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Minireview

Anticoagulants and inhibitors of platelet aggregation derived from leeches

Michel Salzet*

Laboratoire d'Endocrinologie et Immunité des Annélides, UPRESA CNRS 8017, Université des Sciences et Technologies de Lille, SN3, F-59655 Villeneuve d'Ascq Cedex, France

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Abstract Increased life expectancy is associated with aging populations in the developed countries, and we can expect an increased incidence of cardiovascular and inflammatory diseases and cancers. A priority for medical research is to reduce such morbidity. Leeches have been demonstrated to be a useful source of drugs to treat cardiovascular diseases, as they have evolved highly specific mechanisms to feed on their hosts by blocking blood coagulation. Powerful molecules acting at different points in the coagulation cascade or in the inhibition of platelet aggregation have been purified from these animals. Moreover, clinical trials confirm their potential to treat cardiovascular diseases.

Key words: Anticoagulant; Leech; Platelet aggregation inhibitor

1. Introduction

Animals depending on a diet of fresh blood have evolved mechanisms that interfere with the coagulation process of the blood donor. Normally, a nociceptive stress due to injury will lead to an inflammatory response with a large number of leucocytes. It is in the leech's interest to avoid this, as during the months used to digest the blood meal the leucocytes will release blood-degrading enzymes. Thus, the challenge for the leech is to block peripheral nociception during the bite, to reduce local inflammation, and to synthesize anticoagulants that maintain the blood in a fluid state during intake and subsequent digestion [1]. In this regard, a variety of coagulation inhibitors has been isolated from blood-sucking animals such as bats [2], ticks [3,4], leeches [1,5], and hookworms [6]. Here we review the array of anticoagulant molecules and platelet aggregation inhibitors derived from leeches: their therapeutic uses and clinical trials.

2. Coagulation factor inhibitors

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 detail. These are hirudin, a thrombin inhibitor [7], antistasin, a factor Xa inhibitor [8], and decorsin, an antagonist of platelet membrane glycoprotein IIb-IIIa (Table 1) [9]. Although the amino acid sequences and the inhibitory activity of these molecules differ, their three-dimensional structures share the same conformational motif, corresponding to leech antihemostatic protein (LAP: Cys-X6-12-Cys-X-Cys-X3-6-Cys-X3-6-Cys8-14) [10]. Interestingly, their mechanisms of action and the epitopes important for binding to their respective targets are also distinct [10]. This demonstrates the significance of diverse mechanisms in inhibiting the coagulation process, as well as the evolution of slightly different inhibitory processes.

2.1. Thrombin inhibitors

2.1.1. Hirudin. Because thrombin is a key enzyme in the pathogenesis of acute coronary thrombosis, therapy with heparin, an indirect thrombin inhibitor, has developed over the last three decades. The direct anti-thrombins, such as hirudin, have emerged since the studies of Markwardt in 1957 [7]. The presence of hirudin in the salivary glands of the leech, *Hirudo medicinalis*, was first discovered by Haycraft in 1884 [11]. Its role as a potent anti-thrombotic drug was first investigated by Shionoya in 1927 [12], and its structure was established in 1950.

Hirudin is a natural single-chain peptide of 65 residues with three intra-chain disulfide bridges and a sulfated tyrosine residue [13–16]. its N-terminal part is globular and very compact owing to the presence of the three disulfide bridges. By contrast, numerous negatively charged amino acid residues constitute its C-terminal part. Over 100 years after its discovery, its cDNA was cloned and large-scale production of recombinant hirudin (rH) has been achieved in *Escherichia coli* [17], in the yeast *Saccharomyces cerevisiae* [18], and now from *Acremonium chrysogenum* [19]. Compared with low molecular weight heparins (Table 2), hirudin is a strict tight binding inhibitor of thrombin [20]. It does not need a co-factor for its activity. Its development, pre-clinical evaluation and introduction into clinical trials of its recombinant and analogue forms were perfected only 10 years ago [21].

