Leech Thrombin Inhibitors

Michel Salzet*

Laboratoire de Neuroimmunologie des Annélides, ESA CNRS 8017, SN3,IFR 17 INSERM, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cédex, France

Abstract: Serine proteases (SP), such as thrombin, factor Xa, elastase, trypsin are implicated in many clinical disorders such as emphysema, arthritis and cardiovascular diseases. These enzymes, in normal physiological conditions, are regulated by naturally occurring serine protease inhibitors, such as anti-thrombin III involved in thromb in inhibition. Primitive parasitic invertebrates have co-evolved highly specific mechanisms to communicate with their hosts for survival purposes, by blocking host processes such as blood coagulation. Thus a battery of new powerful molecules from blood-sucker animals acting at different points of the coagulation cascade such like factor Xa, thrombin, platelets aggregation inhibitors have been isolated and are now at a clinical level. In this review, we focus our attention on thrombin inhitors.

INTRODUCTION

Evidence is accumulating that demonstrates the significance of proteases in numerous pathological processes. The destruction of the extracellular matrice of articular cartilage and bone in arthritic joints is thought to be mediated by excessive proteolitic activity [1]. In emphysema, gingivitis, tumor invasion and inflammatory infections, it is suggested that tissue destruction is caused by proteases [1]. Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin G) are able to solubilize fibrous proteins such as elastin and collagen [2,3]. Given the specific recognition by proteases of defined amino acid sequences, it may be possible to inhibit these enzymes when they are involved in pathological processes. Thus, the development of non-toxic protease inhibitors extracted from invertebrates for *in vivo* application may be quite important [1]. Moreover, in order to understand pathological phenomena associated with a thrombosis, scientists have developed hematophagous animals as models in which to find new coagulant inhibitor molecules. One of the model, the most studied is blood-sucker leeches. In fact, animals depending on a diet of fresh blood have evolved mechanisms that interfere with the coagulation process of the blood donor. In this regard, a variety of coagulation inhibitors have been isolated and the class the most studied is thrombin inhibitor like hirudin [4].

HIRUDIN

Thrombin is a key enzyme in pathogenesis of acute coronary thrombosis, therapy with heparin, an indirect thrombin inhibitor has been developed since the thirty-last decade (Table 1). The direct antithrombins have emerged since the works of Markward in 1957 [5]. However, since1884, its presence in salivary glands of the leech *Hirudo medicinalis* was already discovered [6]. Its involvement as a potent anti-thrombosis drug was investigated by Shionoya [7]. Its definitive structure was determined in 1950 (Fig. **1**). Hirudin in not a single, homogeneous substance but rather includes variant forms or iso-inhibitors. These variants exhibit point differences in their amino sequences (Fig. **2**, [8]).

STRUCTURE OF HIRUDINS AND HIRULOGS

Hirudins are natural single-chain peptides of 7 kDa with 3 intra-chain disulphide bridges and a sulfated tyrosine residue (Fig. **1**). Among others, three principal variants, designated HV-1, HV-2 and HV-3 have been identified, each consisting of 65 or 66 amino acid residues (Fig. **2**, [8]). A single basic structure is common to all three variants, but amino acid residues at a number of positions on this structure differ. Chemically, hirudins contain no methionine, arginine or tryptophan. Its cDNA was cloned and large scale of recombinant hirudin produced in *E. Coli* [9]. in *Pichia pastoris* [10], in yeast (Fig. **3**) and in the filamentous fungus *Acremonium chrysogenum* [11]. The recombinant peptide (desulfatohirudin, Fig. **4**) and the natural one have the same activity (Tables 3 and 4, [12]). Recombinant hirudins differ from native forms in lacking the sulfate group on the tyrosin at position 63 [13]. Structure of hirudin in solution has been studied in nuclear magnetic resonance and its tertiary structure has been found to consist of a highly packed core and two extended, wing-like domains [14]. One of the latter, centered on amino acid residues 34, contains amino acid mutations, insertions and deletions in an exposed position, resulting in a variety of isoproteins [8]. The dichroism circular studies points to an unusual asymmetric structure of hirudin where basic elements of this structure is a compact, hydrophobic core comprising the

*Address correspondence to this author at Membre de l'Institut Universitaire de France, Laboratoire de Neuroimmunologie des Annélides, ESA CNRS 8017, Université des Sciences et Technologies de Lille, SN3, F-59655 Villeneuve d'Ascq Cedex, France. Tel: 33-3-2033-7706, Fax: 33-3-2004-1130, E-mail: michel.salzet@univ-lille1.fr, http://www.univ-lille1.fr/lea

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Table 1. Comparison of Some Properties of Hirudin with Low Molecular Weight Heparins

Hirudin	Low molecular weight Heparins
Specific and potent inhibitor of thrombin	Inhibits mainly Factor Xa and much less Factor IXa and Xia
Single chain polypeptide of 65 residues, stabilized with 5 disulfide bridges (6.5 kDa)	Heteropolysaccahride, sulfated glycosaminoglucuronanes (5 kDa to 25 kDa)
Anti-thrombin III independent	Anti-thrombin III dependent
Not neutralised by heparinase, endothelium macrophages, fibrin monomer, plasma protein, platelet activator 4, neutral endopeptidase	Neutralised by heparinase, weak endothelim binding
Inactivates clot-bound thrombin	Does not inactivate clot-bound thrombin and factor VII
Prevents thrombin induced aggregation but not other platelet agonists	Inhibits platelet function
Does not induce thrombocytopenia	Can induce thrombocytopenia
Good bioavailibility (80%= after subcutaneous injection)	Bioavaibility after subcutaneous injection is >90%
Fair-dose effect response	Fair-dose effect response
Not immunogenic	Not immunogenic
No liver toxicity	Transient increase of liver enzymes is possible
No increase in vascular permeability	No increase in vascular permeability
No effect on platelets and urinary secreted in intact form	Bind to proteins and cells. Neutralized by anti-heparins. Affect platelets and is metabolized by liver

amino-terminal half of the molecule and an extended highly hrydrophilc carboxy-terminal region [13]. The core region contains alternating polar and non-polar segments and has three disulfide bridges [13]. The quaternary structure suggests that hirudin exists in multimeric form under physiological conditions [13].

