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Leech-Derived Thrombin Inhibitors: From Structures to Mechanisms to Clinical Applications

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Introduction

The European jawed leech *Hirudo medicinalis* and its congeners (*H. verbana*, *H. troctina*, *H. orientalis*, and *H. decora*) have a millennial history of application in the medical praxis; evidence from a painting found in an Egyptian tomb indicated its therapeutic use back to at least 1500 B.C.E.[1] In particular, documented use of leeches in bloodletting goes back to the first century B.C.E. and was boosted by the classical work of the Arabic physician, Avicenna ("The Canon of Medicine"). Leeches were extensively employed in the medieval and early modern medicine and reached their zenith in the 1820s and 1830s; millions of leeches were required annually in Paris alone. The treatment, currently known as hirudotherapy, is experiencing a renaissance nowadays, in particular because of its applications in plastic and reconstructive surgery.[2,3]

The presence of an anticoagulant activity in the saliva of *H. medicinalis* was first described in 1884 by the British physiologist John B. Haycraft, who noticed that "the blood flowing from a leech bite is not readily stopped, often flowing for upwards of an hour after the animal has been removed. The blood within the body of the creature remains fluid for an indefinite time, and when ejected it is found to have lost its coagulability."[4] His work was eventually followed by the preparation of a concentrate that contained the leech anticoagulant activity, termed hirudin, by the German pharmacologist Carl Jacobj.[5] More than half a century later, a small protein that preserved the name was isolated from these extracts and became with time the natural thrombin inhibitor par excellence. (There are actually several hirudin isoforms of about 65 residues; e.g., see refs 6−8 and Figure 1 for a sequence alignment of reported hirudin variants).

Purification, preliminary biochemical characterization, and determination of the amino acid sequence of hirudin by Fritz Markwardt and co-workers in the late 1950s[9,10] and complete elucidation of its covalent structure by Dodt and co-workers[6] led to a biotechnological enterprise that ultimately allowed production of recombinant hirudin and in vivo assessment of its potential as an antithrombotic drug (see below and refs 11 and 12 for recent historical reviews on hirudin and its development for clinical applications).

On the other hand, the inhibitor was thoroughly characterized in several laboratories by biochemical and biophysical means, including determination of NMR structures in solution of both natural and recombinant variants.[13−15] These investigations revealed that hirudin comprises an N-terminal globular domain (residues 1I−49I) stabilized by three disulfide bonds with [1−2, 3−5, 4−6] connectivity, which spontaneously folds in solution.[16] This compact domain is C-terminally extended by a short acidic tail that lacks cysteine residues and is essentially disordered in solution.

The current review summarizes current structural and functional knowledge on hirudin (clan IM, family I14 in the MEROPS classification) and its mechanism of thrombin inhibition. We also discuss structural and functional data on a distantly related inhibitor from family I14, haemadin from the land-living leech *Haemadipsa sylvestris*, as well as the structurally and functionally unrelated inhibitor from the aquatic leech *Theromyzon tessulatum*, theromin (clan IO, family I15). The reader is referred to an excellent recent review for a summary of inhibition strategies employed by antihemostatic factors from hematophagous animals.[17]

Hirudin Is a Noncanonical Inhibitor of Thrombin

Determinations of the crystal structures of human thrombin−hirudin complexes in the early 1990s (mutant Asn^{47I}→Lys from hirudin variant 2[18,19] and from hirudin variant 1, ref 20) were milestones in the investigation not only of thrombin inhibition but of inhibitor−peptidase interactions in general (Figure 2A). This was followed shortly afterward by the structural characterization of the inhibitor complex with the bovine proteinase.[21] The structural work revealed that, against expectations,[22−24] hirudin does not use a basic side chain such as Lys^{47I} to interact with the negatively charged S_1 pocket of the proteinase (Figure 2B). This finding is fully supported by a number of mutagenesis studies,[23,25,26] which are summarized in Supporting Information Table 1. (We employ the standard Schechter and Berger nomenclature: P_m, ..., P_1, P_1', ..., P_n' design substrate/inhibitor residues from N- to C-terminus, where the scissile peptide bond connects residues P_1 and P_1' and where S_m, ..., S_1, S_1', ..., S_n' indicate the cognate (sub)sites on the proteinase surface.)

Instead, direct blockade of thrombin active site is achieved by the rather hydrophobic N-terminal inhibitor residues, Val^{1I} (Ile^{1I}), Val^{2I} (Thr^{2I}) (for variants 1 and 2, respectively), and Tyr^{3I}, which are almost completely buried in the complex. In a further departure from expectations, these residues do not align antiparallel to segment Ser^{214}−Gly^{216}, as previously observed in other serine proteinase−inhibitor complexes (termed canonically binding inhibitors), but form a parallel strand with the backbone atoms of residues Ser^{214}−Gly^{219} instead (Figure 2B; throughout this work we use the trypsin-based numbering system for catalytic domains of serine proteinases). This represented the first experimental observation of a reversible,

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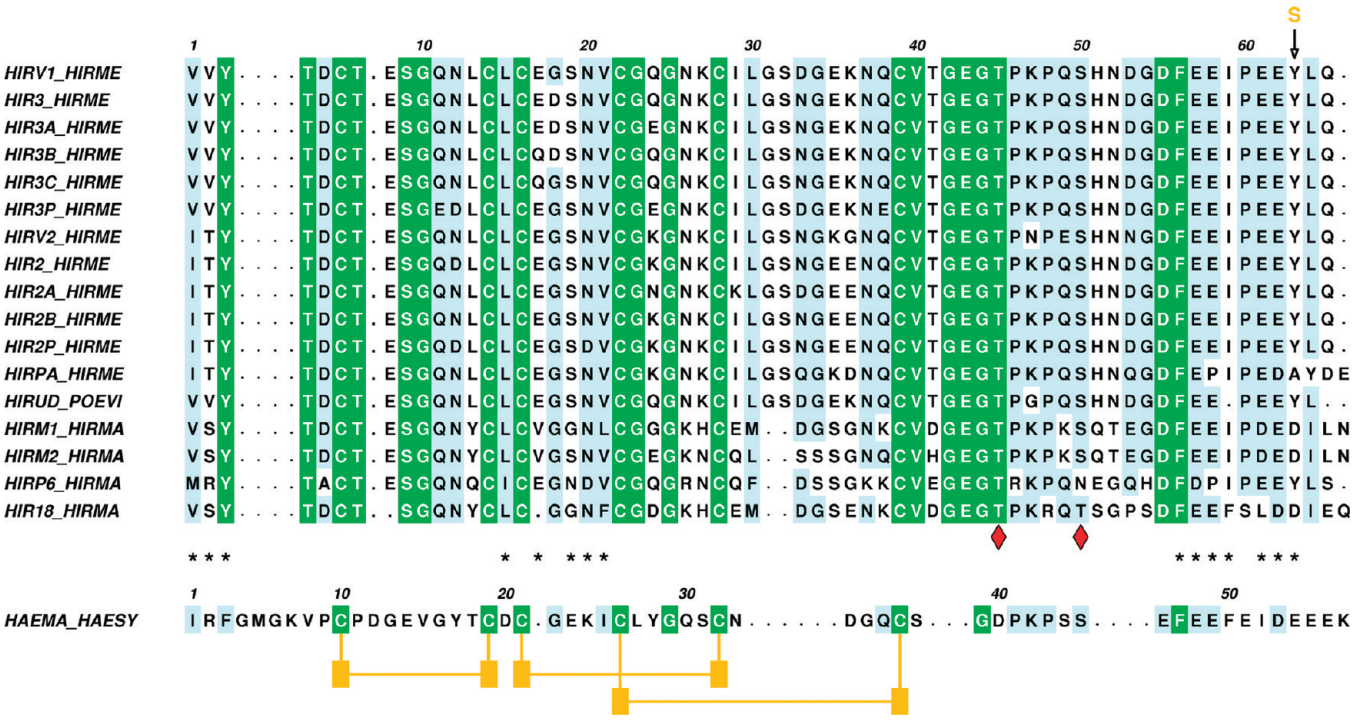

Figure 1. Structure-based sequence alignment of inhibitors that belong to MEROPS family I14. The alignment includes the sequences of all isohirudins from the European medicinal leech *Hirudo medicinalis*, the Indian freshwater leech *Poecilobdella viridis*, and the Asian buffalo leech *Hirudinaria manillensis*, as deposited with the UniProt database. These sequences are compared to haemadin from the land-living Indian leech *Haemadipsa sylvestris*. Residue numbers for both *H. medicinalis* hirudin and haemadin are given, and disulfide bonds are indicated below the alignment. Strictly conserved residues are white with green shading, and conservatively replaced ones are shaded light-blue. Residues involved in important contacts with thrombin are marked with stars. A letter "S" marks the tyrosine residue (Tyr^{63I}) that is commonly sulfated in natural hirudin, and O-glycosylation sites are indicated with red diamonds.

tight-binding peptidase inhibitor that does not follow the "standard" mechanism of proteinase inhibition.

