Structure, Vol. 9, 29–37, January, 2001, ©2001 Elsevier Science Ltd. All rights reserved. PII S0969-2126(00)00551-7

Structural Basis for Inhibition Promiscuity of Dual Specific Thrombin and Factor Xa Blood Coagulation Inhibitors

Herbert Nar,* Margit Bauer, Angela Schmid, Jean-Marie Stassen, Wolfgang Wienen, Henning W.M. Priepke, Iris K. Kauffmann, Uwe J. Ries, and Norbert H. Hauel
Department of Medicinal Chemistry and
Department of Cardiovascular and Metabolic Diseases
Boehringer Ingelheim Pharma KG
88397 Biberach an der Riss
Germany

Summary

Background: A major current focus of pharmaceutical research is the development of selective inhibitors of the blood coagulation enzymes thrombin or factor Xa to be used as orally bioavailable anticoagulant drugs in thromboembolic disorders and in the prevention of venous and arterial thrombosis. Simultaneous direct inhibition of thrombin and factor Xa by synthetic proteinase inhibitors as a novel approach to antithrombotic therapy could result in potent anticoagulants with improved pharmacological properties.

Results: The binding mode of such dual specific inhibitors of thrombin and factor Xa was determined for the first time by comparative crystallography using human α-thrombin, human des-Gla (1–44) factor Xa and bovine trypsin as the ligand receptors. The benzamidine-based inhibitors utilize two different conformations for the interaction with thrombin and factor Xa/trypsin, which are evoked by the steric requirements of the topologically different S2 subsites of the enzymes. Compared to the unliganded forms of the proteinases, ligand binding induces conformational adjustments of thrombin and factor Xa active site residues indicative of a pronounced induced fit mechanism.

Conclusion: The structural data reveal the molecular basis for a desired unselective inhibition of the two key components of the blood coagulation cascade. The 4-(1-methyl-benzimidazole-2-yl)-methylamino-benzamidine moieties of the inhibitors are able to fill both the small solvent accessible as well as the larger hydrophobic S2 pockets of factor Xa and thrombin, respectively. Distal fragments of the inhibitors are identified which fit into both the cation hole/aromatic box of factor Xa and the hydrophobic aryl binding site of thrombin. Thus, binding constants in the medium-to-low nanomolar range are obtained against both enzymes.

Introduction

Activation of the extrinsic or intrinsic pathways of the coagulation cascade leads to the conversion of the zymogen factor X to its activated form, factor Xa. Combined with factor Va and calcium ions on a phospholipid membrane, factor Xa forms the prothrombinase complex which converts prothrombin into thrombin. Thrombin very efficiently initiates fibrin formation, platelet aggregation, and activation of factors V and VIII [1].

Thrombin and more recently, factor Xa have been intensively investigated as targets for antithrombotic therapy [2–7].

Recent preclinical and clinical studies with proteinaceous or small molecule agents have concentrated on selective inhibition of either of the two proteinases [8, 9]. In contrast, anticoagulant strategies of haematophageous animals frequently involve multiple inhibition of the blood coagulation cascade and platelet activation [10–14]. For instance, the soft tick *Ornithodorus moubata* prevents blood clotting through direct inhibition of thrombin by ornithodorin [15], of factor Xa by tick anticoagulant peptide (TAP) [16–18] and inhibition of platelet aggregation by moubatin [19, 20]. Other notorious examples of blood sucking organisms with similar strategies are the medicinal leech Hirudo medicinalis [21] and the Mexican leech Haementeria officinalis [22, 23].

The targeting of several enzymes active within the same physiological pathway therefore appears to be an evolutionarily favored strategy.

Moreover, heparin, a currently widely used anticoagulant, inhibits both thrombin and factor Xa indirectly via complex formation with and modulation of the activity of the serine proteinase inhibitor (serpin) antithrombin III (for review see [24]). This therapy suffers from a number of weaknesses, e.g., lack of oral bioavailability, side effects such as thrombocytopenia, and the necessity of intensive clinical monitoring, most of which are associated with the chemical nature of heparin itself.

A combination of thrombin and factor Xa inhibitory activity in a single, synthetic, orally bioavailable, small molecular weight compound as a novel approach to antithrombotic therapy should therefore result in potent anticoagulants with potentially superior features over currently available therapies.

Thrombin and factor Xa belong to the trypsin family of serine proteinases. Structural data for thrombin [25, 26], factor Xa [27], and trypsin [28] show strong similarities of the S1 specificity pockets of the three enzymes. The S2 and S4 subsites show distinct topologies. While S2 is covered by the 60 insertion loop (the sequence nomenclature used is based on the topological equivalences of the discussed enzymes with chymotrypsinogen [29] and on the thrombin nomenclature suggested by Bode et al. [30]) in thrombin and is hydrophobic in nature, it is smaller and solvent accessible in factor Xa and trypsin. The aryl binding site of thrombin above the conserved residue Trp-215 is lined by residues Leu-99 and Ile-174. In factor Xa, this subsite is built by the

*To whom correspondence should be addressed (email: herbert.nar@bc.boehringer-ingelheim.com).

Key words: blood coagulation; factor Xa; thrombin; crystal structure; inhibitor complexes

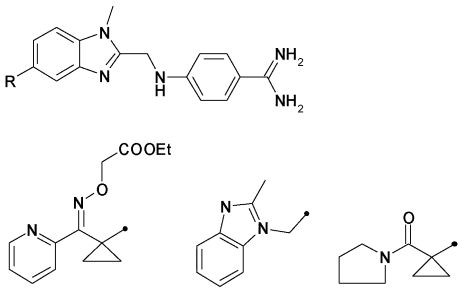

Figure 1. Chemical Structures of the Dual Specific Blood Coagulation Inhibitors Used in this Study

Chemical topology of the 4-(1-methyl-benzimidazole-2-yl)-methylamino-benzamidine scaffold (top) and the distal fragments (bottom) of BIBT0871, BIBR1109 and BIBM1015 (left to right) connected to the scaffold at the position of the substituent R.

corresponding residues Tyr-99 and Phe-174, which together with the indole ring system of Trp-215 form the walls of an aromatic box. In the back of this pocket, amide carbonyl groups from the backbone around residue 99 form a distinct pattern of hydrogen bond acceptors in both enzymes with the thrombin 99-loop being one residue longer than in factor Xa. In the latter enzyme, the 99-loop carbonyls and the side chain of E97 form the so-called cation hole and favor occupation of the aromatic box by basic groups such as amidines or other positively charged substituents [31].

Based on the assumption that simultaneous inhibition of the two key components of the blood coagulation cascade would be therapeutically advantageous and based on the presence of a fair structural similarity of the active sites of the two proteinases, we started a medicinal chemistry program concerned with the search for potent, orally bioavailable, dual specific inhibitors of thrombin and factor Xa.