Synthetic peptides based on the carboxyl end of natural hirudin, hirulog have been performed and possess an antithrombin potency approximately identical to the native hirudin [15]. The chimera is a bi-functional 20-amino acid peptides. It combines the interaction with anion-binding exosite of thrombin with the N-terminal fragment (D-Phe-Pro-Arg-Pro-Gly), which interacts with the catalytic site of thrombin [16]. These peptides include PPACK (D-Phe-Pro-Arg-Chloromethylketone), hirugen (exosite antithrombin peptide, the sulfate C-terminal dodecapeptide of hirudin) and a group of closely related peptides called the hirulogs.

Table 2. Effects of rHirudin Towards Proteolytic Enzymes

Enzymes	Ki (M)
Thrombin	23.1×10^{-14}
Plasmin	22.2×10^{-6}
Factor Xa	22.2×10^{-6}
Trypsin	22.2×10^{-6}
Kallicrein	22.2×10^{-6}
Chymotrypsin	22.2×10^{-6}

Bivalirudin (Angiomax, The Medicines Company) is a synthetic 20 amino acid peptide rationally designed on the basis of structural studies of hirudin, a naturally occurring anticoagulant [17]. Bivalirudin represents a new class of anticoagulant drugs that directly inhibits thrombin, a key component in blood clot formation and extension. With its high binding affinity and specificity for thrombin, bivalirudin acts directly on thrombin, rather than via other clotting factors. The compound has a variety of potential uses as an alternative to heparin in the management of cardiovascular disease and related medical procedures i.e., unstable angina (UA), myocardial infarction (MI) and percutaneous transluminal coronary angioplasty (PTCA).

INTERACTIONS HIRUDIN (HIRULOGS)-THROMBIN

Hirudin instead other thrombin inhibitors has a unique mechanism of interaction on thrombin (Fig. **5**, [13]). Due to its structure, hirudin has a considerable flexibility in its binding activity, which contributes to its strong affinity towards thrombin. Hirudin bind to thrombin in a stoichiometric relationship (1:1). The 10 last amino acid residues of the carboxy terminal tail are necessary for specific binding and inactivation of thrombin (Fig. **5**, [18]). Alteration in amino acids in position 56,57,59,60 and 64 alter the binding efficiency of the molecule [18] and the one in position 56 is critical for its antithrombotic activity [18]. The initial interaction between hirudin and thrombin involves an ionic interaction of thrombin and a multimeric form of hirudin. This initial binding does not produce a conformational change in the molecule. In the second stage

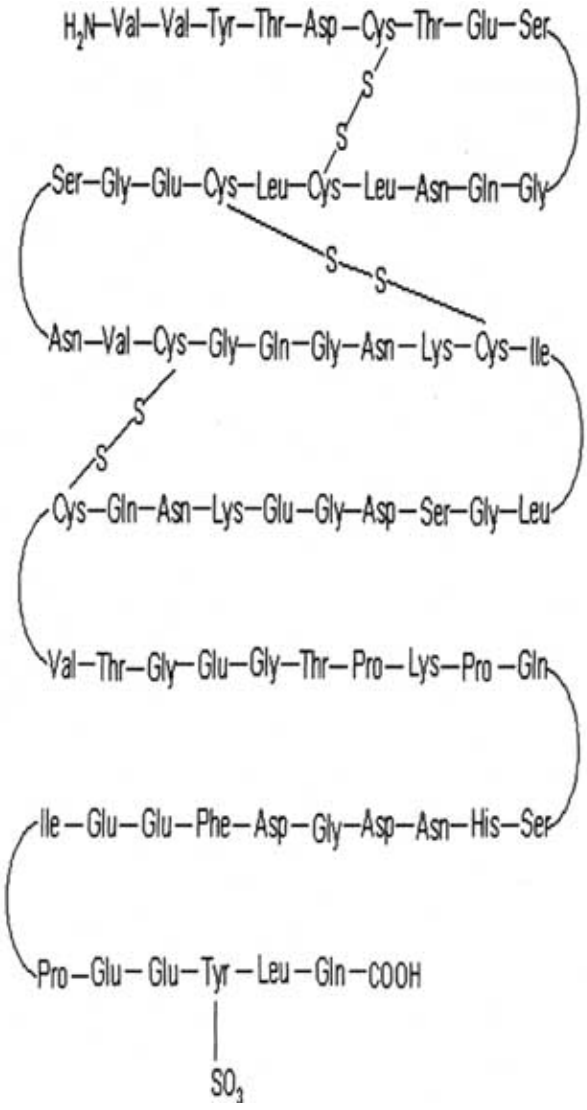

Fig. (1). Structure of hirudin (HV-1).

of interaction, the multimer dissociates to produce monomeric complexes with a 1:1 hirudin-thrombin stoichiometric relationship. At this point thrombin undergoes significant conformational changes that produce its loss of clotting activity. Additional interactions between hirudin and thrombin molecules results in an increased affinity (Ki : 10-100 fmol, [19]). Compared to heparin (table 1), hirudin is a strict, tightness thrombin inhibitor. It does not need co-factor for its activity.