The complex formed between hirudin and thrombin involves both the three N-terminal amino acid residues, which bind near to the active site, and the C-terminal tail that binds to the fibrinogen binding site. Crystal structure studies have allowed researchers to conclude that the last 10 amino acid

*Fax: (33)-3-2004 1130.
E-mail: michel.salzet@univ-lille1.fr

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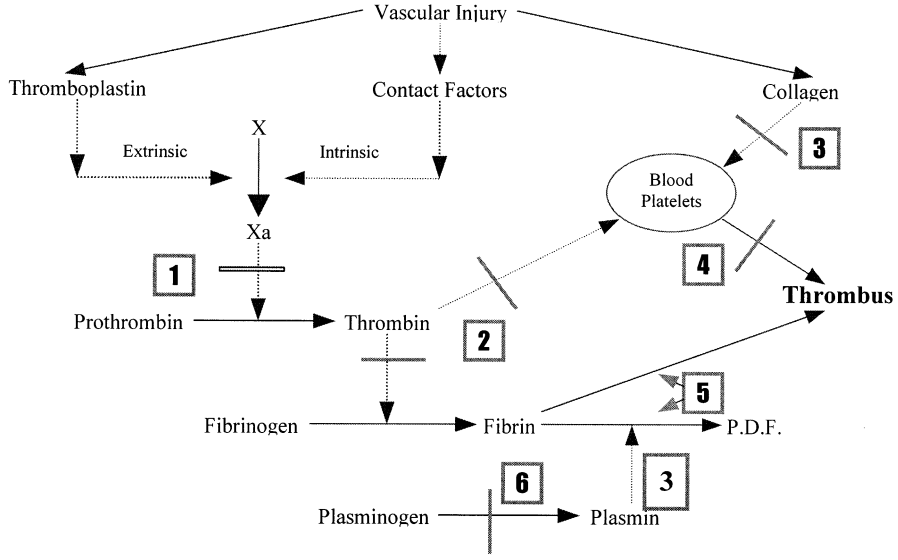

Fig. 1. Targets of leech anticoagulants on homeostasis: up to date from Markwardt. 1: Antistasin, ghilanthen, therosasin; 2: hirudin, haemendin, bufrudin, theromin; 3: calin, LAPP; 4: decorsin, ornatin; 5: hementin, destabilase; 6: hementerin.

residues of the C-terminal region (residues 55–65) react with the anion binding exosite site of thrombin, which is an important region for fibrinogen binding [22]. Residues 1–48 of the N-terminal part are also important for hirudin's action on thrombin [22]. They interact with the catalytic site. These types of interaction explain why hirudin only binds to thrombin and not to other serine proteases.

The development of direct thrombin inhibitors has advanced from the elucidation of hirudin's structure to the development of a recombinant equivalent protein (r-hirudin). Increased interest in thrombin inhibitors is also prompted by reports of heparin-induced thrombocytopenia (HIT type II) using heparin, and the consequent need to treat patients with alternative anticoagulant drugs. These agents produce a direct anticoagulant response by targeting thrombin. In addition, these thrombin inhibitors also inhibit platelet activation by amplifying the coagulation cascade by thrombin activation of factors V and VIII [23–26]. rH has produced promising results in patients with unstable angina [27]. In the series of trials termed Global Use of Strategies to open Occluded Coronary Arteries (GUSTO) IIb, studies on patients with unstable angina show that in vivo thrombin generation and activity are reduced during intravenous infusion of rH. Moreover, two clinical trials in the series Assess Strategies for Ischemic Syndromes (OASIS), have shown that rH is superior to heparin in preventing cardiovascular death, myocardial infarction (MI) and refractory angina or acute MI without ST elevation [28,29]. Desirudin, an rH used in the prevention and management of thromboembolitic disease, binds directly and with high affinity to clot-bound and fluid-phase thrombin [30]. In patients undergoing hip-replacement surgery, desirudin was significantly more effective in reducing the incidence of deep vein thrombosis (DVT) than either un-fractionated or low molecular weight heparin [30]. When rH was compared with heparin, there was a significant decrease in the incidence of death or non-fatal (re) infarction at 24 h in patients with acute MI was reported in GUSTO IIb trials but not in the Thrombolysis and Thrombin Inhibition in Myocardial Infraction (TIMI) 9B trial. Thus, it appears that rH is more effective than heparin in the prevention of DVT in patients undergoing elective replacement [30]. By contrast, the role of rH is less well established in the treatment of acute coronary syndrome [30].