The extended conformation adopted by the three N-terminal hirudin residues allows the side chains of Ile^{1I} (Val^{1I}) and Tyr^{3I} to occupy regions that roughly correspond to the S_2 and S_4 thrombin subsites, respectively, while the second residue approaches the entrance of the S_1 pocket. The free amino terminus of hirudin donates in addition strong H-bonds to the side chains of two catalytic residues (the Oγ atom of Ser^{195} and His^{57} Nε2) and to the carbonyl oxygen of Ser^{214}. These structural findings explained a number of previous functional observations, such as the inactivity of a recombinant hirudin variant with an eight-residue N-terminal extension,[27] the extraordinarily reduced affinity of molecules in which the start Met is not removed, or when another residue is added before the natural N-terminus,[28,29] and the relevance of a positively charged, free amino terminal end for thrombin binding.[29] This is in addition to several site-directed mutagenesis studies of hirudin N-terminal peptide,[29−36] which are discussed below in connection with the mechanism of thrombin inhibition by another leech-derived inhibitor from family I14, haemadin. Altogether, interactions mediated by the first three N-terminal residues represent ~30% of the binding energy[32] and allow anchoring of this peptide across the active site cleft in spite of suboptimal usage of the specificity-determining S_1 subsite.

Several additional interactions between thrombin and the hirudin core domain, in particular residues Leu^{15I}−Val^{21I}, significantly contribute to strengthening complex formation, bringing about one-third of the binding energy.[37] For instance, both Asp^{5I} and Ser^{19I} are hydrogen-bonded to Arg^{221A}, while the carboxylate of Glu^{17I} approaches the guanidinium group of Arg^{173}, and Val^{21I} engages in important van der Waals interactions with the side chains of Ile^{174} and Glu^{217}. The work of Stone and co-workers revealed that the aliphatic Leu^{15I} side chain is particularly important for thrombin binding: it stabilizes residues from both the N-terminal inhibitor peptide (Tyr^{3I}) and the Cys^{16I}−Cys^{22I} loop (Val^{21I}) and contacts also the side chain of Arg^{221A}. Not surprisingly, these Leu^{15I}-mediated interactions contribute significantly to the stability of the complex; interactions with the side chains of Glu^{17I}, Asn^{20I}, and Val^{21I} are also relevant for thrombin binding (Supporting Information Table 1).

Finally, the C-terminal hirudin peptide (sometimes termed hirugen) binds in a rather extended conformation across a surface area in thrombin enriched in basic residues, commonly termed anion-binding exosite I or fibrinogen (Fbg) recognition exosite (Figure 2C). This interaction has to be expected from the complementarity of electrostatic surface potentials and was predicted from studies with truncated variants and synthetic peptides, as well as from the identification of accessible lysine residues in the complex.[38−40] It is important to note that most natural hirudin variants are sulfoproteins, in which the side chain of Tyr^{63I} is sulfated.[41] The high inhibition constant of about 20 fM measured for natural hirudin[42] depends on this post-translational modification; desulfated hirudin and non-sulfated recombinant forms possess 10-fold lower affinities for thrombin[23,25,26,42] and accordingly lower activities in APTT[a]

[a]Abbreviations: ACS, acute coronary syndromes; APTT, activated partial thromboplastin time; DVT, deep vein thrombosis; Fbg, fibrinogen; FXa, activated factor X; HSA, human serum albumin; HIT, heparin-induced thrombocytopenia; PAR, protease activated receptor; STA, staphylokinase; tPA, tissue plasminogen activator; TT, thrombin time.

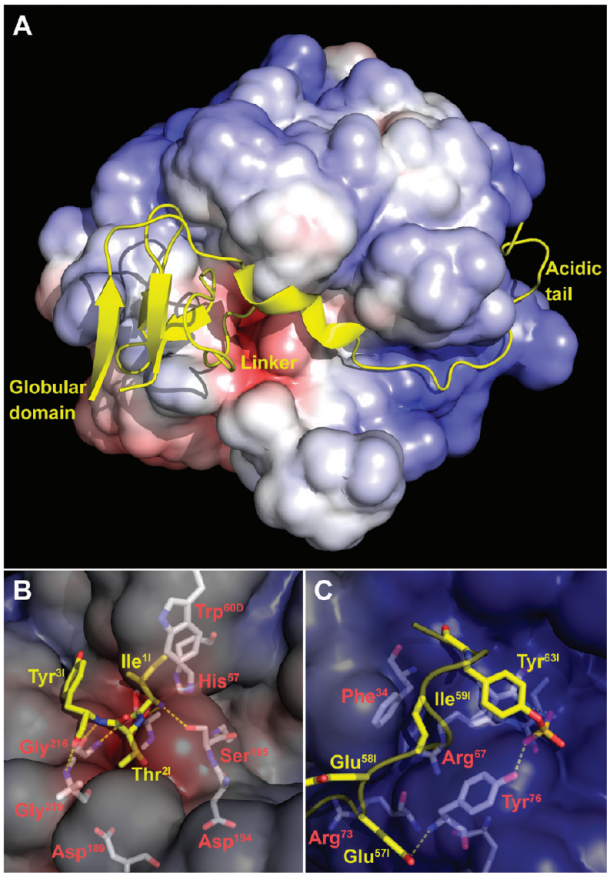

Figure 2. Three-dimensional structure of thrombin complex with hirudin. (A) Schematic representation of the overall structure of the thrombin−hirudin complex (PDB entry 4HTC). Thrombin is represented by its electrostatic surface potential, while hirudin is shown as a yellow ribbon. The major structural elements of the inhibitor molecule are labeled. Notice that the globular domain covers the active site cleft of the proteinase, while the C-terminal tail runs along the surface of the basic exosite I. (B) Close-up of thrombin active site region in complex with hirudin (PDB entry 4HTC). Only the N-terminal inhibitor tripeptide is shown for simplicity, along with a few thrombin side chains. Notice that the thrombin S_1 pocket and in particular Asp^{189} are not involved in important interactions with the inhibitor, but areas that roughly correspond to subsites S_2 and S_4 are occupied by the side chains of Ile^{1I} and Tyr^{3I}, respectively. (C) Close-up of thrombin exosite I, showing interacting residues from the acidic tail of sulfohirudin (PDB entry 2PW8).

and TT assays.[43] A recent crystal structure of thrombin bound to a biosynthetic, sulfated hirudin variant shows that the increased affinity of sulfohirudin for thrombin is due to a strong salt bridge with the ε-amino group of Lys^{81} and an extended hydrogen-bond network that links sulfate oxygen atoms with the hydroxyl group of Tyr^{76} and, through bridging water molecules, to main chain atoms of Asn^{78} and Glu^{80} (Figure 2C).[44]

The most important ionic contributions to complex formation involve this C-terminal peptide, which accounts for about 32% of the binding energy at zero ionic strength.[45] Mutagenesis studies have shown that all the negatively charged residues from the C-terminal tail are relevant for complex formation (refs 25, 46, and 47 and Supporting Information Table 1); they appear to be essential for orienting the inhibitor toward the basic exosite I. In addition to contacts mediated by sulfated Tyr^{63I}, a solvent-exposed ion pair is formed between the strictly conserved Asp^{55I} carboxylate and the basic side chain of Arg^{73}. However, and somehow counterintuitive, additional direct electrostatic interactions are scarce at this hirudin−exosite I interface. In the complex with hirudin variant 2, Glu^{49I} engages in a salt bridge with the side chain of Lys^{60F}, while the imidazole moiety of His^{51I} might partially compensate the negative charge of Glu^{39} (ref 19). Position 49 is almost invariably occupied by a polar but not charged glutamine (Figure 1), and replacement of Gln^{49I} by the charged glutamate did not improve the kinetic constants of hirudin variant 1.[37] Further, His^{51I} is not defined by electron density in the thrombin−sulfohirudin structure.[44] Thus, the general relevance of these contacts for thrombin recognition is questionable. Residues Glu^{61I} and Glu^{62I} even point away from the thrombin surface, while Glu^{57I} and Glu^{58I}, although directed toward the basic side chains of Arg^{75} and Arg^{77A}, do not engage in direct salt bridges with these critical exosite I residues. Nevertheless, a recent analysis of electrostatic interactions using point mutants of both thrombin and hirudin indicates that Glu^{57I} interacts directly with Arg^{75}/Arg^{77A} in the thrombin−hirudin complex,[46] as previously suggested.[19] This scarcity of direct polar interactions contrasts with the multiple contacts made by the aromatic phenyl moiety of the strictly conserved Phe^{56I}, which occupies a shallow apolar depression at the "lower" end of exosite I essentially spanned by side chains of residues Met^{32}, Phe^{34}, Leu^{40}, Arg^{67}, and Arg^{73}. The important role of this conserved residue for thrombin binding has been confirmed in several investigations.[48−51] Another hydrophobic hirudin residue, Ile^{59I}, engages in van der Waals interactions with Gln^{38}, Leu^{40}, and Tyr^{76}; the relevance of these interactions was also demonstrated in studies with synthetic peptides[48] and site-directed mutagenesis.[51]

Of particular note for later pharmacological applications (discussed below), it was demonstrated that the C-terminal peptides of different hirudin variants are able to inhibit various thrombin functions such as Fbg clotting,[48,50,52−54] PAR-mediated platelet activation,[55,56] and binding to the endothelial receptor, thrombomodulin.[57]

The structural and mutagenesis studies mentioned above along with intrinsic fluorescence measurements[46,58] have allowed dissection of the mechanism of hirudin binding to thrombin. Briefly, complex formation entails (1) steering of the inhibitor toward thrombin exosite I, mediated by its electrostatic complementarity with hirudin C-terminal tail. This is followed by (2) binding of this acidic peptide across exosite I through specific ionic but in particular multiple van der Waals interactions. This rate-limiting step appears to require and/or induce conformational changes in thrombin,[50,59] which probably include large rearrangements of the 149 (or "autolysis") loop.[19] Accordingly, hirudin behaves as a slow-binding inhibitor in high ionic strength solutions.[42] Formation of this initial complex (3) locates the N-terminal hirudin tripeptide at an appropriate distance and conformation to allow rapid insertion into the active site cleft of the proteinase, supported by additional interactions of core domain residues.