The comparison of activity of hirulog with (d-Phe)-Pro-Arg-Pro-Gly pentapeptide and the C-terminal tyr-sulfated dodecapeptide of hirudin (S-Hir53-64) shown that the presence of both active components is necessary for the inhibition of the fibrinogenolytic activity and activity towards chromogenic substrates [20]. Moreover experiments conducted with sulfated tyrosin-hirulog showed three to four times greater inhibition activity [20]. This conduct, pharmaceutical company to design new hirudin chimers likes hirudisins, a hirudin derivatives containing IIb/IIIa receptor

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HV-1 :  vvYTDCTESGQNLCLCEGSNVCGqGNKCILGSdGekNQCVTGEGTPkPqSHNdGDFEEIPEE-YLQ

HV-2 :  ITYTDCTESGQNLCLCEGSNVCGKGNKCILGSnGkgNQCVTGEGTPnPeSHNnGDFEEIPEE-YLQ

HV-3 :  ITYTDCTESGQNLCLCEGSNVCGKGNKCILGSqGKdNQCVTGEGTPkPqSHNqGDFEpIPEdayde
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Fig. (2). Hirudins variants (HV-1, HV-2, HV-3) sequence alignment. In caps are amino acid residues in common in the three variants (-) indicates a gap.

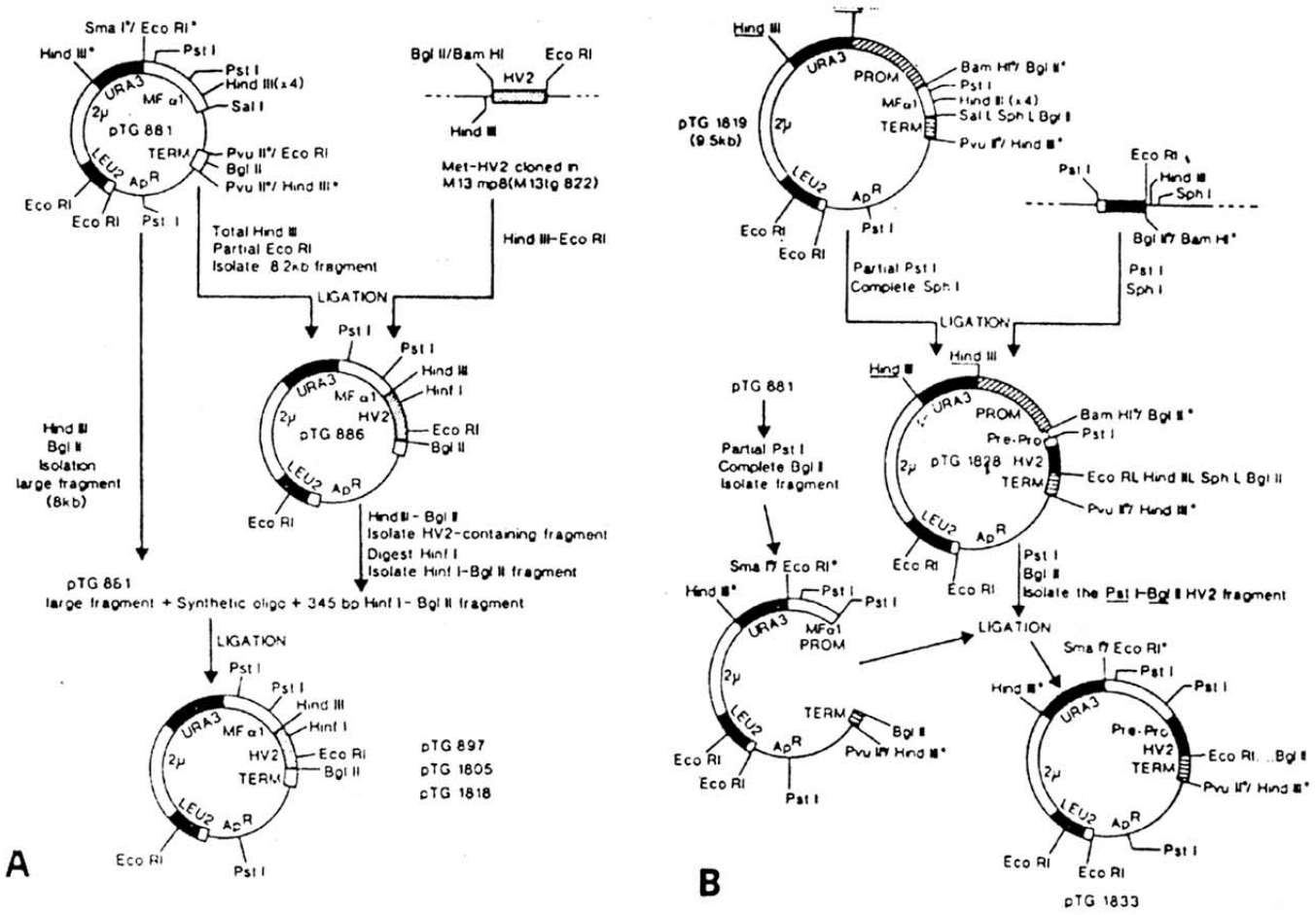

Fig. (3). Vector construction for extracellular expression of Hirudin in yeast [55].

and thrombin inhibition have been developed [31] Fragment 1-47 of hirudin HV-2 in which Val1 has been replaced by tert-butylglycine, Ser2 by Arg, and Tyr3 by beta-naphthylalanine, to give the BugArgNal analogue has been performed by De Filippis team [22]. The results of chemical and conformational characterization indicate that the synthetic peptide is able to fold efficiently with the correct disulfide topology (Cys6-Cys14, Cys16-Cys28, Cys22-Cys37), while retaining the conformational properties of the natural fragment. Thrombin inhibition data indicate that the effects of amino acid replacements are perfectly additive if compared to the singly substituted analogues (De Filippis V, Quarzago D, Vindigni A, Di Cera E, Fontana A, 1998, Biochemistry 37:13507-13515), yielding a molecule that inhibits the fast or slow form of thrombin by 2,670- and 6,818-fold more effectively than the natural fragment, and that binds exclusively at the active site of the enzyme with an affinity (Kd,fast = 15.4 pM, Kd,slow = 220 pM) comparable to that of full-length hirudin (Kd, fast = 0.2 pM, Kd, slow = 5.5 pM). Moreover, BugArgNal displays absolute selectivity for thrombin over the other physiologically important serine proteases trypsin, plasmin, factor Xa, and tissue plasminogen activator, up to the highest concentration of inhibitor tested (10 microM).