In our design of these inhibitors, we used 4-(1-methyl-benzimidazole-2-yl)-methylamino-benzamidine as the basic scaffold (Figure 1). This molecular fragment turns out to be ideally suited to meet the steric requirements for fitting equally well into the S1 and S2 pockets of thrombin and factor Xa. The exploration of distal substituents on the inhibitor scaffold fitting into the S4 subsites of both enzymes generated molecules that simultaneously inhibit very efficiently both thrombin and factor Xa. The most potent example of this series, BIBM1015, exhibits inhibition constants in the low nanomolar range.

Here, we describe, for the first time in a comparative fashion, the binding mode of two inhibitors of this familiy in thrombin, factor Xa and trypsin. As it turns out, the molecules bind in two distinct conformations to thrombin and factor Xa/trypsin, respectively. The crystal structures explain the structural cause for the dual inhibition properties and experimentally verify that trypsin may serve as a potent active site surrogate for factor Xa as previously suggested [32].

Results

While for thrombin and trypsin an abundance of cocrystal structures have been reported [33], to date only few crystal structures of active site inhibited factor Xa have been solved and refined at low or medium resolution and published [31, 34, 35].

We have determined crystal structures of two related synthetic inhibitors, BIBT0871 and BIBR1109 (Figure 1), in complex with their target proteinases, human α-thrombin and human des-Gla (1–44) factor Xa, as well as their complexes with bovine trypsin.

The crystal structure analyses were carried out at 1.9–2.0 Å resolution (with the exception of the FXa-BIBR1109 complex at 3.0 Å) resulting in high quality structures with the expected low residual errors for atomic positions (Table 1). The inhibitors are clearly defined in the experimental electron densities in all cases (Figure 2). For BIBT0871, the acetic ester substituent at the oximether is not in contact with the proteins and faces solvent channels in the crystals and is therefore disordered.

General Binding Mode of the Inhibitors

The inhibitors are benzamidine-based compounds that bind to the specificity pocket of all three enzymes in a similar way and form the expected twin-twin interaction of their amidine moiety with the side chain of Asp-189 at the bottom of the S1 subsite. The distal substituents of the inhibitors are aromatic ring systems that were designed to fit into the aryl binding sites of thrombin and factor Xa. There is a N-methyl-benzimidazole scaffold that bridges the benzamidine and the distal aromatic rings that is positioned above the peptide backbone of residues 214–216 and is not able to form the canonical hydrogen bonds with it.

Factor Xa – BIBT0871 Complex

The exocyclic torsion angles within the diatomic bridge connecting the benzamidine and benzimidazole ring systems (at the aniline nitrogen $-17°$, at the carbon atom $101°$) suggest that the inhibitor is bound in a low energy conformation (Figure 3). As a result, the N-H vector is pointing toward the side chain of Ser-195 forming a hydrogen bond interaction with the serine hydroxyl group. The 1-methyl-benzimidazole scaffold lies flat on the protein surface above Gly-216 and places the methyl substituent into the S2-subsite. The S4-subsite of factor Xa is occupied by the pyridinyl moiety of BIBT0871, which is positioned such that it forms an aromatic stacking interaction with Phe-174 (ring distance 3.6 Å) and an edge-on interaction with Trp-215 (distance 3.8 Å).

The structure of the enzyme active site is changed slightly concomittant to inhibitor ligation, suggesting an induced fit type binding mechanism. The factor Xa S1-subsite in the BIBT0871 complex is narrower compared to that of the self-complexed form of factor Xa, which, in the particular tetragonal crystal form described [27], is filled with the C-terminal arginine residue of the EGF2 domain of a neighboring, symmetry equivalent molecule. However, a comparison with the other inhibitor bound structures of factor Xa [31, 34] reveals that the narrow S1-subsite circumference is probably the relaxed conformation and that the rigid body motion of residues 190–194 around the oxyanion hole may be induced by the particular interaction with the EGF2 C-terminal peptide chain in the self-complexed form [27].

Table 1. Data Collection and Refinement Statistics

	Factor Xa		Thrombin		Trypsin	
	BIBT0871	BIBR1109	BIBT0871	BIBR1109	BIBT0871	BIBR1109
Data Collection						
Temperature (K)	100	293	100	100	100	100
Space group	$P2_12_12_1$	$P2_12_12_1$	C2	C2	$P2_12_12_1$	$P2_12_12_1$
Cell, a, b, c (Å)	56.2, 72.7, 76.7	57.4, 73.2, 79.7	69.3, 70.9, 72.8	69.6, 70.9, 72.9	63.2, 69.3, 63.8	63.1, 69.4, 63.8
β (°)			100.3	100.4		
Max resolution	1.9	3.0	2.0	1.9	1.9	1.9
No. of observations	102,600	54,275	53,146	43,458	60,354	75,541
Unique reflections	25,371	6,623	22,489	25,611	21,559	22,243
Completeness (%)	99.3	94.4	94.8	92.2	94.9	97.8
Rm (%)	5.5	12.2	5.5	5.7	4.6	6.9
Refinement						
Resolution range (Å)	20.–1.9	20.–3.0	20.–2.0	20.–1.9	8.–1.9	8.–1.9
No. of reflections	25,049	6,609	22,064	25,246	21,314	21,927
R, RF (%)	23.7/27.2	21.8/27.8	21.0/26.4	20.9/28.9	17.6/22.1	17.4/21.6
Protein atoms	2,267	2,267	2,359	2,359	1,629	1,629
Ca, SO4	1, –	1, –	–, –	–, –	1, 1	1, 1
Inhibitor atoms	38	32	38	32	38	32
Water molecules	213	–	195	313	168	176
Rmsd bonds (Å)	0.007	0.005	0.008	0.009	0.008	0.008
Rmsd angles (°)	1.27	1.53	1.76	1.80	1.78	1.81
Bav ($Å^2$)	37.2	22.0	25.6	28.6	21.4	19.8
Rmsd bonded B ($Å^2$)	1.4	3.9	2.8	2.5	2.2	1.8
PDB entry code	1G2L	1G2M	1G30	1G32	1G34	1G36

To create space for the N-methyl group and the benzene ring of the central benzimidazole scaffold at the S2-subsite, the side chain of Tyr-99 is moved over by a rotation of 55° around its side chain $\chi 2$ torsion angle, which leads to a slight expansion of the S2-subsite. This creates a small depression into which the methyl group perfectly fits.

The 99-loop at the back of the S4-subsite is shifted backward slightly by 1.1 Å generating the space necessary for accommodating the pyridine ring.