Haemadin from the Land-Living Indian Leech Targets Thrombin Exosite II

The success of preliminary clinical investigations with *H. medicinalis* hirudin spurred the search for novel anticoagulants from other related jawed leeches. This led to the discovery and characterization of four hirudin variants from the "buffalo leech" *Hirudinaria manillensis*, which is more specialized for mammalian parasitism: HM1, HM2 (also known as bufrudin), P6, and P18 (hirullin P18).[60−66] At the sequence level, these "isohirudins" show only minor differences to

hirudin variants from *H. medicinalis* (Figure 1), most notably post-translational modification of Thr^{45I} or Thr^{50I} with a short linear, O-linked trisaccharide Fuc-Galβ(1→3)GalNAc (numbering for *H. medicinalis* hirudin).[63] These isohirudins are thus expected to bind thrombin essentially as observed in all reported crystal structures;[18−20,44] slight differences in the main chain conformation of the C-terminal tail might accommodate, for example, a smaller aspartate found instead of the sulfated Tyr^{63I} residue in some *H. manillensis* isoforms. The same considerations apply to the hirudin variant isolated from the Indian freshwater leech, *Poecilobdella viridis*.[67]

In light of this remarkable similarity of isohirudins, it came as a surprise when Strube and co-workers reported data on a novel, tight-binding and specific thrombin inhibitor ($K_i \approx 100$ fM) that had been isolated from the land-living Indian leech *Haemadipsa sylvestris*.[68] The protein, termed haemadin, has 57 amino acid residues and is thus notably smaller than all hirudin variants (Figure 1). Most unexpectedly, haemadin's amino acid sequence did not display significant similarities to other peptidase inhibitors and was in particular only marginally related to hirudin. In spite of the lack of important sequence similarity, haemadin shares with hirudin the same overall domain organization: a globular N-terminal domain stabilized by three disulfide bridges, followed by a highly acidic C-terminal peptide. Therefore, it was assumed that haemadin-mediated thrombin inhibition would represent a simple variation of the previously observed noncanonical mechanism exhibited by hirudin: insertion of N-terminal residues into the proteinase active-site cleft and occupancy of exosite I by the C-terminal tail.

Determination of the crystal structure of the human thrombin−haemadin complex revealed that only the first half of this prediction was right (Figure 3).[69] Indeed, the N-terminal haemadin peptide binds in a noncanonical manner across the active site of the proteinase, with residue Ile^{1I} occupying the S_2 subsite, while its free N-terminus donates H-bonds to both Ser^{214} carbonyl oxygen atom and His^{57} Nε2. However, in contrast to hirudin, Arg^{2I} in haemadin inserts deeply into the S_1 specificity pocket and engages not only in a direct salt bridge with thrombin's Asp^{189} carboxylate but also in hydrogen bonds to the main chain oxygen atoms of Ala^{190} and Gly^{219}. These features would result in a higher affinity of haemadin core domain for the proteinase, compared to hirudin, in line with the 7-fold lower K_i of *H. manillensis* variant P6, when compared to the P18 isoform[63] (see also Figure 1 and Supporting Information Table 1). Further, the inhibitory potencies of the Val^{2I}→Arg mutant of *H. medicinalis* hirudin variant 1 (ref 32) and that of the Ser^{2I}→Arg mutant from the core domain of *H. manillensis* HM2 are increased 9- and 24-fold compared to their wild-type counterparts, respectively.[34,35] Notably, the presence of a second basic residue in haemadin's unique N-terminal peptide (Lys^{7I}) reinforces binding to the acidic active site region of thrombin (Figures 3A and Figure 3B). A second N-terminal residue, Met^{5I}, is located approximately equidistant from glutamate residues Glu^{146}, Glu^{192}, and Glu^{217} and engages in several important van der Waals interactions with their side chains.

It is also noteworthy that the third residue in haemadin is a phenylalanine instead of the strictly conserved Tyr found in hirudin (Figure 1). This would also contribute to an increased inhibitory potency when compared to the founder member of the I14 family because of the hydrophobic character of thrombin's S_4 subsite. Along these lines, Tyr^{3I}→Phe mutants of both *H. medicinalis* and *H. manillensis* hirudin possess 2- to

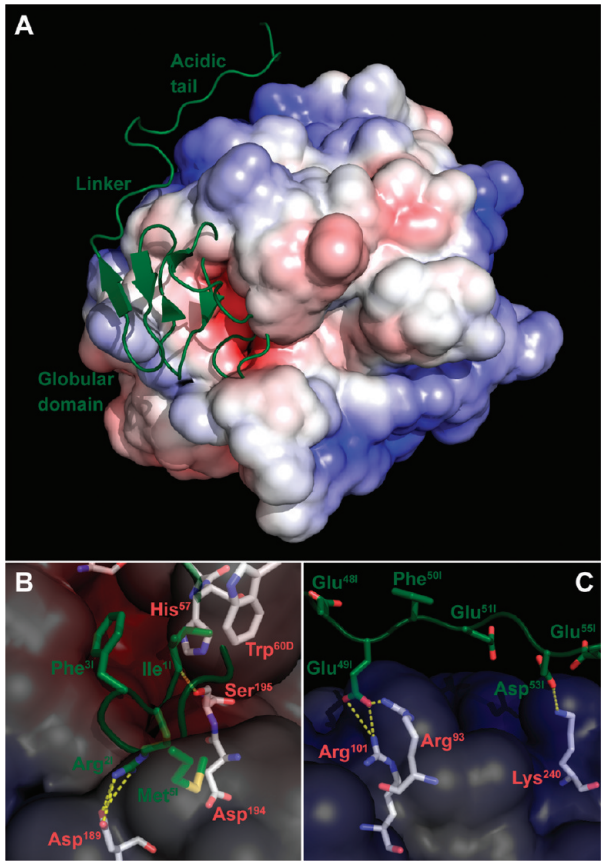

Figure 3. Three-dimensional structure of the thrombin−haemadin complex. (A) Thrombin is given with its electrostatic surface potential, while the inhibitor is depicted as a green ribbon (PDB entry 1E0F). (B) Close-up of thrombin active site region in complex with the N-terminal haemadin peptide. Notice a much better adaptation to thrombin active site architecture, compared to hirudin (Figure 2B), and in particular occupancy of the S_1 pocket by the Arg^{2I} side chain, which engages in salt bridges with the Asp^{189} carboxylate. (C) Close-up of thrombin exosite II, showing interacting residues from the acidic haemadin tail.

2.5-fold lower K_i values for thrombin than the corresponding wild-type inhibitors, and the same mutation increases the affinity of the HM2 core domain by 10-fold.[30,31] Similar improvements in potency were reported for the Tyr^{3I}→Trp variant of hirudin HV1,[31] and the equivalent mutant of the core domain from *H. manillensis* isohirudin was 5-fold more active than the natural fragment.[33] The results of these investigations suggest that the more hydrophobic residues Phe/Trp are better suited to the S_4 subsite. However, a recent study pointed to a more subtle dependence on the desolvation free energy change upon complex formation and the electric dipole moment of the side chain at third position.[36] More strikingly, replacement of the three N-terminal residues of this truncated hirudin by *tert*-butylglycine, arginine, and β-naphthylalanine (3-(2-naphthyl-L-alanine), respectively, increases inhibitory potency by 3 orders of magnitude; substitutions of the second and third amino acid residues led to particularly large changes in free energies and dissociation constants (Supporting Information Table 1).[34,35]

Altogether, comparison with these natural and engineered hirudin variants indicates that haemadin's core domain and in particular its N-terminal peptide might be considered the result of a natural optimization procedure that selected for the sequence that better fits all major thrombin subsites.

Similar to thrombin−hirudin complexes, residues from haemadin core domain also contribute to complex formation. In particular, there are close van der Waals contacts between the aliphatic side chain of Ile^{25I} (equivalent to Val^{21I} in hirudin, Figure 1) and thrombin side chains from Ile^{174} and Glu^{217}, as well as between Tyr^{17I} and residues from the 60-loop. This is in addition to a few side chain-to-main-chain H-bonds (e.g., from the guanidinium group of Arg^{173} to the carbonyl and the carboxamide oxygen atoms of Leu^{25I} and Gln^{30I}, respectively).