Table 3. Clinical Aspect of Hirudin

Specific and potent inhibitor of thrombin. No needs of cofactors
Inert pharmacodynamicaly, not immunogen, no effect on platelets, plasmatic proteins or enzymes
No complicated bleeding after high active doses of antithrombics
High disponibility after sub-coetaneous administration
No endogenous modulation, urinary excreted under active form
No binding on organs
Control of hirudotherapy by measuring the thrombin time
Efficacious on patients having anti-thrombin III deficiency
Can also be used when a default of platelets

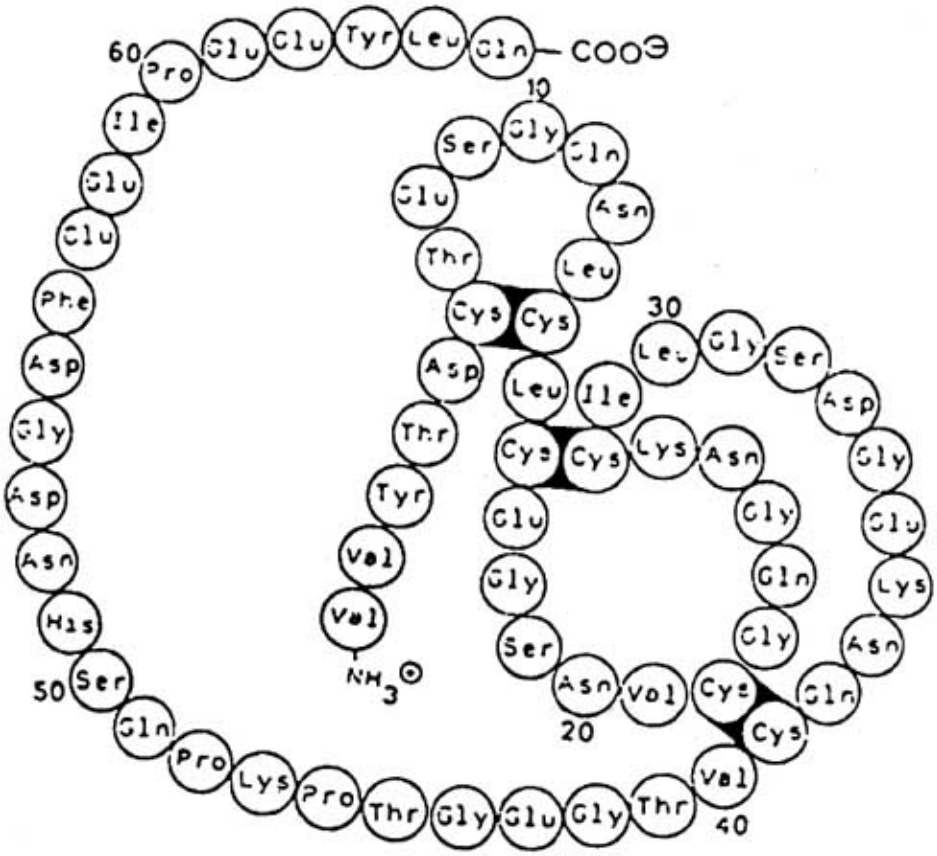

Fig. (4). Desulfato-hirudin recombinant structure [12] (specific activity : 12 000 ATU/mg, 69363 D, pI 3.7-3.8).

PHARMACOKINETICS OF HIRUDIN AND HIRULOGS

The pharmacokinetic of hirudin have been studied in healthy human volunteers. Findings are close to the one found on animals. Patients received a subcutaneous injection of 600 to 1000 antithrombin units (ATU) per kilogram of body weight or an intravenous dose of 100 ATU/kg. The subcutaneous doses produced rapidly rising plasma hirudin concentration pealed 90 to 180 min post-injection and then decreased steadily. Intravenous administration was followed by a rapid distribution phase, then by an elimination phase, the course being described by an open two compartments model [23]. Hirudin had an apparent volume of distribution of 0.33 L/Kg after subcutaneous administration and 0.24 L/Kg after intravenous administration [24] Intact hirudin is rapidly eliminated by renal excretion. Urinary excretion of hirudin accounted for 61.2% of a subcutaneous dose after 24 hours, with 50% being excreted during the first 6 hours after injection and no further excretion of intact hirudin after 24 hours. Following intravenous dos, 39% to 44% of the dose was recovered in the urine [24]. In healthy volunteers, after administration of single intravenous doses of 0.01 to 0.1 mg/Kg, a mean elimination half-life was about 1 hour, the mean total clearance of 190 mM/min and a volume of distribution of 14 L [25]. Administration of hirulog has shown a more rapid clearance and shorter half time than hirudin. With intravenous infusion, hirulog had plasma half time of 36 minutes [25].