2.1.2. New molecules discovered. Besides hirudin [7], other thrombin inhibitors have been isolated from leeches. These include granulin-like peptide [31], bufrudin [32], theromin

Table 1
Clinical phases of molecules extracted from leeches

Products	Activity	Therapy target	Clinical level	Company
Eglin	inhibitor of elastase/ chymotrypsin	emphysema; non-steroidal anti-inflammatory	gene cloned in *E. coli*, pre-clinical development	CIBA/Geigy, Basel, Switzerland
Gelin	inhibitor of elastase/ chymotrypsin	gingivitis and periodontitis; dermatitis; non-steroidal anti-inflammatory	clinical trials in humans for oral application and for dermatis	EuroBioPharm, Ridderkerk, The Netherlands
Ghilanten	inhibitor of factor Xa	anticoagulant, antimetastatic	primary structure studies; evaluation in animal metastatic models	Merrell Dow Research Inst., Cincinnati, OH, USA
Hementin	fibrinogenolytic enzyme	inhibits platelet aggregation	gene cloning; pre-clinical development	Biopharm, Charleston, USA
Orgelase	hyaluronic acid-specific hyaluronidase	MI; drug delivery; ophthalmology	pre-clinical development; including ex vivo studies for drug delivery through human skin	Biopharm, UK
Hirudin	thrombin inhibitor	MI	phase IV development	Transgene, France

Table 2
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
Anti-thrombin III-independent	Anti-thrombin III-dependent
Not neutralized by heparinase, endothelium macrophages, fibrin monomer, plasma protein, platelet activator 4, neutral endopeptidase	Neutralized by heparinase, weak endothelium binding
Inactivates clot-bound thrombin	Does not inactivate clot-bound thrombin and factor VII
Prevents thrombin-induced aggregation but not other platelet agonist	Inhibits platelet function
Does not induce thrombocytopenia	Can induce thrombocytopenia
Good bioavailability (80% after subcutaneous injection)	Bioavailability 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

[33] and haemadin [34]. Haemadin and theromin are completely new thrombin inhibitors having no sequence homology with any other inhibitory factor yet sequenced throughout the animal kingdom. Haemadin has been isolated from the leech, *Haemadipsa sylvestris* [34]. It is a peptide of 5 kDa and with a K_i of 100 fM, less active than both hirudin (21 fM) and theromin (12 fM).

The most potent inhibitor is theromin (K_i of 12 vs. 21 fM for hirudin), which has been isolated from the gut leech *Theromyzon tessulatum* [33]. 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 [33]. Theromin's N-terminal sequence is highly anionic and its C-terminal part very compact, owing to the 10 cysteine residues present there. Hirudin's N-terminal sequence interacts with the catalytic site of thrombin, and amino acid residues 46–48 (PKP) are important for the link between hirudin and thrombin [22]. The same sequence has been found in haemadin [34] and in *H. manillensis* antithrombin peptides [32]. 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, containing about 24% cysteine residues, is compared with potentially homologous inhibitors, there is moderate similarity to antistasin-type protease inhibitors. This concerns mainly cysteine residues. However, considering the low level of general sequence identity between theromin and peptides of this family, it is difficult to claim theromin as a new member of the antistasin-type family. However, sequence comparisons have been carried out between theromin and four different serine protease inhibitors isolated from the leech *T. tessulatum*: cytin, therin, therostasin, and tessulin [35–38]. This revealed that two of the four, therostasin [38] and tessulin [35], 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 identity from residues 2 to 28, with the exception of Val/Leu substitution at position 26 for theromin. The rest of the sequences are less similar to each other. However, another region is also well conserved between therostasin and theromin, namely a consensus sequence in residues 40–53 of theromin (^{40}DANGCESFCTCNTR^{53}). Interestingly, the putative active sites of the trypsin inhibitor, tessulin (^{25}CLCKEPC^{31}) [36], and the factor Xa inhibitor, therostasin (^{33}AQCRIYC^{39}), are not conserved in the thrombin inhibitor, theromin [33]. 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, 9 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) [38], and the trypsin inhibitor tessulin (MCENTECPRACPGE) [36]. This 9 kDa thrombin inhibitor peptide possesses a p*I* 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. Clotting fibrinogen assays performed on different species of *Theromyzon* (*T. binannulatum*, *T. cooperi*, *T. garjaewi*, *T. maculosum* and *T. sexoculatum*) confirm the presence of thrombin inhibitor(s) in these gut leeches (US patent PCT/EP94/01404). Taken together, 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 observed that it is not a tightly binding inhibitor for thrombin (US patent PCT/EP94/01404). We suspect that this molecule might be a more specific inhibitor of another protease. Interestingly, theromin significantly diminishes the level of human granulocyte and monocyte activation induced by lipopolysaccharides (LPS) in a dose-dependent manner (1 U/ml). Moreover, in contrast to hirudin, which at concentrations from 0.1 to 2 μM induced vasodilatation of PGF2 α-precontracted ring segments of porcine pulmonary arteries with intact endothelium, theromin has no effect on human saphenous vein endothelial cells [33]. Taken together, these recent data indicate that these new leech thrombin inhibitors still offer prospects for future development of practical applications.