Thrombin–BIBT0871 Complex

In contrast to the conformation observed in factor Xa, BIBT0871 utilizes a different conformation about the diatomic bridge connecting the benzamidine and the benzimidazole (Figure 4). Here, the corresponding torsion angles are $-138°$ at the aniline nitrogen and $-16°$ at the carbon atom, relative to the situation in factor Xa a slightly more unfavorable conformation. Consequently, the inhibitor N-H bond vector points toward bulk solvent and the methylene group is placed close to His-57 and Ser-195 on the protein surface. The plane of the aromatic N-methyl-benzimidazole scaffold is oriented perpendicular to the protein surface at Gly-216 and the methyl substituent is positioned between residues His-57, $Tyr\text{-}60^A$, and $Trp\text{-}60^D$ deep within the S2-subsite. The pyridinyl group is centered in the aryl binding pocket where it forms an edge–on aromatic interaction with Trp-215 and alkyl-π-interactions with Ile-174.

Compared to apo-thrombin, pronounced structural rearrangements of the protein are induced by ligand binding. Most prominently, due to the conformation around the aniline nitrogen, the position of the neighboring methylene group forces the side chain of Ser-195 to adopt a different conformation with the hydroxyl group moved by rotation about the $\chi 1$ torsion angle to avoid sterically unfavorable contacts. The His-57 side chain is shifted by 1.1 Å toward the protein interior and thereby conserves its hydrogen bonding with Ser-195. The 60-loop is shifted by about 2 Å toward the catalytic triad residues His-57 and Ser-195 and closes in on the hydrophobic N-methyl-benzimidazole fragment of the inhibitor within the active site cleft of the enzyme. The occupation of the S4 subsite with the pyridyl substituent forces the 99-loop of thrombin to move back slightly with respect to the unliganded form of the proteinase.

Factor Xa/Thrombin–BIBR1109 Complexes

BIBR1109 differs from BIBT0871 in the distal fragment, which is a second 1-substituted benzimidazole system (Figure 1.). In complex with thrombin and factor Xa this inhibitor binds in an identical conformation in both cases with the benzene ring of the benzimidazole occupying the position of the pyridyl ring and the imidazole ring the site of the oximether of BIBT0871 (Figure 2).

Trypsin Complexes

Trypsin has been utilized as a readily crystallizable active site surrogate for factor Xa and other trypsin-like serine proteinases [32, 36, 37]. We have determined the crystal structures of both synthetic inhibitors as being bound to trypsin to check the validity of this approach by direct comparison to the experimentally determined structure of factor Xa in complex with the same inhibitors.

As it turns out, the bound conformations of the inhibitors are identical in trypsin and factor Xa (Figure 5). Here, as in factor Xa, the NH . . . Oγ Ser-195 interaction is formed by adoption of the identical conformation about the diatomic bridge. Structural adaptations of the proteinase upon ligand binding are minimal in trypsin. This may be due to the larger size of its S2-site due to

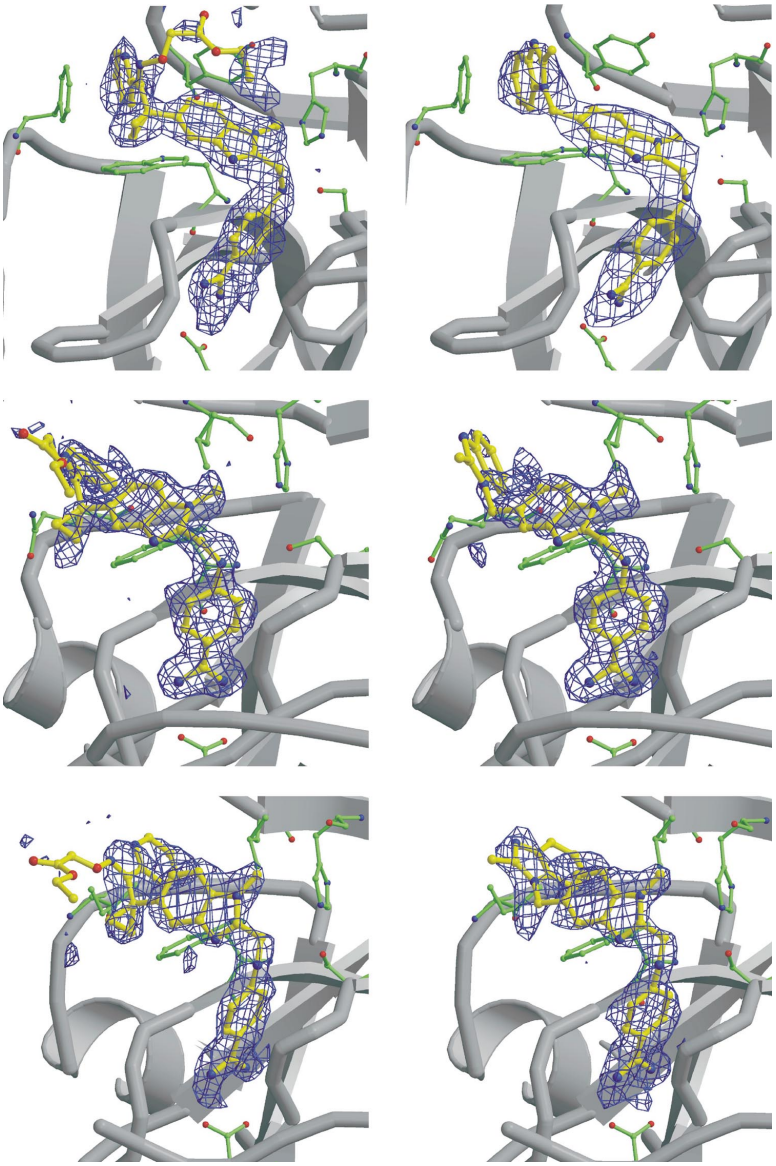

Figure 2. Experimental Evidence for the Bound Conformation of the Ligands to their Protein Receptors

Difference electron density maps (contoured at 2σ) of BIBT0871 (left column) and BIBR1109 (right column) in the active sites of factor Xa, trypsin and thrombin (from top to bottom) superimposed on the final structures.

the absence of Tyr-99 (which is Leu-99 in trypsin) and because the aryl binding site is not confined by spacious residues but rather flat and solvent accessible.

Discussion

Two inhibitors with a mixed inhibition profile toward the blood coagulation proteinases thrombin and factor Xa were cocrystallized with the proteinases thrombin, factor Xa, and trypsin.