PHARMACODYNAMICS OF HIRUDIN AND HIRULOGS

Hirudin significantly prolonged the partial thromboplastin, thrombin and Quick-test times in human subject. Thrombin time has the most sensitive reponse, but this response is not clearly correlated with plasma concentrations [24]. Doses ranging from 0.01 to 0.1 mg/Kg, the maxium anticoagulant effect was achieved with doses of 0.07 mg/Kg or higher [26]. No change in platelet count, fibrinogen concentrations or fibrinolytic factor was reported with hirudin [26]. In the case of hirulog a rapid dose-dependent prolongation of APTT, PT and thrombin time (TT) is produced [26]. The antithrombin activity of hirulog at doses of 0.25 mg kg^{-1} h^{-1} demonstrated by statistically decreases in plasma levels of fibrinopeptide A [27].

CLINICAL DEVELOPMENT

Its development, pre-clinical evaluation and introduction into clinical trials of hirudin, recombinant and its analogs have been performed since 10 years [28,29], Table 3). The complex forming by hirudin and thrombin is very close and

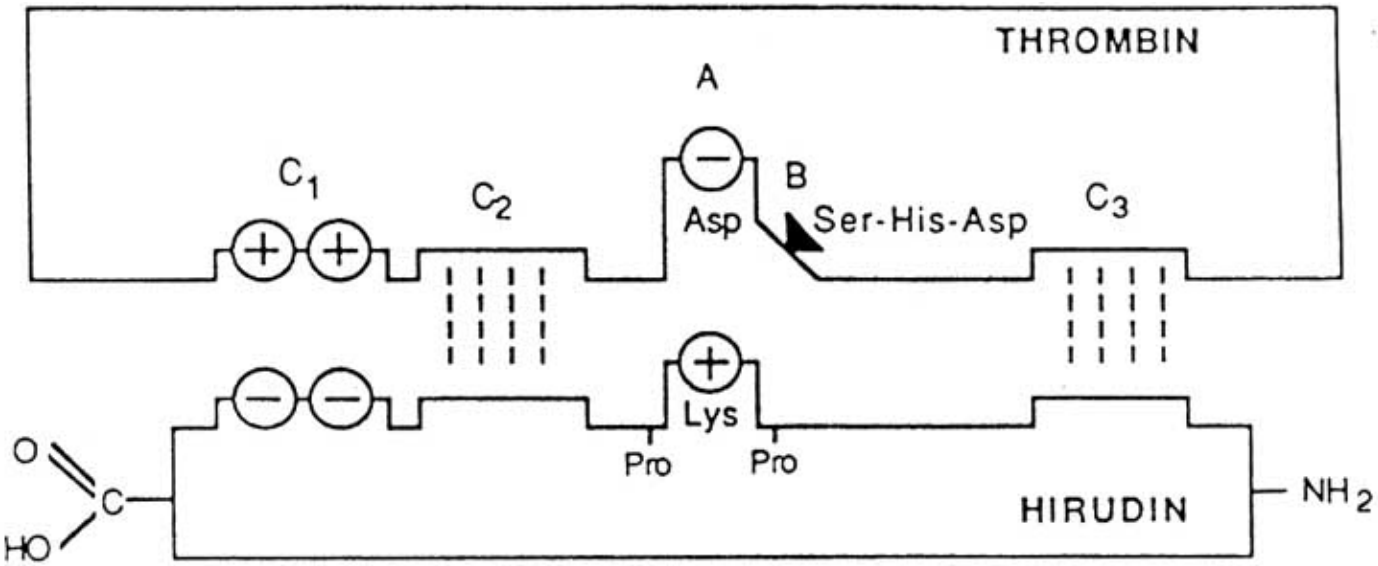

Fig. (5). Schemes of thrombin-hirudin complex [56].

is extended to a large area around the active site. Its half-life time in healthy young volunteers is 50 to 65 min [29]. In contrast, in older patients with established coronary artery disease with normal renal function (serum creatinine 1 $\pm$ 0.2 mg/dl), the plasma $t_{1/2}$ of hirudin is found to be 2 to 3 hours [30]. Bleeding times were not significantly prolonged in these two trials and only mildly prolonged during administration of recombinant-hirudin [31]. Recombinant-hirudin appears to be weak allergen and hirudin-specific IgE antibodies are scarce in 163 volunteers receiving twice at a 1-month interval the recombinant molecule [32]. No antibodies were detected 2 weeks after the infusion. Moreover, experiences with recombinant-hirudin in preventing or treating venous thrombo-embolism are very preliminary (Table 2). However, recombinant-hirudin (r-hirudin) achieved promising results in patients with unstable angina or important coronary angioplasty. A trial with 166 patients were randomized to a 72- to 120-hour infusion of heparin (n=50) or recombinant hirudin (n=116) at 5 escalating dosages from 0.05 mg/kg/h to 0.3mg/kg/h) [33]. Results shown that hirudin improved the angiographic endpoints compared to high dose of heparin [33]. However, inpatients with acute mycoardial infarction, 3 important clinical trials were stopped because of an excess of bleeding complications [33].

The development of direct thrombin inhibitors has growth up since the structural elucidation of hirudin to the development of a recombinant equivalent protein (r-hirudin). The increased interest in thrombin inhibitors is also prompted by reports of heparin-induced thrombocytopenia (HIT type II) with heparin and the need to anticoagulate patients with alternate drugs. These agents produce a direct anticoagulant response by targeting thrombin. In addition, the amplification of the coagulation cascade by thrombin activation of factors V and VIII, these thrombin inhibitors also inhibit platelet activation [29-33]. Recombinant-Hirudin (rH) yielded promising results in patients with unstable angina [33]. In GUSTO (Global Use if Strategies to open Occuled Coronary Arteries) IIb Trials on patients with unstable angina results shown that in vivo thrombin generation and activity are reduced during intravenous infusion of rH. Moreover, in OASIS (Assess Strategies for Ischemic Syndromes) 2 clinical trials, investigators shown that rH is superior to heparin in preventing cardiovascular death, myocardial infarction (MI) and refractory angina or acute myocardial infarction without ST elevation [34, 35]. Desirudin, a rH used in the prevention and management of thromboembolitic disease binds directly and with high affinity to clot-bound and fluid phase thrombin [36]. In patients undergoing HIP replacement surgery, desirudin was significantly more effective in reducing the incidence of deep vein thrombosis (DVT) than either unfractionated or low molecular weight heparin [36]. A significant reduction with rH compared with heparin in the incidence of death or non-fatal (re) infraction at 24 hours in patients with acute MI was reported in GUSTO IIb trials but not in the TIMI (Thrombolysis and Thrombin Inhibition in Myocardial Infraction) 9B trial. So, it appears that rH is most effective than heparin in the prevention of DVT in patients undergoing elective replacement [36]. By contrast in the treatment of acute coronary syndrome, the role of rH is less certain. In clinical study, hirulog was tolerated and then were no major hemorrhagic or thrombotic complications even with concomitant administration of aspirin [27].