2.2. Factor Xa inhibitors

The complementary roles of platelets and thrombin in the pathophysiology of acute coronary syndromes suggest that,

Table 3
Comparison of active site sequences of antistasin-type protease inhibitor

	P4	P3	P2	P1	P1′	P2′	P3′	Inhibitors of	Reference
Antistasin I	V	R	C	R	I	H	C	factor Xa, trypsin	[40]
Antistasin II	I	N	C	R	K	T	C	factor Xa, trypsin	[40]
Ghilanten	V	R	C	R	V	Y	C	factor Xa, trypsin	[41]
Guamerin I	I	R	C	M	I	F	C	elastase	[62]
Guamerin II	I	R	C	M	I	F	C	chymotrypsin	[63]
Hirustasin	V	H	C	R	I	R	C	cathepsin G, trypsin	[64]
Tessulin	C	L	C	K	E	P	C	chymotrypsin, trypsin	[37]
Therin	Y	L	C	K	M	A	C	trypsin	[35]
Therostasin	A	Q	C	R	I	Y	C	factor Xa	[38]

for treatment to be effective, both mediators must be targeted [39]. The direct utilization of factor Xa inhibitors is also a promising route for anti-thrombotic therapy [39].

2.2.1. The antistasin protein. The first factor Xa inhibitor found in leeches was antistasin, a 15 kDa protein isolated from the salivary glands of the Mexican leech, *Haementeria officinalis* [40]. A homologous protein, ghilanthen, has also been found in *Haementeria ghiliani* [41]. Antistasin consists of 119 amino acid residues, of which residues 1–55 (domain I) are 56% similar to residues (56–110) (domain II). Of the nine C-terminal amino acids (111–119; domain III), four are positively charged [40]. The reactive site is located in domain I [41–43]. The cDNA has been cloned [42], and the recombinant protein has been produced in an insect baculovirus host vector system [43]. Pharmaceutical studies have been performed, and the data show that the protein is still active 30 h after injection in animals. Moreover, when tested in different thrombosis models, antistasin was superior to heparin [44]. For example; recombinant antistasin (rA) has been shown to reduce restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits [44]. Moreover, chimeric peptides with only domain I have been tested [40]. Neither domain II nor III contain any intrinsic factor Xa inhibitory activity, nor do they contribute to the activity of domain I. The most potent synthetic peptide derived from antistasin corresponds to amino acids 27–49 with a disulfide bridge (ATS29–47). This inhibited factor Xa with a K_i of 35 nM [45]. The shortest peptide displaying anticoagulant activity was D-RCRVHCP, which increased clotting times by 50% at micromolar concentrations [45]. Based on these results and the understanding of the molecular mechanisms implicated in factor Xa inhibition, new anticoagulants have been produced (e.g. DX-9065a) [46]. Tolerability, pharmacokinetic and pharmacodynamic studies of DX-9065a have demonstrated that this synthetic inhibitor has a good correlation between linear pharmacokinetics and pharmacodynamics after intravenous administration in humans [47]. In vitro characterization of RPR130737, a novel synthetic factor Xa inhibitor, showed a selectivity of 1000-fold over thrombin in terms of activated protein C, plasmin, tissue-plasminogen partial thromboplastin time, and prothrombin time in a dose-dependent fashion [47].

As well as antistasin, we also isolated therostasin from *T. tessulatum*. This is a potent naturally occurring tight binding inhibitor of mammalian factor Xa (K_i 34 pM) [38]. Therostasin is a cysteine-rich protein consisting of 82 amino acid residues with 16 cysteine residues. Sequence analysis reveals that

Table 4
Molecules isolated from leeches and implicated in inhibition of the coagulation cascade or in the inhibition of platelet aggregation

