site model. While the results of such a method are not quantitative, and cannot for example provide a relative free energy of binding in ligand−receptor interactions directly, further theoretical calculations such as molecular dynamics or free energy perturbation[28] can benefit from

the initial alignments and complexes predicted by BCSPL. Such a methodology will indeed prove useful in aiding structure-based ligand design as we have shown here.

Supporting Information Available: Structural diagrams of the inhibitor−thrombin complexes and the hydrogen bond lengths between inhibitors and thrombin (20 pages). Ordering information is given on any current masthead page.

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