NEW LEECH THROMBIN INHIBITORS

Most of the polypeptides belonging to the family of thrombin inhibitors, i.e. Hirudin, granulin-like peptide, bufridin, Hillurin, Haemadin, Theromin, have been isolated from jawed leeches, such as the amphibian parasite leech *Hirudo medicinalis* [37], Hirudo nipponia [38], the mammalian parasite *Hirudinaria manillensis* [56] or the land living *Haemadipsa sylvestris* [39,40]. Only theromin has recently been isolated from the gut-leech *Theromyzon tessulatum* (Table 4, [41]). Haemadin from *Haemadipsa*

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>Hirudin
------------------------vvytdctesgqnlclCE--gsnvcgq
gnkcilgsdgeknqcvtGEGTPKPQSHNDGDFEEIPEEYLQ
>Hirullin [57]
------------------------vsytdctsgqnyclc----ggnfcgd
gkhcemdgsenkcvdgeGTPKRQTSGPSDFEEFSLDDIEQ-
>Haemadin
--------------------IRFGmgkvpcpdgevgytcDC--gekicly
gqscndgqcsgdpkpssEFEEFEIDEEEK------------
>Theromin
ECENTECPRACPGEYEFDEDGCNTcvckgcddaqcrcssDANGcesfctc
ntrcsaadecnprctck-----------------------
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Fig. (6). Sequences comparison of leech thrombin inhibitors. In caps amino acid residues with high similarities between the different sequences.

sylvestris and theromin are completely novel therombin inhibitor with no sequence homologies with any factor inhibitor yet sequences throughout the animal kingdom (Fig. **6**).

Unlike the European leech *Hirudo medicinalis*, the Asian jawed leech Hirudinaria manillensis is specialized for feeding on mammalian blood. In the salivary glands of both these leeches, there is a potent inhibitor of thrombin, called hirudin, which acts as an anticoagulant. Purification of a variant of hirudin, called bufrudin, from the head portions of Hirudinaria has been performed by Sawyer team [57]. Comparison of the primary structure of bufrudin, with hirudin HV1, show about 70% sequence identity with deletion of two amino acids, but the key amino acids at the C-terminus, involved in the inhibition of thrombin, are conserved. However, similar sequence comparison of bufrudin with hirullin P18 [58], a hirudin variant isolated from the same leech species but from whole leech, instead of heads, reveals even less sequence identity of about 60%. Hirullin P18 is a 61-amino acid hirudin-related protein having potent antithrombin activity. Similar to hirudin, it contains a highly acidic C-terminus, but has a significantly different sequence from any other known hirudin variant. The C-terminal fragment acetyl-hirullin P18[41-62] possesses an antithrombin potency similar to that of acetyl-desulfatohirudin[45-65]. Additionally, like the hirudin fragment analog, it inhibits fibrin-clot formation by binding to a non-catalytic site on thrombin. Sequential shortening of the hirullin P18 C-terminal fragment demonstrates the critical nature of Phe51, which corresponds to the important Phe56 residue of hirudin. Although the sequences of hirullin P18[54-61] and hirudin[59-65] have substantial differences, the C-terminal functional domain represented by hirullin P18[50-61] appears to be comparable to hirudin [55-65] in terms of its functional role in antithrombin activity. Comparison between bufridin and hirullin suggested that the conformation of the C-terminal portion of bufrudin may be significantly different from hirullin P18, but similar to hirudin HV1, upon its interaction with thrombin. These results indicate that, as with Hirudo leech, various isoforms of hirudin also exist in Hirudinaria leech, with a significant change occurring in the structure of the molecule during the evolution of leeches.

Table 4. **Thrombin Inhibitor Family Active Site and Substrates Isolated from Leeches *i.e.*Theromyzon Tessulatum, Hirudo Medicinalis and Haemadipsa Sylvestris.(φ : No Inhibition)**

Enzymes	Theromin	Hirudin	Haemadin
Chymotrypsin	φ	φ	φ
Trypsin	φ	φ	φ
Cathepsin G	φ	φ	φ
Plasmin	φ	φ	φ
Urokinase	φ	φ	φ
Elastase	φ	φ	φ
Thrombin	12 fmol	21 fmol	100 fmol
Factor Xa	φ	φ	φ

Haemadin has been isolated from the leech, *Haemadipsa sylvestris* [39]. This peptide is a slow, tight-binding inhibitor of thrombin with an apparent molecular mass of about 5 kDa. Its inhibitory activity, called haemadin, is thrombin specific since it does not inhibit other proteases like trypsin, chymotrypsin, factor Xa, or plasmin. NH2-terminal amino acid sequence analysis (residues 1-45) does not reveal any homology to known serine protease inhibitors, including the thrombin-specific inhibitor hirudin. The haemadin cDNA cloned by polymerase chain reaction techniques codes for a polypeptide of 57 amino acid residues preceded by 20 residues of a signal peptide sequence. A synthetic gene coding for the mature haemadin was expressed in Escherichia coli. Recombinant haemadin displays a similar inhibition constant and specific activity as its natural counterpart. Although there is no obvious sequence identity between haemadin and hirudin, both proteins