The inhibitor structures allow for efficient occupation of the S1- and S4- subsites of the enzymes, however, functional groups for the formation of the canonical hydrogen bonding interactions observed for peptide derived inhibitors [25, 38] are absent. In contrast to published factor Xa specific inhibitors [31], the 1-methylbenzimidazole moiety provides a methyl group for the occupation of the small S2-subsite of this proteinase, which predominantly cleaves peptide sequences with glycine at P2 [1, 39, 40].

The bound conformations of the inhibitors are identical in factor Xa and trypsin but distinct from the binding mode utilized when the inhibitors bind to thrombin (Figure 6).

While the active site of trypsin is rather flat in the S2- and S4-subsite areas and allows for a relaxed bound inhibitor conformation, the clefts of thrombin and factor Xa are rather deep and impose severe restrictions on the available space to be occupied by the inhibitor.

Thus, to occupy the proximal S2-pocket of thrombin with its N-methyl-benzimidazole group while maintaining the favorable ionic interactions in the specificity pocket, the inhibitor has to adopt the observed thrombin bound conformation which places the NH group into bulk solvent without the possibility of forming a hydrogen bond with Ser-195 OH. This conformation of the proximal inhibitor fragment in turn, however, allows an optimal occupation of the distal aryl binding site.

In factor Xa, the main restriction imposed on the inhibitor is the maintenance of the S1-subsite interaction of

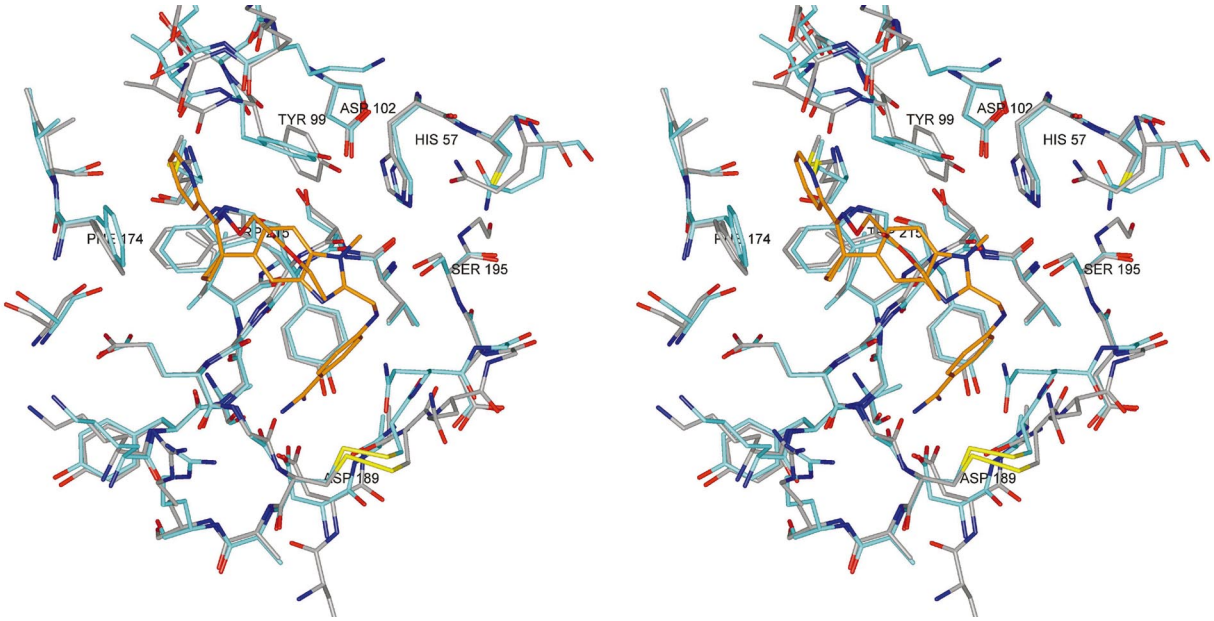

Figure 3. Structure of Ligand Bound Factor Xa and Comparison with the Unliganded Proteinase

Stereo representation of BIBT0871 (orange carbon atoms) in complex with factor Xa (blue carbon atoms) and superimposed on the active site structure of apo-factor Xa (gray carbon atoms). Changes of the protein structure mainly involve a shift of residues 190-194 forming the lower rim of the specificity pocket and side chain reorientations of Tyr-99 and Phe-174. The former may be a results of a distortion of apo-factor Xa in the self-inhibited crystal structure, the latter probably reflects real inhibitor induced changes to create space in the S2-subsite region (Tyr-99) and to optimize aromatic interactions in the S4 subsite (Phe-174).

the benzamidine with the Asp-189 carboxylate and further to put the methyl substituent of the benzimidazole into the S2-subsite as a P2 surrogate. This is possible while maintaining the favorable interaction of the aniline NH with Ser-195 OH. The resulting trajectory for the distal substituent into the aromatic S4-pocket is different from the thrombin case, but seems equally well suited for optimal occupation of this site.

Inhibition constants toward thrombin and factor Xa for the two cocrystallized inhibitors are reported in Table 2. While the Ki's are submicromolar for the target enzymes, no selectivity against trypsin is observed. The

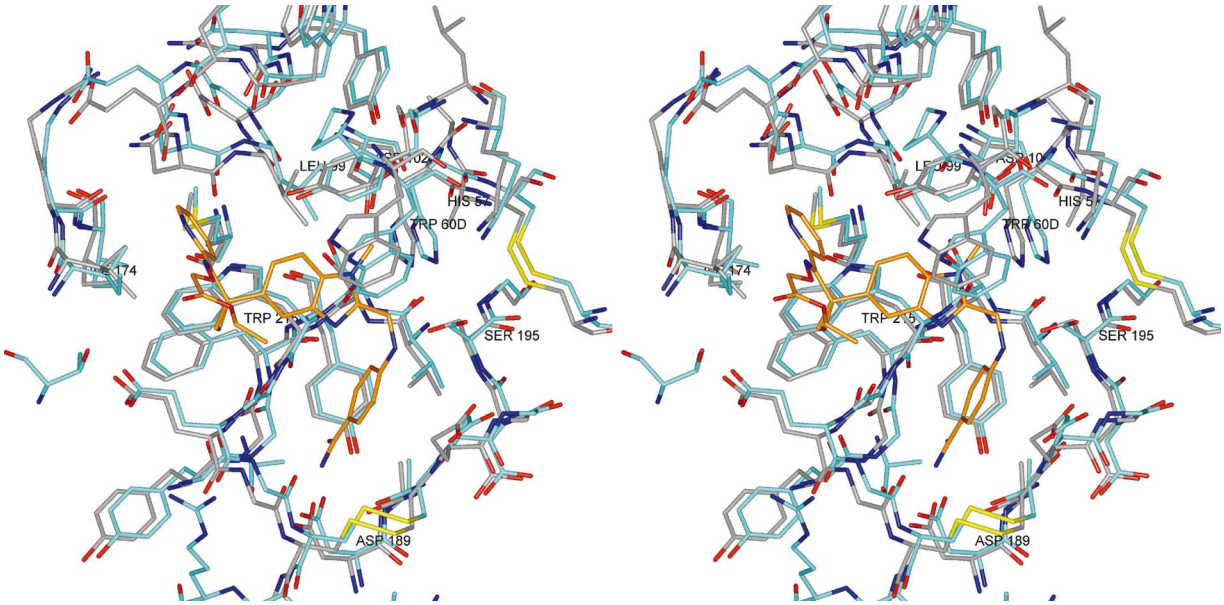

Figure 4. Structure of Ligand Bound Thrombin and Comparison with the Unliganded Proteinase