However, in contrast to all expectations, the two I14 family inhibitors do differ in a critical point: the C-terminal haemadin peptide covers exosite II, not the fibrinogen-binding exosite (Figure 3A and Figure 3C). This dramatic change in quaternary structure is achieved by a different orientation of the haemadin core domain compared to hirudin, which rotates by about 12° to the "left" (as seen in the standard orientation), their centers of mass being shifted by approximately 6 Å. Accordingly, we showed that haemadin is capable of inhibiting not only free α-thrombin but also the thrombomodulin-bound proteinase.[69] Occupancy of exosite II by the haemadin C-terminal tail explains the fact that this peptide is markedly shorter than the equivalent stretch in hirudin but also notably more acidic.

We have assessed the contribution of haemadin tail to exosite II binding by determining the effect of ionic strength on the kinetics of α-thrombin inhibition using appropriate proteinase mutants.[70] As expected, nonionic binding contributions were found to be virtually the same for all of the studied thrombin variants, while the ionic binding energy contributions varied from molecule to molecule. These ionic interactions contribute 17 $kJ \cdot mol^{-1}$ to the Gibbs free energy of human thrombin−haemadin complex formation (up to 20% of the total binding energy at zero ionic strength), which corresponds to a maximum of six salt bridges. In addition, we have shown that haemadin core domain (residues Ile^{1I}−Asp^{40I}) is no longer a tight-binding inhibitor and displays a 20000-fold reduced affinity for thrombin, while covalent modification of thrombin active site Ser^{195} reduces affinity for haemadin 72000-fold. These findings indicate that the inhibitor core domain is more important than the acidic C-terminal tail for thrombin binding, similar to what has been reported for hirudin.

Inhibitors from Clan IO, Family I15: Theromin and the Factor Xa Inhibitors Antistasin and Therostasin

The surprising divergence between hirudin variants from *Hirudo medicinalis* and *Hirudinaria manillensis*, on the one hand, and haemadin from the land-living leech *Haemadipsa sylvestris*, on the other, suggests that differences in habitats and feeding behaviors might reflect themselves in essentially different mechanisms of thrombin inhibition. For example, and in contrast to those leeches, the small rhynchobdellid leech *Theromyzon tessulatum* (also known as duck leech) is known to feed only three times during its lifetime, after which the salivary gland is lost by apoptosis.[71] This important physiological variation points to the evolution of different antihemostatic mechanisms in this aquatic leech. Indeed, *T. tessulatum* is the source of the most potent thrombin inhibitor known to date, theromin (K_i = 12 fM).[72] Protein analysis revealed that theromin is a homodimer of small, cysteine-rich monomers (16 out of its 67 amino acid residues are cysteines); limited sequence similarities indicate that each monomer comprises two roughly similar substructures (see below). A granulin (or epithelin)-like thrombin inhibitor isolated from *Hirudo nipponia* might actually represent the theromin ortholog in the Korean leech. Indeed, this so-called "leech granulin" has been described as a small ($M_r \approx 6$ kDa), acidic (pI 3.75), cysteine-rich polypeptide (~20% cysteine residues);[73] all these values correspond closely to those of a theromin monomer.

Theromin shares no sequence similarity with any other thrombin inhibitor reported so far but is closely related to a potent, tight binding inhibitor of factor Xa (K_i = 34 pM) that is also expressed and stored in the salivary glands of *T. tessulatum* and called therostasin.[74] Salzet and co-workers have isolated in addition to these anticoagulant factors a trypsin inhibitor from the rhynchobdellid leech, tessulin, which possesses an N-terminal domain almost identical to those of theromin and therostasin.[75] On the other hand, PSI-BLAST searches reveal clear similarities of the two halves of theromin/therostasin to the C-terminal subdomains of a number of leech-derived inhibitors that belong to MEROPS clan IO, family I15. (According to Mittl and co-workers,[76] and although they are not proper globular domains, the two modules found in most inhibitors of the family will be termed domains N and C, respectively; see below and Figure 4 for a structure-based sequence alignment of I15 family members.) The type-example and founding member of the family is a FXa inhibitor with antimetastatic properties previously characterized from the Mexican leech *Haementeria officinalis*, antistasin;[77,78] its orthologue from the Amazon leech, *Haementeria ghilianii*, has been termed ghilanten.[79−81] Curiously, the structural arrangement of theromin/therostasin is also found in a FXa inhibitor isolated from the primitive metazoan, *Hydra magnipapillata*, and also (somehow unluckily) termed antistasin;[82] it contains six repeats of domain C (Figure 4). In addition to these anticoagulant roles, members of the I15 family display a wide range of inhibitory activities. Neutrophil elastase is targeted by inhibitors found, for example, in *Hirudo nipponia* (guamerin)[83] and in the Nile leech *Limnatis nilotica* (fahsin),[84] while kallikrein inhibitors from *H. medicinalis* (hirustasin)[85] and *H. nipponia* (piguamerin)[86] have been reported. Altogether, these peptidase inhibitors are likely to act in synergy to counteract the hemostatic and inflammatory systems of their preys. This versatility of I15 family members contrasts with the thrombin specificity of I14 inhibitors hirudin and haemadin and suggests that the latter inhibitors are a more recent evolutionary development in leeches.

To date, no structural information is available for the *T. tessulatum* I15 family inhibitors. However, the structure of the founder member of the family, antistasin from *Haementeria officinalis*, has been solved by X-ray crystallography at high resolution (1.9 Å).[87] In addition, structures of hirustasin have been determined both free (at atomic resolution, 1.2−1.4 Å; ref 88) and bound to its target proteinase, tissue kallikrein.[76] Further, the structures of *H. medicinalis* bdellastasin bound to bovine and porcine trypsin have been solved (Figure 5A).[89] Finally, the structure of the chymotrypsin complex with guamerin has been recently reported.[90] These structural investigations revealed that I15 family inhibitors lack a proper hydrophobic core and possess only sparse secondary structural elements; their tertiary structures are maintained instead by 10 cysteine residues that form five disulfide bridges with an absolutely conserved pattern of [1−3, 2−4, 5−8, 6−9, 7−10] (or [abab cdecde]). These cross-links cause the polypeptide chain to fold into the two similar N and C domains, as defined by Mittl and co-workers;[76] the shapes of the resulting structures range from flat to bricklike or wedgelike. The intricate cross-linked structures of antistasin-like inhibitors appear to guarantee high physicochemical stability; for example, guamerin is