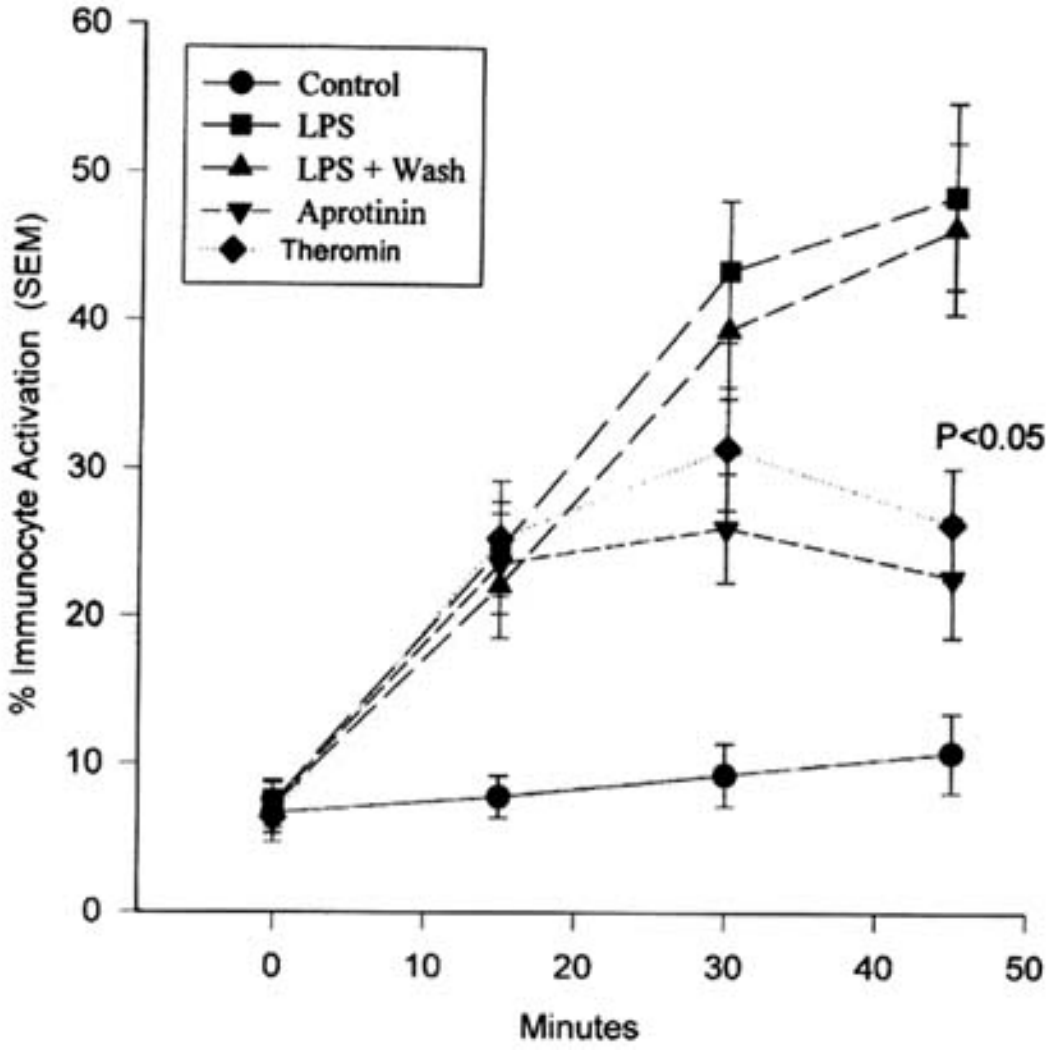

Fig. (7). Demonstration of the dual nature of LPS activation of human monocytes. LPS (1 U/ml) stimulates monocytes in a manner that can be diminished by aprotinin (10^{-6} M) and Theromin (10^{-6} M). The percentage activation is noted by computer-assisted microscopy where the number of cells exhibiting form factors below 0.50 is compared to those exhibiting this factor at 0.70 and above. Details of the assay are found in the text.

seem to share common mechanisms for thrombin inhibition [39]. A crystal structure of the human alpha-thrombin- haemadin complex has recently been realized [40]. The N-terminal segment of haemadin binds to the active site of thrombin, forming a parallel beta-strand with residues Ser214-Gly216 of the proteinase. This mode of binding is similar to that observed in another leech-derived inhibitor, hirudin. In contrast to hirudin, however, the markedly acidic C-terminal peptide of haemadin does not bind the fibrinogen-recognition exosite, but interacts with the heparin-binding exosite of thrombin. Thus, haemadin binds to thrombin according to a novel mechanism, despite an overall structural similarity with hirudin. Haemadin inhibits both free and thrombomodulin-bound alpha-thrombin, but not intermediate activation forms such as meizothrombin.