Stereo representation of BIBT0871 (orange carbon atoms) in complex with thrombin (blue carbon atoms) and superimposed on the active site structure of apo-thrombin (PDB-code: 1HAH; gray carbon atoms). Ligand-induced changes involve rearrangements of the catalytic residues Ser-195 and His-57, a shift of residue Trp-60^D above the inhibitors methyl-benzimidazole moiety and a reorientation of the 99-loop in the back of the S4-subsite.

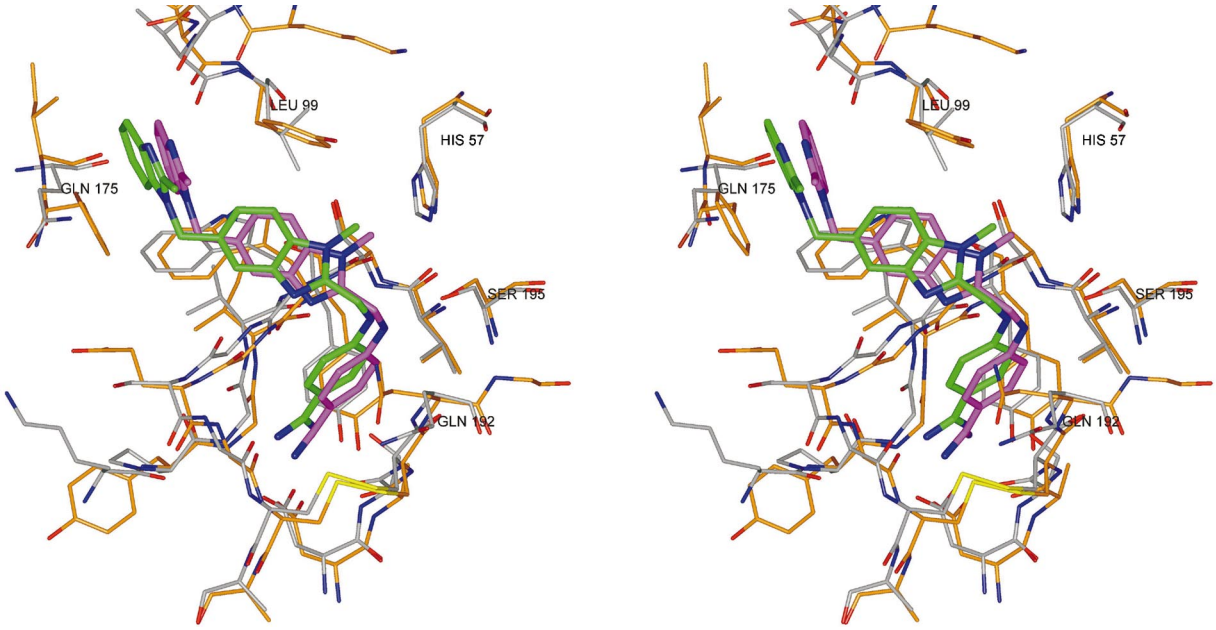

Figure 5. Comparison of the Inhibitor Conformations as Bound to Factor Xa and Trypsin

Stereo representation of BIBR1109 (green carbon atoms) in complex with trypsin (gray carbon atoms) and superimposed on the active site structure of BIBR1109 (magenta carbon atoms) in complex with factor Xa (orange carbon atoms). The inhibitor conformations are identical between the two structures.

observed selectivity of the inhibitors toward the fibrinolytic enzymes (plasmin, urokinase, and tPA) of 2–3 orders of magnitude may be sufficient for excluding any effect compromising their anticoagulant activity in vivo.

The lack of selectivity toward trypsin, present in the gastrointestinal duct, is anticipated to be of no concern for the following reason. The strong hydrophilic character of the compounds results in low oral bioavailabilities of the presented compounds (data not shown). However, modification of the active principle to a prodrug form by chemical masking of the charged groups produces biologically inactive molecules that are resorbed after oral administration. The prodrugs will not interfere with the trypsin activity in the gastrointestinal duct, but are converted to the active compounds by enzymes present in the gastrointestinal wall, blood, or liver.

The factor Xa inhibition was determined to be about 3- to 15-fold more efficient relative to thrombin inhibition. In the light of the described structures, the potency against factor Xa is in part based on a favorable solvent shielded NH–Oγ Ser-195 interaction. This is in line with the observed structure activity relationships for other classes of substituted benzamidines with hydrogen bond donors in the para-position [41]. The potency against thrombin, in contrast, is based mainly on a large area of buried hydrophobic surface with the 1-methyl-benzimidazole fitting snugly into the S2-pocket of the enzyme.

In a search for more potent inhibitors of this family, we were able to identify a suitable substitution of the aromatic residues binding to the S4-pockets of thrombin and factor Xa by a sterically less demanding pyrrolidine amide as realized in BIBM1015 (Figure 1 and Table 2). Due to the conserved 4-(1-methyl-benzimidazole-2-yl)-methylamino-benzamidine moiety, the binding mode of this compound to factor Xa and thrombin is identical to the described binding modes of BIBR1109 and BIBT0871 as shown by its cocrystal structures with thrombin and trypsin (data not shown). BIBM1015 exhibits Ki values of 15 nM for factor Xa and 20 nM for thrombin and represents one of the most potent dual inhibitors reported to date. It serves as a starting point for an optimization program for combined factor Xa/thrombin inhibitors in our group.

The comparative analysis of 3D structures of inhibitors bound to thrombin, factor Xa and trypsin further deepens the understanding of the scope and limitations of the active site surrogate approach for trypsin-like proteinases using trypsin cocrystal structures. They show that for the presented and similar types of synthetic inhibitors, trypsin may indeed be used as an active site model for factor Xa, but it may not yield relevant results for the thrombin bound conformations.