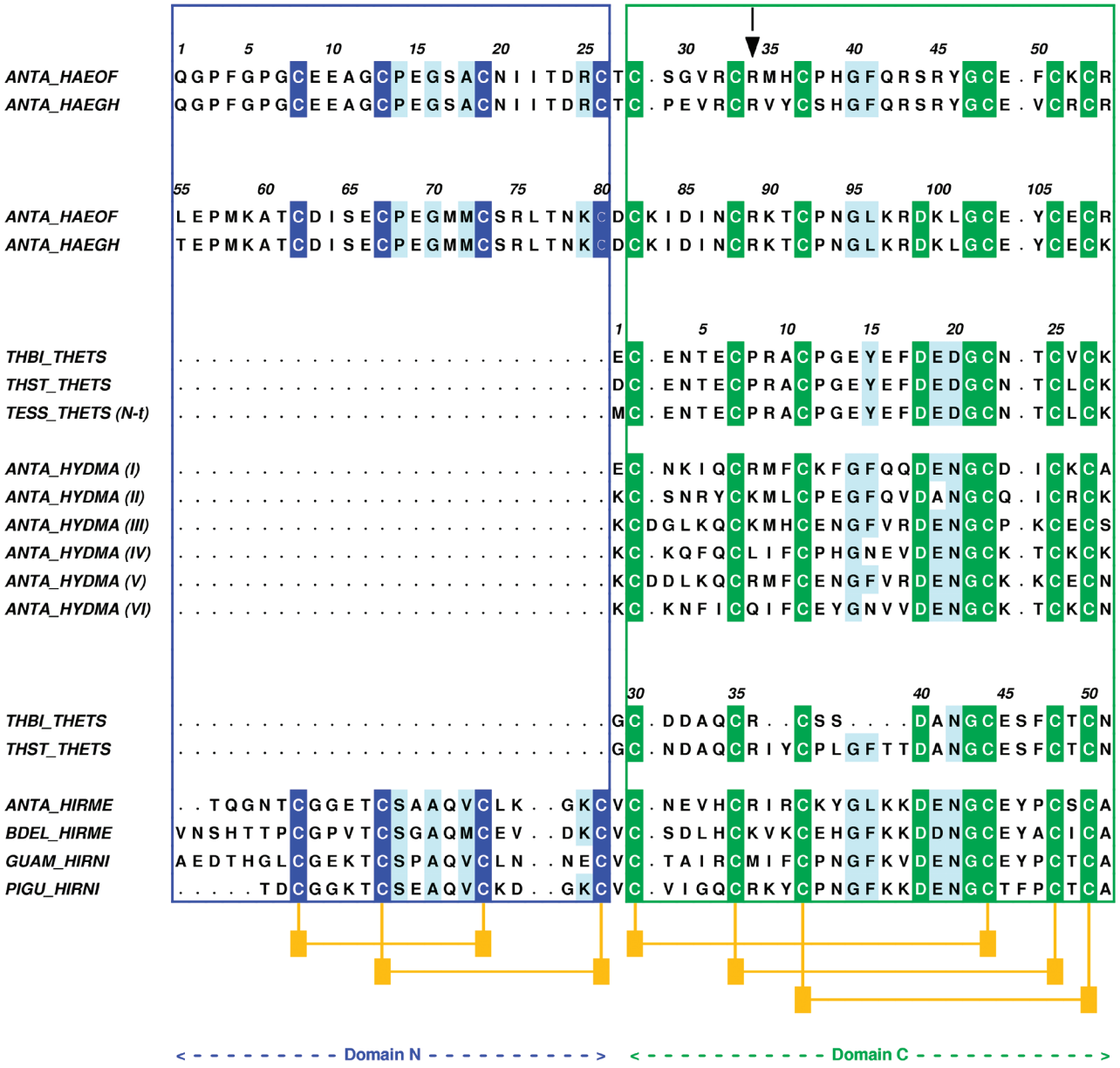

Figure 4. Structure-based sequence alignment of antistasin family inhibitors (clan IO, family I15). The sequences of thrombin, FXa and trypsin inhibitors isolated from the rhynchobdellid leech *Theromyzon tessulatum* (theromin, THBI_THETS; therostasin, THST_THETS, and thessulin, TESS_THETS, respectively) are aligned to the type-example of the family, *Haementeria officinalis* antistasin (ANTA_HAEOF), its orthologue from the Amazon leech, *H. ghilianii* (ghilanten; ANTA_HAEGH), as well as other members of the I15 family: hirustasin (ANTA_HIRME) and bdellastasin (BDEL_HIRME), from *Hirudo medicinalis*, and guamerin (GUAM_HIRNI) and piguamerin (PIGU_HIRNI) from *H. nipponia*. Also included in the alignment below the N-terminal domains of the *T. tessulatum* inhibitors are the six independent modules of *Hydra magnipapillata* antistasin (ANTA_HYDMA). These are numbered I to VI and correspond to residues 20−47, 53−80, 90−118, 119−146, 153−181, and 182−209 of the full-length protein. The unrelated C-terminal sequences of several inhibitors have been omitted. In theromin and therostasin these peptides might be involved in intermolecular disulfide bridges. Numberings correspond to the mature sequences of antistasin and theromin, respectively. Residues conserved in domains N and C are white with dark-blue or green shadowing, respectively. Conservative substitutions are shaded light-blue. Notice that *T. tessulatum* inhibitors lack domain N, which is conserved in most other family I15 members. The P_1 residue is marked with an arrow, and disulfide bonds are indicated. For theromin/therostasin, the predicted [1−4, 2−5, 3−6] pattern is based on similarities to domains C from other family members (see text for details).

stable at pH values ranging from 1 to 11 and heat-stable up to 90 °C.[83] Nevertheless, they are intrinsically quite flexible; structural comparisons of free and complexed inhibitors have indicated that proteinase binding requires a major reshaping of the reactive site loop and might be accompanied by a change in the relative orientation of the two subdomains as well. Altogether, these rearrangements allow inhibitors of the I15 family to optimally adapt to the active site cleft of their cognate proteinases.[88,89]

A remarkable feature of antistasin-like inhibitors is the conservation of an Asp-Xxx_2-Gly-Cys-Xxx_{2-3}-Cys-Xxx-Cys sequence in domain C, as the conserved glycine residue adopts torsion angles typical of a left-handed helix in antistasin,[87] hirustasin,[76,88] bdellastasin,[89] and guamerin.[90] This conformation is usually "forbidden" for residues of L-amino acids with non-hydrogen side chains, explaining its conservation in an otherwise highly variable family. Further, the conserved aspartate residue within this substructure plays an important

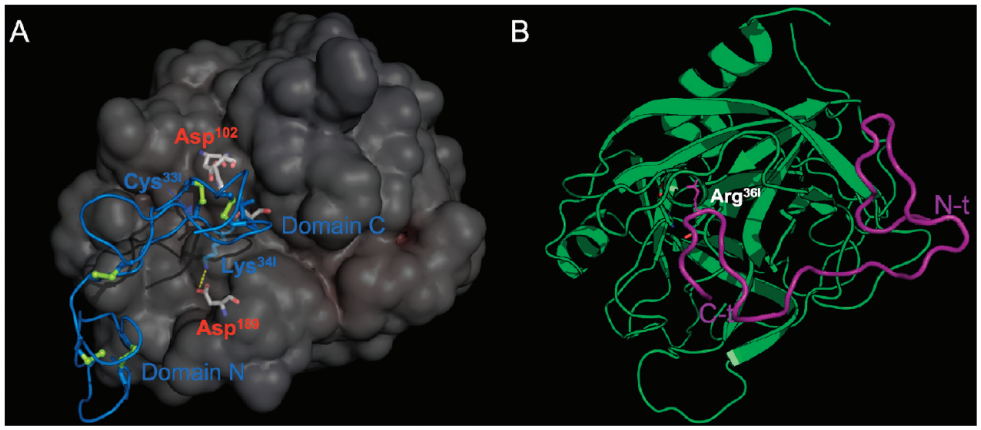

Figure 5. Structure and inhibition mechanism of I15 family inhibitors. (A) Three-dimensional structure of bovine trypsin bound to *H. medicinalis* bdellastasin (PDB entry 1C9T). The serine proteinase is depicted as a solid surface (gray), and the inhibitor is represented as a blue ribbon. Disulfide bridges and other selected side chains are shown. Notice in particular the substrate-like binding mode of the inhibitor. (B) Schematic representation of the predicted mechanism of theromin-mediated thrombin inhibition. The thrombin molecule is depicted as a green ribbon, and theromin is given as a purple ribbon.

structural role by accepting H-bonds from the amide nitrogen atoms of three consecutive downstream residues, the Gly-Cys-Xxx triplet. This substructure might thus form part of a folding "hot spot" within the I15 family of peptidase inhibitors.

The wealth of structural and functional information accumulated for other members of the family allows hypothesizing about the mechanism of theromin-mediated thrombin inhibition. First, we note that the conserved pattern of disulfide bonds and the significant sequence homology strongly suggest that theromin and therostasin comprise two nonidentical copies of domain C (Figure 4). Thus, the 12 N-terminal cysteine residues of theromin and therostasin are most likely linked with [1−4, 2−5, 3−6, 7−10, 8−11, 9−12] connectivity, while the four C-terminal Cys residues might engage in intermonomer cross-links. This would in turn suggest that the two theromin/therostasin domains are fairly independent in solution, albeit the presence of only two hinge residues might point to limited interdomain contacts.

The structural investigations listed above, along with thorough functional and mutagenesis studies of antistasin,[91−95] have verified that inhibitors from family I15 target cognate serine proteinases in a canonical, substrate-like manner, with the P_1 residue immediately following the second cysteine from domain C. For theromin/therostasin, the equivalent residue in the N-terminal domains of the inhibitors is a proline (Figure 4), which is unlikely to engage in active site interactions. By contrast, the reactive site loops from the C-terminal domains of the *T. tessulatum* inhibitors might be expected to engage in canonical interactions with the active sites of thrombin/FXa. For therostasin this assignment is easily comprehensible, as the P_1 residue would be an arginine, and the sequence of the reactive site loop is otherwise quite similar to canonically binding inhibitors, in particular to piguamerin (Figure 4). By contrast, theromin possesses a reactive site loop that is drastically trimmed when compared to those of all other I15 family members, and it is thus unclear whether the inhibitor could engage thrombin via a canonical mechanism. Conceivably, these deletions in the C-terminal domain would generate a flatter reactive site loop that is better adapted to the restricted proteinase active-site cleft, perhaps allowing interactions between the Arg^{36I} side chain and Asp^{189} at the bottom of thrombin's S_1 pocket.

On the other hand, it is noteworthy that the N-terminal modules of theromin and therostasin are highly acidic; in the former, the Gly^{13I}−Asp^{20I} hirudin-like sequence found between the third and fourth Cys residues might be expected to interact with one of the thrombin exosites. Interestingly, the reduced inhibitor retains anti-thrombin activity, suggesting that each monomer possesses independent thrombin-binding sites.[72] However, it cannot be excluded that the two monomers interact with a single proteinase moiety in vivo. The hypothetical mechanism of thrombin inhibition, based on exosite-mediated interactions of the N-terminal subdomain and contacts with active site cleft residues through the C-terminal region, is schematically represented in Figure 5B. Similar exosite interactions could be envisioned for the FXa−therostasin pair. Along these lines, it is noteworthy that residues Glu^{15I}−Ser^{17I} from antistasin domain N form an epitope that interacts with the FXa exosite comprising basic residues Arg^{222}, Lys^{223}, and Lys^{224} (ref 87). Alternatively, "slippage" of the reactive site loop in domain N could allow interaction of the Pro^{8I}−Arg−Ala^{10I} sequence in theromin with thrombin subsites S_2−S_1'. Validation of the proposed models will have to await experimental determination of the crystal structures of thrombin−theromin and FXa−therostasin complexes.