A cysteine-rich (approximately 20%), low molecular weight (MW 6 kDa) polypeptide has been isolated from the Korean blood-sucking leech, *Hirudo nipponia* [38]. From its amino acid composition and N-terminal amino acid sequence analysis, the new protein is similar to granulin (or epithelin), and so it has been named leech granulin. The leech granulin behaved as a thrombin inhibitor in the purification steps of size-exclusion, ion-exchange, chromatofocusing, and reverse-phase high-performance liquid chromatography. The leech granulin is an acidic peptide (pI 3.75) containing high cysteine residues with a unique sequence similar to granulins or epithelins isolated from other organisms. For the first time, a granulin-like polypeptide having thrombin inhibitory activity has been isolated from a leech species

· The most potent inhibitor is yet known is theromin (Ki of 12 fM *vs.* 21 fM for hirudin). This peptide has been isolated from the gut leech *Theromyzon tessulatum* [41]. This is a homodimer of 67 amino acid residues, with 16 cysteines engaged in eight disulfide bridges. Compared with Hirudin, both peptides are anionic and rich in cysteine residues [41]. Theromin's N-terminal sequence is highly anionic and its C-terminal part very tight, owing to the 10 cysteine residues present there. The Hirudin N-terminal sequence is known to interact with the catalytic site of thrombin and amino acid residues 46 to 48 (PKP) are important for the link between Hirudin and thrombin [22]. The same sequence has also been found in Heamadin [39]and *H. Medicinalis* anti-thrombin peptides [37]. These types of interactions explain why Hirudin only binds to thrombin and not to other serine proteases. By contrast, Theromin does not possess such a dramatic signature in its sequence. In fact, Theromin has no significant sequence homology with any other animal thrombin inhibitor so far isolated. However, if Theromin which contains about 24% cysteine residues, is compared with potentially homologous inhibitors, low identity is observed with antistasin-type protease inhibitors and concerns mainly cysteine residues. But, considering the low level of general sequence identity between Theromin and peptides of this family, it is difficult to consider Theromin as a new member of the antistasin-type family. However, sequence comparisons carried out for Theromin with the four different serine protease inhibitors isolated from the leech *T. tessulatum i.e.* Cytin, Therin, Therostasin and Tessulin [42-45]. This revealed that two of the four *i.e.* Therostasin [45], and Tessulin [43], have a high degree of sequence identity with theromin (70 % and 52% respectively). Furthermore, if Theromin is aligned with these potentially homologous inhibitors, the three proteins show an identity from residues 2-28 with the exception of Val/Leu substitution at position 26 for theromin (Fig. **2**). The rest of the sequences are less similar to one to another. However, between Therosastin and Theromin, another region is also well conserved *i.e.* a consensus sequence has been found in residues 40-53 of Theromin (^{40}DANGCESFCTCNTR^{53}). Interestingly, the putative active sites of the trypsin inhibitor, Tessulin (^{25}CLCKEPC^{31}) [43] and the factor Xa inhibitor, Therostasin (^{33}AQCRIYC^{39}) [45] are not conserved in the thrombin inhibitor, Theromin. Thus, the observed similarities could be the result of an evolutionary divergence from an ancestral gene, arising after gene duplication, able to generate several peptides acting towards specific substrates. We can also add to the above *Theromyzon* molecules, the three other thrombin inhibitors discovered in *Theromyzon* by Hamberger and collaborators (US patent PCT/EP94/01404). In fact, the Merck Company 1994 deposited foreign patent applications regarding three thrombin inhibitors of mass 3 kDa, 9 kDa and 14 kDa. Interestingly, the N-terminal sequence of the 9 kDa inhibitor, EDDNPGPPRACPGE (US patent PCT/EP94/01404), shows homology with those of Theromin (ECENTECPRACPGE), the factor Xa inhibitor Therostasin (DCENTECPRACPGE) [45], and the trypsin inhibitor Tessulin (MCENTECPRACPGE) [43]. This 9 kDa thrombin inhibitor peptide possesses a PI of 4.9, a clotting time in a fibrinogen test of >600 s/5 µl, and a specific activity at the final step of purification of 25 IU for thrombin inhibition and 0.2 IU for Factor Xa inhibition and clotting fibrinogen assays performed on different species of *Theromyzon* (*T. binannulatum*, *T. cooperi*, *T. garjaewi*, T. *maculosum* and *T. sexoculatum*) confirms the presence of thrombin inhibitor(s) in these gut leeches (US patent PCT/EP94/01404). These data reveal that *Theromyzon* possesses different isoforms of thrombin inhibitor. However, the 9 kDa protease inhibitor possesses thrombin and factor Xa inhibitory activities and, when we compared its activity to the one we obtained for Theromin, we can observed that it is not a tight binding inhibitor for thrombin. We suspect that this molecule might be a more specific inhibitor of another protease. Interestingly, Theromin, in a dose-dependent manner, significantly diminishes the level of human granulocyte and monocyte activation induced by lipopolysaccharides (1U/ml, Fig. **7**). Moreover, in contrast to hirudin, which at concentrations from 0.1 to 2 µM, induced vasodilation of PGF2 alpha-precontracted ring segments of porcine pulmonary arteries with intact endothelium, theromin has no effect on human saphenous vein endothelial cells [41].

CONCLUSION

Thrombin inhibitors isolated from invertebrates like leeches has revealed clinical development to threat cardiovascular diseases. Beside this class of molecules, factor Xa inhibitor, platelet aggregation inhibitors have also been isolated from leeches [46,47]. Among the different anticoagulant molecules in leeches, involved in the inhibition of the coagulation cascade or in inhibition of the platelet aggregation (Fig. **1**), three substances have been investigated in great detail *e.g.* Hirudin (thrombin inhibitor, [48-51]), antistasin (factor Xa inhibitor, [52]) and decorsin (antagonist of platelet membrane glycoprotein IIb-IIIa, [53]). Although the amino acid sequences of these molecules differ and their inhibitory activity, their three-dimensional structures share the same conformational motif corresponding to the Leech Antihemostatic Protein (LAP: Cys-X6-12-Cys-X-Cys-X3-6-Cys-X3-6-Cys8-14) [54]. Interestingly, their mechanisms of action and epitopes important for binding to their respective targets are distinct as well [54].

Taken together, leeches are really a reservoir of new powerful natural peptides to treat inflammation, coagulation, thrombosis, emphysema, cancer, all aging diseases.

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