The present work shows that inhibitors exhibiting a mixed activity profile toward thrombin and factor Xa utilize different binding conformations albeit similar molecular interactions with the proteinases. By elucidating these distinct binding modes of identical inhibitors as being bound to the two enzymes, most useful information is obtained for the design of a new generation of potent antithrombotic drugs directed against the two key proteinases of the blood coagulation cascade.

Biological Implications

The search for potent orally bioavailable anticoagulants as effective drugs in thromboembolic disorders such as deep vein thrombosis and in the prevention of venous

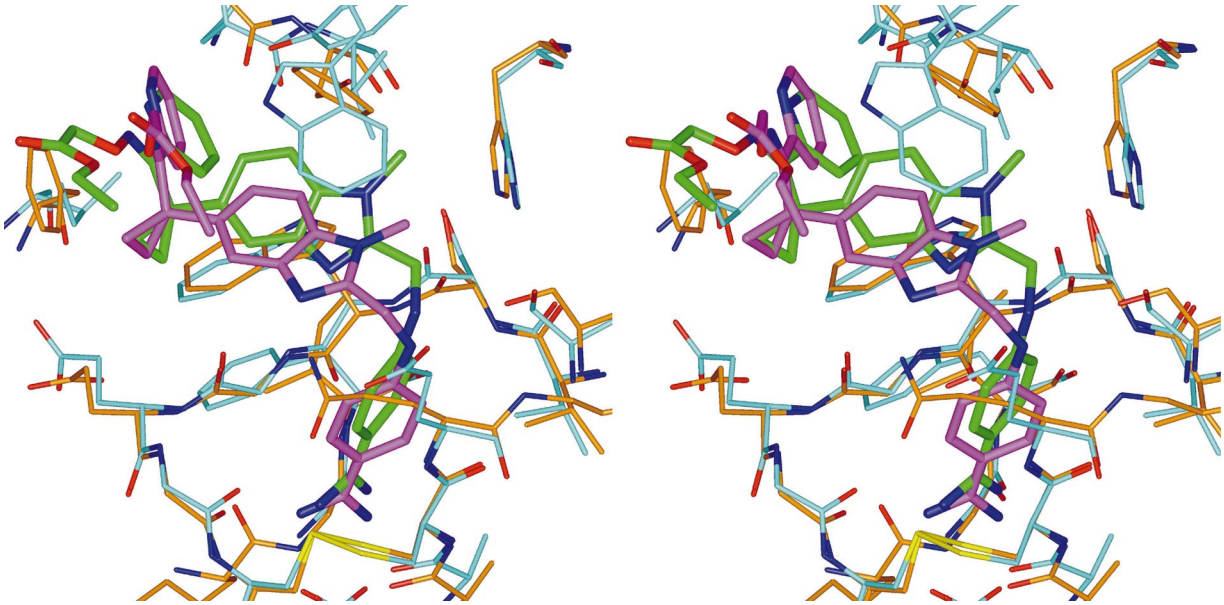

Figure 6. Comparison of the Inhibitor Conformations as Bound to Factor Xa and Thrombin

Stereo representation of BIBT0871 (green carbon atoms) in complex with thrombin (blue carbon atoms) and superimposed on the active site structure of BIBT0871 (magenta carbon atoms) in complex with factor Xa (orange carbon atoms). This superposition reveals the distinct conformations of the inhibitor structures proximal to Ser-195, which places the benzimidazole fragments in two different positions. Nevertheless, the distal aromatic substituents are in very similar positions in the S4-subsite of the two enzymes.

and arterial thrombosis is currently a major focus of pharmaceutical research. In the past, selective inhibitors of thrombin or other actors in the blood coagulation cascade such as factor Xa, factor IXa, or factor VIIa have been sought and, in the case of thrombin and factor Xa, are being studied in the early clinical development phases.

A combination of the effects of thrombin and factor Xa inhibitors as a novel approach to the therapy of thromboembolic disorders appears to be an attractive goal in antithrombotic research for various reasons. First, blood sucking animals have developed strategies to prevent blood clotting by simultaneous inhibition of two or more procoagulatory factors suggesting that mimicking such an evolutionarily developed approach by the invention of synthetic agents with a similar inhibition profile may be similarly successful in therapeutic applications. Second, several effective therapeutic approaches, such as the treatments of HIV (protease and reverse transcriptase inhibitors) [42], hypertension [43], stroke and infections, are based on the administration of combinations of two or more compounds. Third, heparin and low molecular weight heparin, the most important anticoagulatory agents in today's clinical use, represent examples of coagulation inhibitors that combine in themselves multiple mechanisms of action, albeit through an indirect action on thrombin and factor Xa via the serpin antithrombin III. Fourth, a potent direct dual specific inhibitor may be therapeutically effective at lower plasma concentrations compared to selective inhibitors. Finally, a compound that combines two modes of action in a single chemical entity carries additional advantages over the combination of separate compounds by facilitating clinical testing and registratory matters.

The search for anticoagulant drugs has been efficiently guided by the knowledge derived from known 3D structures of the targeted serine proteinases. Based on the similarity of the active site structures of thrombin and factor Xa and based on the above reasoning, we have set out to explore whether it is possible to design

Table 2. Ki, Selectivity Data

Ki (nM)	BIBT0871	BIBR1109	BIBM1015
Factor Xa	57 (3)	40 (1)	15 (1)
Thrombin	140 (10)	780 (50)	20 (2)
Trypsin	110 (5)	67 (3)	102 (10)
Two-chain urokinase	44,000	16,000	6,500
Plasmin	6,800	9,200	13,000
Factor XIa	9,000	4,100	8,200
Factor VIIa/tissue factor	>40,000	>40,000	>40,000
Tissue type plasminogen activator	>50,000	>50,000	>30,000

Data are the Ki mean values and standard deviations (in parentheses) calculated from 12 independent determinations for the target enzymes factor Xa, thrombin and trypsin (6 for the selectivity data against the bottom 5 proteases).

inhibitors that are similarly effective against both enzymes.

Here, we describe the crystal structures of two inhibitors of this research program in complex with thrombin, factor Xa and trypsin, the prototype of this class of trypsin-like serine proteinases which is often used as a model system to study the structures of factor Xa inhibitors as bound to the enzyme active site. This work shows how a particular inhibitor is able to inhibit two distinct albeit related enzymes using two different conformations evoked by the enzyme active site structures. Moreover, the identity of the inhibitor conformations in complex with trypsin and factor Xa is a validation of the active site surrogate approach for this pair of proteinases.

The cocrystal structures provide most useful information for the design of a new generation of more potent orally bioavailable coagulation inhibitors.

Experimental Procedures

Synthesis

BIBT0871, BIBR1109, and BIBM1015 were synthesized according to [44]. The detailed syntheses will be published elsewhere.