Leech-Derived Thrombin Inhibitors as Antithrombotic Agents

Cardiovascular diseases such as myocardial infarction, stroke, and deep vein thrombosis (DVT) remain the leading cause of morbidity and mortality in industrialized countries; pathological thrombus formation is responsible for most of these episodes. Given the drawbacks of the drugs currently available to treat prothrombotic conditions such as heparin, it is comprehensible that natural inhibitors of clotting factors from hematophagous animals have long been tested as potential anticoagulants.

For historical reasons, hirudin has played a prominent role in the development of new antithrombotic agents (for reviews on the development of recombinant hirudins as antithrombotics and their therapeutic use, see refs 11, 96, and 97). Two recombinant forms of the inhibitor are currently available as anticoagulants: lepirudin, which received first marketing approval from the European Medical Evaluation Agency (EMEA) in March 1997 and was approved by the U.S. Food and Drug Administration (FDA) a year later under the trade name Refludan, and desirudin, approved by the FDA in April 2003 under the brand name Iprivask. These antithrombotic

agents have been authorized to treat patients with heparin-induced thrombocytopenia (HIT) and for prophylaxis of DVT after major orthopedic surgery, respectively. Hirudin thus represents the only parasite-derived anticoagulant approved so far for clinical use in humans.[98,99]

Clinical studies have suggested in addition an advantage of hirudin over unfractionated and low molecular weight heparin in preventing DVT in some patient groups; in patients with established vein thrombosis, the leech-derived inhibitor also reduced the rate of new pulmonary embolism and the extension of venous thrombosis compared to heparin. Interestingly, hirudin was found to be more effective than heparin in preventing ischemic events in the acute phase in patients with unstable angina, although this clinical benefit was no longer significant after 30 days in the GUSTO-IIb study.

However, full-length hirudin suffers from the disadvantage of possessing a quite narrow therapeutic window, in particular when used in combination with the thrombolytic agents, tPA (alteplase) or streptokinase (drawbacks and precautions are discussed in detail in a recent review).[100] Besides, hirudin's considerably higher cost compared to heparin does not justify routine use and has limited its clinical applications.[96] Several strategies to improve the pharmacological parameters of hirudin have been followed so far. For instance, considering the role of the Arg-Gly-Asp (RGD) motif for Fbg binding to platelet integrin receptors, chimeric variants in which this sequence is grafted into the hirudin framework have been developed. The first reported chimeras of this class were obtained by replacing the Ser^{32I}-Asp-Gly-Glu^{35I}, solvent-exposed and poorly conserved sequence of the natural inhibitor, by Arg-Gly-Asp-Ser or Lys-Gly-Asp-Ser to yield variants termed hirudisin and hirudisin-1, respectively.[101] These recombinant molecules retain full antithrombin activity (in fact, hirudisin was 2-fold more potent than recombinant hirudin) and possess in addition disintegrin activity. Presentation of the RGD motif within a disulfide-constrained loop proved to be beneficial, as hirudisin inhibits ADP-induced platelet aggregation 3- to 5-fold stronger than the linear GRGDS- or RGDS peptides, apparently through direct interactions with the Fbg receptor, integrin $\alpha_{IIb}\beta_3$.

The recently determined solution NMR structure of hirudisin revealed significant differences in the surface electrostatic charge distribution compared to wild-type hirudin, especially around the RGD segment.[102] These differences are expected to improve interactions with thrombin exosite I but also to be beneficial to the additional function as an antiaggregant. Indeed, this chimera has been recently shown to be 2−3 times more effective than the natural inhibitor in preventing thrombosis in an experimental rabbit model.[103] The authors conclude that hirudisin could be used for the prevention of thrombosis after carotid artery anastomosis or in other thrombotic events.

Because clinical applications of full-length hirudin are restricted by the risk of bleeding and rapid clearance of the inhibitor from circulation, another strategy to improve its pharmacological parameters is the coupling to a carrier protein such as albumin. Along these lines, a recently reported chimera has been generated by appending human serum albumin (HSA) to the N-terminus of hirudin variant 3.[104] The inhibitor is linked to the carrier protein by a short peptide containing an optimized plasmin cleavage site, thus generating in practice a plasmin-activatable thrombin inhibitor. Indeed, as expected from blockade of hirudin N-terminal peptide, the recombinant chimera shows no thrombin inhibitory activity in the absence of plasmin. However, the fibrinolytic proteinase liberates active hirudin in a time- and concentration-dependent manner. This HSA−hirudin chimera ("long-lasting hirudin") was cleared much more slowly than the free inhibitor from circulation and otherwise showed encouraging preliminary results in vivo.

Other approaches are directed to generate recombinant proteins with both anticoagulant and fibrinolytic properties. For instance, a chimera has been generated by joining a hirudin molecule and the bacterial fibrinolytic agent staphylokinase (STA) via a long linker containing a coiled-coil dimerization motif.[105] This hirudin−STA chimera was shown to retain both antithrombin and fibrinolytic activities and might thus offer an advantage over other fibrinolytic agents by reducing the risk of reocclusion. Finally, a STA chimera that contains in addition the fibrin specificity-conferring kringle 2 domain from tPA and the C-terminal hirudin tail has been constructed and reported to be a more potent and faster-acting thrombolytic agent with better antithrombin and antiaggregant properties than previously tested variants, including free recombinant STA.[106] No clinical trials involving any of these chimeras have been reported so far, and they would share with recombinant hirudin the drawback of a high cost.

Hirudin as Template for the Design of Novel Antithrombotic Agents

In spite of the success of full-length hirudin and the interesting prospects for hirudin-based chimeras discussed in the previous section, more promising alternatives to hirudin and, in general, to natural proteinaceous inhibitors appear to be small synthetic molecules that incorporate and/or mimic functions critical for thrombin recognition. This strategy has been crowned with success with the development of shorter synthetic hirudin derivatives as antithrombotic agents that possess improved pharmacological properties.

A number of bivalent synthetic peptides, collectively termed hirulogs, are the best characterized hirudin-derived inhibitors. Hirulogs comprise an N-terminal tetrapeptide (D-Phe-Pro-Arg-Pro) that ideally fits the thrombin active site cleft in a substrate-like, antiparallel manner, connected to the nonsulfated C-terminal tail from *H. medicinalis* hirudin variant 2 (Asn^{53I}−Leu^{64I}) via polyglycine spacers (Gly_{2n}, with n = 1−4).[107] The variant with a four-glycine linker, known as hirulog-1, displays a thrombin inhibition constant (K_i) of about 2 nM [107−110] and eventually reached the market under the trade name Angiox or Angiomax (bivalirudin) (see below). Incorporation of a stable phosphono derivative of L-phenylalanine in hirulog-1 (4′-phosphono-L-phenylalanine, which mimics the natural L-Tyr-O^4-$sulfate^{63I}$ residue) resulted in a more potent anticoagulant agent, hirufos.[111]

In other related drug design projects, the D-Phe-Pro-Arg sequence has been replaced by related tripeptide boronate inhibitors that feature either an arginine-like isothiouronium group or an uncharged bromopropyl residue at position P_1; the hirudin tail is connected to the D-Phe residue through a flexible, reverse-oriented glutaric acid linker. The name borologs has been coined to design these potent thrombin inhibitors (K_i of 2 and 780 pM, respectively).[112] Finally, in a recent investigation the D-Phe-Pro-Arg motif has been replaced by a Phe-Asn-Pro-Arg tetrapeptide.[113] These authors took further advantage of the genetically encodable Xxx-Yyy-Pro-Arg-(P_1)-Pro(P_1')-Gln-Zzz active site-directed sequence to perform a systematic search for preferred residues at positions P_4, P_3, and P_3' through phage display technology; selected

sequences exhibited potent inhibition of thrombin-mediated Fbg clotting.