Determination of Inhibition Constants

For determination of the inhibition constants (Ki), the inhibition of factor Xa, thrombin, trypsin, two-chain urokinase, plasmin, factor XIa, factorVIIa/tissue factor complex, tissue-type plasminogen activator, was studied at three different substrate concentrations and seven different concentrations of the inhibitors at 37°C and in a final volume of 235 μl in 0.1 mol/l Tris-buffer (pH 8.0) containing 0.15 M sodium chloride and 1 mg/ml human serum albumin (Behringwerke, Marburg, Germany). The inhibitor was first mixed with the enzyme and incubated 10 min before the reaction was started by addition of the respective substrate solution. The absorbance was monitored for 150 s at 30 s intervals in a spectrophotometer (Spectra Max Molecular Devices, Sunnyvale, CA). After subtraction of the background (measured at 620 nm) from the signal (measured at 405 nm) the steady-state reaction rates were used for construction of Dixon plots and the Ki value was calculated.

Enzyme Preparation, Crystallization, Data Collection, and Structure Determination

Human α-thrombin and Factor Xa were obtained from Enzyme Research Laboratories (South Bend, IN, USA). Bovine Trypsin was purchased from Sigma (Deisenhofen, Germany).

Unliganded thrombin was crystallized in complex with the exosite binding hirudin fragment residues 55–65 [45]. Crystals of bovine β-trypsin complexed with benzamidine were grown from small seed crystals of the "open" crystal form [37] in 1.7–1.9 M ammonium sulfate (pH 6). α-Thrombin and β-trypsin cocrystals were generated by soaking crystals with mother liquor containing 1 mM of inhibitor. Factor Xa was proteolytically modified by chymotryptic digestion [46] using agarose immobilized chymotrypsin (Sigma, Deisenhofen, Germany) and crystallized from a solution of 3 mg/mL factor Xa in 10 mM Tris/HCl, 20 mM sodium chloride, 2 mM calcium chloride, pH 7.5 equilibrated against 20% PEG 4000, 0.1 M malate/imidazole, 0.1 M sodium acetate (pH 6.0). Small rod-like crystals appeared within days and grew to a maximal size of 500 × 50 × 50 μm. Data were collected on a MAR Research imaging plate (X-Ray Research, Hamburg, Germany) mounted on a Rigaku RU200 rotating anode generator and processed and scaled with HKL [47]. For the factor Xa-BIBT0871 complex, data collection was performed on the BW6 beamline at DESY, Hamburg. The starting models for structure determination were taken from the PDB, Brookhaven (1MTS for β-trypsin, 1HAH for α-thrombin and 1FAX for factor Xa). Model building and refinement were carried out with MAIN [48] and either REFMAC [49], X-PLOR [50], or CNS [51].

Data collection and refinement parameters are summarized in Table 1. The coordinates and structure factors have been deposited with the Protein Data Bank (PDB entry codes are listed in Table 1).

Acknowledgments

We thank Hilde Wiedenmayer, Ingrid Christ, Josef Eiband, and Georg Schadler for their excellent technical assistance. We greatly acknowledge the help of Hans Bartunik for access to and during data collection on the MPG/GBF wiggler beamline BW6/DORIS at DESY.

Received: July 31, 2000
Revised: October 26, 2000
Accepted: November 3, 2000

References

1. Davie, E.W., Fujikawa, K., and Kisiel, W. (1991). The coagulation cascade: initiation, maintenance, and regulation. Biochemistry *30*, 10363–10370.
2. Komiyama, Y., Pedersen, A.H., and Kisiel, W. (1990). Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. Biochemistry *29*, 9418–9425.
3. Sanderson, P.E., and Naylor-Olsen, A.M. (1998). Thrombin inhibitor design. Curr. Med. Chem. *5*, 289–304.
4. Leung, D., Abbenante, G., and Fairlie, D.P. (2000). Protease inhibitors: current status and future prospects. J. Med. Chem. *43*, 305–341.
5. Al-Obeidi, F., and Ostrem, J.A. (1999). Factor Xa Inhibitors. Exp. Opin. Ther. Patents *9*, 931–953.
6. Walenga, J.M., Jeske, W.P., Hoppensteadt, D., and Kaiser, B. (1999). Factor Xa inhibitors: Today and beyond. Curr. Opin. Cardiovascular. Pulmonary & Renal Investigational Drugs *1*, 13–28.
7. Ewing, W.R., Pauls, H.W., and Spada, A.P. (1999). Progress in the design of inhibitors of coagulation factor Xa. Drugs of the Future *24*, 771–787.
8. Stuerzebecher, J., et al. (1989). Synthetic inhibitors of bovine factor Xa and thrombin: Comparison of their anticoagulant efficiency. Thromb. Res. *54*, 245–252.
9. Kaiser, B. (1997). Factor Xa versus factor IIa inhibitors. Clin. Appl. Thrombosis/Hemostasis *3*, 16–24.
10. Cupp, E.W., Cupp, M.S., Ribeiro, J.M., and Kunz, S.E. (1998). Blood-feeding strategy of Haematobia irritans (Diptera: Muscidae). J. Med. Entomol. *35*, 591–595.
11. Markwardt, F. (1994). Coagulation inhibitors from blood-sucking animals. A new line of developing antithrombotic drugs. Pharmazie *49*, 313–316.
12. Stassens, P., et al. (1996). Anticoagulant repertoire of the hookworm Ancylostoma caninum. Proc. Natl. Acad. Sci. USA *93*, 2149–2154.
13. Stark, K.R., and James, A.A. (1996). Salivary gland anticoagulants in culicine and anopheline mosquitoes (Diptera:Culicidae). J. Med. Entomol. *33*, 645–650.
14. Rigbi, M., Orevi, M., and Eldor, A. (1996). Platelet aggregation and coagulation inhibitors in leech saliva and their roles in leech therapy. Semin. Thromb. Hemost. *22*, 273–278.
15. van de Locht, A., et al. (1996). The ornithodorin-thrombin crystal structure, a key to the TAP enigma? EMBO J. *15*, 6011–6017.
16. Wei, A., et al. (1998). Unexpected binding mode of tick anticoagulant peptide complexed to bovine factor Xa. J. Mol. Biol. *283*, 147–154.
17. Waxman, L., Smith, D.E., Arcuri, K.E., and Vlasuk, G.P. (1990). Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. Science *248*, 593–596.
18. Vlasuk, G.P. (1993). Structural and functional characterization of tick anticoagulant peptide (TAP): a potent and selective inhibitor of blood coagulation factor Xa. Thromb. Haemost. *70*, 212–216.
19. Waxman, L., and Connolly, T.M. (1993). Isolation of an inhibitor selective for collagen-stimulated platelet aggregation from the soft tick Ornithodoros moubata. J. Biol. Chem. *268*, 5445–5449.
20. Keller, P.M., et al. (1993). Cloning of the cDNA and expression

of moubatin, an inhibitor of platelet aggregation. J. Biol. Chem. *268*, 5450–5456.
21. Eldor, A., Orevi, M., and Rigbi, M. (1996). The role of the leech in medical therapeutics. Blood Rev. *10*, 201–209.
22. Lapatto, R., et al. (1997). X-ray structure of antistasin at 1.9 A resolution and its modelled complex with blood coagulation factor Xa. EMBO J. *16*, 5151–5161.
23. Theunissen, H.J., et al. (1994). Mutational analysis of antistasin, an inhibitor of blood coagulation factor Xa derived from the Mexican leech Haementeria officinalis. Thromb. Res. *75*, 41–50.
24. Stubbs, M.T., and Bode, W. (1995). The structural basis for thrombin inhibition by hirudin and serpins. In Thrombin. Buchanan, M.R., Brister, S.J., Ofosu, F. A., eds. (Boca Raton: CRC), pp. 19–51.
25. Bode, W., Turk, D., and Karshikov, A. (1992). The refined 1.9-A X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human alpha-thrombin: structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. Protein Sci. *1*, 426–471.
26. Stubbs, M.T., and Bode, W. (1993). A player of many parts: The spotlight falls on thrombin's structure. Thromb. Res. *69*, 1–58.
27. Padmanabhan, K., et al. (1993). Structure of human Des(1–45) factor Xa at 2.2-A resolution. J. Mol. Biol. *232*, 947–966.
28. Bode, W., and Schwager, P. (1975). Single calcium-binding site of crystalline bovine beta-trypsin. FEBS Lett. *56*, 139–143.
29. Hartley, B.S., and Shotton, D.M. (1971). The Enzymes, (P.D. Boyer, ed.), Academic Press, New York and London.
30. Bode, W., et al. (1989). The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J. *8*, 3467–3475.
31. Brandstetter, H., et al. (1996). X-ray structure of active site-inhibited clotting factor Xa. Implications for drug design and substrate recognition. J. Biol. Chem. *271*, 29988–29992.
32. Stubbs, M.T., Huber, R., and Bode, W. (1995). Crystal structures of factor Xa specific inhibitors in complex with trypsin: structural grounds for inhibition of factor Xa and selectivity against thrombin. FEBS Lett. *375*, 103–107.
33. Vacca, J.P. (1998). Thrombosis and coagulation. Annu. Rep. Med. Chem. *33*, 81–90.
34. Kamata, K., Kawamoto, H., Honma, T., Iwama, T., and Kim, S.H. (1998). Structural basis for chemical inhibition of human blood coagulation factor Xa. Proc. Natl. Acad. Sci. USA. *95*, 6630–6635.
35. Maignan, S., et al. (2000). Crystal structures of human factor Xa complexed with potent inhibitors. J. Med. Chem. *43*, 3226–3232.
36. Turk, D., Sturzebecher, J., and Bode, W. (1991). Geometry of binding of the N alpha-tosylated piperidides of m-amidino-, p-amidino- and p-guanidino phenylalanine to thrombin and trypsin. X-ray crystal structures of their trypsin complexes and modeling of their thrombin complexes. FEBS Lett. *287*, 133–138.
37. Bode, W., Turk, D., and Sturzebecher, J. (1990). Geometry of binding of the benzamidine- and arginine-based inhibitors N a lpha-(2-naphthyl-sulphonyl-glycyl)-DL-p-amidinophenylalanyl-pipe ridine (NAPAP) and (2R,4R)-4-methyl-1-[N alpha-(3-methyl-1,2,3,4-tetrahydro-8- quinolinesulphonyl)-L-arginyl]-2-piperidine carboxylic acid (MQPA) to human alpha-thrombin. X-ray crystallographic determination of the NAPAP-trypsin complex and modeling of NAPAP-thrombin and MQPA-thrombin. Eur. J. Biochem. *193*, 175–182.
38. Wu, T.P., et al. (1993). The structure of a designed peptidomimetic inhibitor complex of alpha-thrombin. Protein Eng. *6*, 471–478.
39. Matthews, D.J., and Wells, J.A. (1993). Substrate phage: selection of protease substrates by monovalent phage display. Science *260*, 1113–1117.
40. Harris, J.L., et al. (2000). Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc. Natl. Acad. Sci. USA *97*, 7754–7759.
41. Choi-Sledeski, Y.M., et al. (1999). Sulfonamidopyrrolidinone factor Xa inhibitors: potency and selectivity enhancements via P-1 and P-4 optimization. J. Med. Chem. *42*, 3572–3587.
42. Wiznia, A., et al. (2000). Combination nucleoside analog reverse transcriptase inhibitor(s) plus nevirapine, nelfinavir, or ritonavir in stable antiretroviral therapy- experienced HIV-infected children: week 24 results of a randomized controlled trial—PACTG 377. Pediatric AIDS Clinical Trials Group 377 Study Team. AIDS Res. Hum. Retroviruses *16*, 1113–1121.
43. Taylor, A.A., and Sunthornyothin, S. (1999). The case for combining angiotensin-converting enzyme inhibitors, and calcium-channel blockers. Curr. Hypertens. Rep. *1*, 446–453.
44. Ries, U., et al. (2000). Preparation of amidinophenylaminomethylenzimidazoles as antithrombotics. *PCT Int. Appl. WO 0001704* 140 pp.
45. Skrzypczak-Jankun, E., et al. (1991). Structure of the hirugen and hirulog 1 complexes of alpha-thrombin. J. Mol. Biol. *221*, 1379–1393.
46. Skogen, W.F., Esmon, C.T., and Cox, A.C. (1984). Comparison of coagulation factor Xa and des-(1–44) factor Xa in the assembly of prothrombinase. J. Biol. Chem. *259*, 2306–2310.
47. Gewirth, D. (1994). The HKL Manual, 3rd Edition, Yale University, New Haven CT.
48. Turk, D. (1992). Weiterentwicklung eines Programms fuer Molekuelgraphik und Elektronendichte Manipulation und seine Anwendung auf verschiedene Protein-Strukturaufklaerungen, TU Muenchen, Muenchen.
49. CCP4. (1994). The CCP4 Suite: programs for protein crystallography. Acta Crystallogr. D *50*, 760–763.
50. Bruenger, A. (1988). X-PLOR, Version 3.1, A System for X-Ray Crystallography and NMR, Yale University Press, New Haven and London.
51. Brunger, A.T., et al. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. D Biol Crystallogr *54*, 905–921.