Even though the Arg−Pro bond is in general not readily attacked by trypsin-like serine proteinases, hirulogs and related bivalent inhibitors are slowly degraded by thrombin[108] and cleared from plasma by a combination of proteolytic cleavage and renal excretion. This partial resistance to proteolysis has motivated the development of a number of noncleavable hirulog derivatives. For instance, two series of compounds in which the Arg−Pro scissile peptide bond in "first generation" hirulogs is replaced by the nonhydrolyzable functions of β-homoamino acids (ArgΨ[CH_2CONH]-Xxx, hirulog **2** series) or reduced peptide bond analogues (ArgΨ[CH_2N]-Xxx, hirulog **3**) have been presented.[114] Similar peptidomimetic functions have been employed to design thrombin inhibitors termed hirutonins, in which an active site-directed N^{α}-acetyl-D-Phe-Pro-Arg moiety is fused to the Gln^{49I}−Gln^{65I} hirudin tail via ketomethylene groups (ArgΨ[CO(CH_2)$_n$CO]-Xxx, n = 1−4).[115,116] Replacement of the Gln^{49I}−Gly^{54I} linker in the congener with n = 2, hirutonin-2, by a variable number of ω-aminopentenoyl units and trimming of the hirudin tail to the minimum sequence required for exosite I binding (Asp-Phe-Glu-Pro-Ile-Pro-Leu) resulted in lower molecular weight inhibitors with subnanomolar K_i values, hirutonins 5−7.[116] Alternatively, the P_3 to P_1 residues of the activation cleavage site on the thrombin receptor PAR1 (Asp-Pro-Arg) have been linked to the hirudin tail via a transition-state analogue α-ketoamide. The resulting potent bivalent inhibitor **8** (CVS995, $K_i \approx 1$ pM) features in addition an N-terminal "cap" of 2-prolylpentanoyl.[117] A different strategy to avoid inhibitor cleavage relies on the use of an N^{α}-(methyl)Arg residue at the P_1 position; the active site-directed D-cyclohexylalanine-Pro-N^{α}(Me)Arg substructure was linked to a hirugen-like C-terminal segment via a flexible Xxx-$(Gly)_5$ linker.[118] However, the structural analysis revealed slow cleavage of the N^{α}(Me)Arg-Thr bond in the most potent member of the series, I-11.[119]

Instead of utilizing an ideal substrate-like binding peptide for occupying the thrombin active-site cleft, a different approach that reproduces the parallel binding mode of I14 family inhibitors has been followed to develop true hirudin peptidomimetics, termed hirunorms.[120,121] Hirunorms feature a number of noncoded amino acid residues, including a D-Ala-β-Ala-β-Ala linker. In addition, two α-aminoisobutyric acid residues replace Glu^{61I} and Glu^{62I} in order to favor 3_{10}-helix formation, while β-cyclohexylalanine is found instead of Leu^{64I}. In the most potent member of the series, hirunorm IV ($K_i \approx 90$ pM), the three N-terminal hirudin residues are replaced by cyclohexylglycine, arginine, and β-(2-naphthyl)-alanine, respectively, which are known to improve thrombin affinity because of a better occupancy of S_1, S_2, and S_4 subsites (see above and Supporting Information Table 1). The price paid for conserving the parallel binding mode, thus avoiding proteolysis, is a relatively high molecular weight of hirunorms compared to hirulogs and hirutonins. This drawback, however, might be compensated by the ∼10- and 3-fold higher specific activities of hirunorm IV for increasing clotting times of normal human plasma when compared to hirudin and hirulog-1, respectively.[120]

Alternatively, nonsubstrate type compounds such as dansyl-Arg-D-pipecolic acid (Dns-Arg-D-Pip) have been used as potent, active site-directed elements; in other variants the toxic dansyl group has been replaced by 4-*tert*-butylbenzenesulfonyl (Bbs) or 1- or 2-naphthalenesulfonyl (Nas).[122] These moieties are connected to the hirudin tail through linkers containing 12-aminododecanoic acid (μAdod) and/or 4-aminobutyric acid units (γAbo), rendering the bivalent inhibitors resistant to thrombin hydrolysis. The affinity of these inhibitors has been further improved by performing a "methyl scan" of linker residues (i.e., the incorporation of methyl groups at all possible positions within the linker). This strategy eventually allowed identification of L-Ala and D-Ala as preferred residues at positions P_1' and P_3'; these residues increased thrombin affinity 20- and 25-fold, respectively.[123] In this manner, by exploitation of S′ subsites on the thrombin surface, a bivalent thrombin inhibitor has been practically transformed into a trivalent inhibitor.

Medium-to-high-resolution crystal structures of thrombin bound to hirulog 1 (PDB entry 2HGT),[124] a noncleavable analogue with a β-homoarginine residue at position P_1, hirulog 3 (1ABI),[125] borologs 1 and 2 (1A3B and 1A3E, respectively),[112] the PAR1-based noncleavable inhibitor **8** (1DIT),[117] the variant with an active site-directed D-cyclohexyl-Pro-N^{α}(Me)Arg fragment (1EBI),[119] hirutonins 2 and 6 (1IHS and 1IHT, respectively),[126] and a derived peptidyl pyridinium methyl ketone inhibitor with D-cyclohexylalanine replacing D-Phe in the active site-directed moiety P596 (1HBT)[127] have been reported. This is in addition to thrombin complexes with hirunorms IV (PDB entry 4THN)[128] and V (PDB 5GDS).[129] Finally, four inhibitors that feature either Dns-Arg-D-Pip (P498 and P500; coordinates not deposited with the PDB),[130] or Bbs-Arg-D-Pip active site-directed moieties (P628 and P798; PDB entries 1EOL and 1EOJ, respectively)[131] have been structurally characterized in their complexes with the proteinase. Altogether, these structural investigations have fully validated the various rational drug design strategies employed by revealing the expected interactions at both the active site and exosite I (Figures 6 and 7).

In another approach for inhibitor development, a chimeric peptide composed of an RGD integrin-binding motif followed by the hirudin tail (Trp-Gly-**Arg-Gly-Asp**-Ser-Ala-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu; RGD motif and hirudin tail are bold and underlined, respectively) has been shown to retain both anticoagulant and antiaggregant activities.[132] In a similar manner, dipeptides Asp-Phe and Asp-Ser were incorporated after the proteolytically resistant ketomethylene pseudopeptide bond of hirutonins.[133] These modified hirutonins inhibited, on the one hand, thrombin amidolytic activity ($K_i \approx 35$ nM) and prevented Fbg clotting and, on the other hand, interfered with thrombin-induced platelet aggregation by competing with fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. However, this disintegrin activity was not higher than that of the linear RGDS peptide and was achieved at the expense of an approximately 10-fold decrease in direct antithrombotic function. In a further study, the hirutonin chimera that features the RGDS sequence was shown to block shear-induced platelet adhesion to von Willebrand factor and subsequent aggregate formation.[134] Neither noncleavable hirudin peptidomimetics nor RGD−hirugen chimeric peptides have been pursued further for clinical applications, although at least for the noncleavable hirutonin, BCH-2763, in vivo efficacy has been shown in rodent and/or canine models of arterial or venous thrombosis.[135−138]

Demonstration that bivalirudin/hirulog produces potent dose-dependent antithrombotic effects in primate models without compromising hemostasis[139] paved the way for a number of clinical trials conducted in the 1990s, which confirmed the expected efficacy of the hirudin-derived inhibitor as an antithrombotic agent. For instance, the HERO study

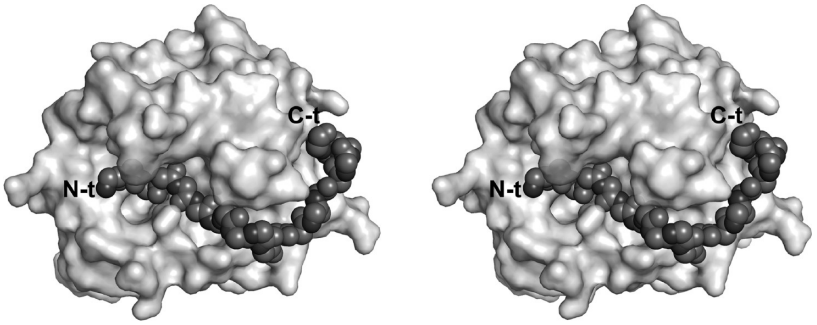

Figure 6. Structure-based drug design of hirudin peptidomimetics. Stereoview of the crystal structure of thrombin (light-gray solid surface) bound to the noncleavable inhibitor, hirulog 3 (PDB entry 1ABI; only main chain atoms of the bivalent inhibitor are shown as van der Waals spheres). Notice that the hirudin-derived peptide covers exosite I essentially as in the full-length inhibitor (compare Figure 2A and Figure 2C). The design procedure basically replaces the bulky globular domain by a short active site-binding peptide.

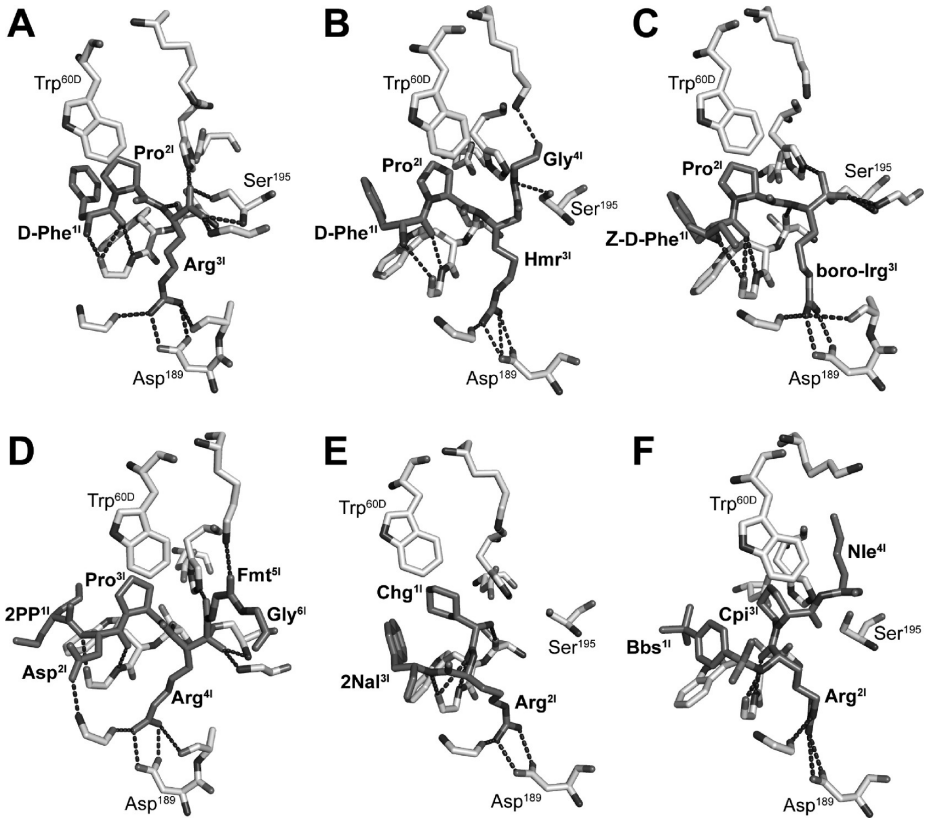

Figure 7. Different approaches for generating hirudin-like, bivalent thrombin inhibitors. For simplicity, only the active site regions of thrombin and the more N-terminal groups of some structurally characterized hirudin peptidomimetics are shown. C-Terminal tails of all inhibitors run approximately as shown in hirulog 3 (Figure 6). Selected hydrogen bonds are indicated. (A) Hirulog 1/bivalirudin (PDB entry 2HGT). Notice that the Arg^{3I}–Pro^{4I} bond is cleaved in this structure. (B) Noncleavable derivative, hirulog 3 (PDB entry 1ABI). Notice that residue Arg^{3I} has been replaced by β-homoarginine (Hmr). Notice also that this inhibitor is equivalent to hirulog **2a** in the original investigation.[114] (C) Borolog 1 (PDB entry 1A3B). Position P_1 is occupied by the arginine-like, isothiouronium side chain (boro-Irg), and the boron atom is covalently linked to Ser^{195} Oγ. (D) PAR1-based inhibitor with an α-ketoamide transition state mimetic, **8** (PDB entry 1DIT): 2PP, 2-propylpentanoyl; Fmt, α-ketoamide bond (interpreted as a formate residue). (E) True hirudin peptidomimetic hirunorm IV (PDB entry 4THN). Notice the different path of the inhibitor main chain, compared to substrate-like inhibitors depicted in panels A–D: Chg, cyclohexylglycine; 2Nal, β-(2-naphthyl)alanine). (F) Inhibitor with improved occupancy of S′ pockets, P628 (PDB entry 1EOL): Bbs, 4-*tert*-butylbenzenesulfonyl; Cpi, carboxypiperidine (D-pipecolic acid); Nle, norleucine.

showed that hirulog was more effective than heparin at achieving early patency (TIMI 3 flow) when used as an adjunct to aspirin and streptokinase, without increasing the risk of major bleeding.[140] Bivalirudin was finally approved under the commercial name Angiox or Angiomax for patients with acute coronary syndromes (ACS) scheduled for urgent or early intervention and was introduced in December 2000.[141–143] Since then, important positive clinical data have been accumulated for this bivalent thrombin inhibitor, which has been used to treat over 1.25 million patients so far (for a recent authoritative review on bivalirudin and its clinical applications, see ref 144). For instance, bivalirudin alone maintained efficacy in reducing ischemic events in patients with moderate or high risk for ACS while significantly reducing bleeding complications when compared to heparin plus platelet inhibitors.[145] Of particular note, patients with major bleeding had

significantly higher 30-day rates of mortality (7.3% vs 1.2%) and composite ischemia (23.1% vs 6.8%) versus those without major bleeding, which clearly favors the routine use of the specific thrombin inhibitor.[146]

Concluding Remarks and Future Perspectives

We have been taking advantage of leeches' extraordinary ability to interfere with blood coagulation for thousands of years now, long before we even started to understand the basic principles that govern blood clot formation and dissolution. In this long period of time, we have moved from the straightforward use of leeches for bloodletting, as practiced already in ancient times, to the synthesis of sophisticated inhibitors based upon the molecular mechanism of thrombin inhibition by one the major weapons from the antihemostatic arsenal, hirudin. However, in spite of the success of both hirudin and hirulog (bivalirudin) as antithrombotic agents, we believe that their drawbacks and limitations, as well as the clinical relevance of cardiovascular disease, incite the continuous search for novel thrombin inhibitors.

We hope that current and future information about the structure of thrombin−inhibitor complexes and detailed mechanistic studies will suggest novel strategies for the design of antithrombotics with improved pharmacological properties. Along these lines, a large number of hirudin peptidomimetics other than hirulog are awaiting for clinical trials to demonstrate their potential usefulness in the treatment of different cardiovascular conditions. This is in addition to a large number of crystal structures of thrombin bound to different small-molecule inhibitors, which have been solved using crystals grown in the presence of hirugen. The structures of these ternary complexes are therefore ideal starting points for designing novel bivalent inhibitors. On the other hand, demonstration of simultaneous binding to active site and exosite II of thrombin by haemadin suggests straightforward ways for the development of inhibitors that, for example, target both free and thrombomodulin-bound forms of the proteinase. Furthermore, entirely novel perspectives for inhibitor design might be expected from the mechanism of thrombin inhibition by the I15 family member, theromin. Finally, contemplating the amazing diversity of structural frameworks and inhibition mechanisms shown by thrombin inhibitors, we are tempted to speculate about the presence of still uncharacterized polypeptides that exhibit unique binding modes to thrombin. Recent developments such as the use of multifunctional micellar nanoparticles to target hirulog to atherosclerotic plaques[147] offer unforeseen possibilities at the crossroads of nanotechnology and antithrombotic drug design.

Glossary

antistasin — An FXa inhibitor isolated from the Mexican leech *Haementeria officinalis* and is the type-example and founding member of the I15 family of peptidase inhibitors. Somehow unluckily, the same term designs a related inhibitor from freshwater polyp, *Hydra magnipapillata*, which also possesses anti-Xa activity.

bivalirudin — Bivalent hirudin. It is the commercial name of the synthetic hirudin peptidomimetic hirulog-1.

borolog — Hirulog derivative that incorporates a tripeptide boronate inhibitor instead of the D-Phe-Pro-Arg moiety.

desirudin — Abbreviation for 63-desulfohirudin. It is one of the recombinant hirudin forms currently available as anticoagulants and is produced as a recombinant protein in *Saccharomyces cerevisiae* and then desulfated with the help of an arylsulfatase. It is marketed under the trade names Iprivask and Revasc.

haemadin — A highly potent and specific thrombin inhibitor ($K_i \approx 100$ fM) isolated from the land-living leech *Haemadipsa sylvestris*. Although it belongs to the same I14 inhibitor family as hirudin, haemadin possesses a different thrombin inhibition mechanism from that of the founder member of the family.

hirudin — A small protein (65 amino acid residues) isolated from several leeches such as *Hirudo medicinalis* and *Hirudinaria manillensis*. Hirudin is a highly potent and specific thrombin inhibitor ($K_i \approx 20$ fM for variants with a sulfated Tyr^{63I}) that belongs to MEROPS family I14. Two nonsulfated recombinant forms have been introduced in the medical praxis as lepirudin and desirudin.

hirudisin — Recombinant hirudin variant that incorporates an RGD integrin-binding motif within one of the exposed loops that are not involved in interactions with thrombin, thus conferring disintegrin activity in addition to the natural antithrombotic function.

hirugen — A peptide that designs the C-terminal, acidic tail of hirudin (usually, residues 45−65).

hirulog — Synthetic thrombin inhibitor generated by coupling the "ideal" active-site binding peptide, D-Phe-Pro-Arg-Pro, to the nonsulfated C-terminal tail from *H. medicinalis* hirudin variant 2 (Asn^{53I}−Leu^{64I}) via polyglycine linker. Hirulog-1 has been introduced in the medical praxis under the name bivalirudin.

hirunorm — Synthetic, bivalent thrombin inhibitor characterized by the conservation of the noncanonical, parallel binding mode of I14 family inhibitors.

hirutonin — Noncleavable hirulog derivative in which the active site- and exosite I-binding peptide is linked via a ketomethylene group.

lepirudin — One of the recombinant hirudin forms approved as antithrombotic agents. Its trade name is Refludan.

theromin — The most potent natural thrombin inhibitor isolated so far (from the rhynchobdellid leech *Theromyzon tessulatum*; K_i = 12 fM) and belonging to family I15 of peptidase inhibitors.

therostasin — An I15 family inhibitor identified in the duck leech *T. tessulatum*, which potently inhibits FXa.

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Supporting Information Available: Table summarizing the results of all site-directed mutagenesis studies on hirudin variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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