Species	Molecule	Biological action	Type of leech	Reference
H. medicinalis	calin	platelet aggregating inhibitor factor	jawed	[51]
	bdellins	trypsin and plasmin inhibitors	jawed	[65]
	bdellastasin	trypsin and plasmin inhibitor	jawed	[66]
	destabilase	fibrin de-polymerase factor	jawed	[57]
	LDTI	tryptase, trypsin, chymotrypsin	jawed	[67]
	hirudin	inhibitor	jawed	[13]
	hirustasin	thrombin inhibitor trypsin, chymotrypsin, cathepsin G, kallikrein inhibitor	jawed	[64]
Hirudo nipponia	granulin	thrombin inhibitor	jawed	[31]
	guamerin I	elastase inhibitor	jawed	[62]
	piguamerin	trypsin and kallikrein inhibitor	jawed	[68]
Hirudinaria manillensis	bufrudin	thrombin inhibitor	jawed	[32]
	gelin	elastase, cathepsin G, chymotrypsin, subtilisin inhibitor	jawed	[69]
H. sylvestris	haemadin	thrombin inhibitor	jawed	[34]
Whitmania endulata	guamerin II	elastase, chymotrypsin inhibitor	jawed	[63]
Macrobdella decora	decorsin	GPIIb/IIIa inhibitor	jawed	[9,55]
H. officinalis	antistasin	factor Xa inhibitor	proboscis	[40]
	LAPP	platelet aggregating inhibitor factor	proboscis	[56]
H. ghilianii	ghilanten	factor Xa inhibitor	proboscis	[41]
	hementin	fibrinolytic enzyme	proboscis	[70]
Haementeria lutzi	hementerin	fibrigenolytic enzyme	proboscis	[60]
Placobdella ornata	ornatin	GPIIb/IIIa inhibitor	proboscis	[56]
T. tessulatum	cytin	chymotrypsin inhibitor	proboscis	[37]
	tessulin	trypsin, chymotrypsin inhibitor	proboscis	[36]
	therin	trypsin inhibitor	proboscis	[35]
	theromin	thrombin inhibitor	proboscis	[33]
	therostasin	factor Xa inhibitor	proboscis	[38]

therostasin is completely novel. Its cDNA (825 bp) encodes a polypeptide of 82 amino acid residues preceded by 19 residues representing a signal peptide sequence. As with the other known inhibitors of factor Xa, therostasin is expressed and stored in cells of the leech salivary glands [38]. This peptide is also different from the factor Xa inhibitor isolated from *Haementeria depressa*, lefaxin [48]. Amidolytic activity of lefaxin on chromogenin substrate revealed an apparent K_i of 4 nM. This protein, of 30 kDa with an isoelectric point of 5.7, has sequence similarity with the nitric oxide carrier protein myohemerythrin from the annelid *Nereis diversicolor* [49].

2.2.2. Antistasin-type molecules. The P1 residue in serine protease inhibitors most often reflects the specificity of the proteinase that is being inhibited; i.e. lysine or arginine residues for trypsin-like enzymes and phenylalanine and leucine for chymotrypsin-like enzymes [50]. For factor Xa inhibitor, P1 is often an arginine-like residue, as in antistasin and ghilanthen. Based on these considerations and because several molecules have the same-spacing in the 10 cysteine residues, a novel family has been defined: the antistasin-type family (Table 3). Molecules of this family display different activities towards serine proteases, and it appears that the major differences in specificity are caused by differences in the sequence surrounding the reactive site (Table 3). Moreover, some of the molecules of this family, such as therin and tessulin, show anti-inflammatory activity, [35,36]. Experiments conducted with LPS to mimic a septic injury show that LPS stimulates immunocytes in a process that exhibits primary and secondary levels of stimulation. The LPS-stimulated secondary effect requires enzyme-processed secretory products released from the immunocytes [35,36]. Therin, tessulin or therostasin applied alone diminish the immunocyte activation level; administered in combination they act synergistically. This indicates that they also exert an enzyme-inhibitory action on inflammation. They might have a high medical interest as therapeutic agents to curtail emphysema, cancer and inflammation.

2.3. Molecules acting on a different point of the coagulation cascade or on platelet aggregation inhibition

In addition to the targets discussed above (thrombin and factor Xa), leeches have developed an arsenal of highly specific and powerful weapons acting at different steps of the coagulation cascade, on the fibrinolysis pathway, and on platelet aggregation and activation (Fig. 1, Table 4).

Calin (isolated from *H. medicinalis*) specifically blocks adhesion and aggregation of platelets induced by collagen [51]. Calin induces an anti-thrombotic effect in a thrombosis model rich in platelets [52]. Similarly, in proboscis leeches, a protein of 126 amino acid residues, termed the leech antiplatelet protein (LAPP), has been discovered in *H. officinalis* [53]. The use of the recombinant protein in sections of human arteriosclerotic coronary arteries blocked platelet aggregation [54]. However, direct injection of LAPP to baboons did not prevent the experimental development of thrombi [55].

Decorsin [56] and ornatin [57] are two powerful antagonists of the GPIIb/IIIa protein. By their RGD sequence, these peptides are inhibitors of platelet aggregation by being competitors of the GPIIb/IIIa for its receptor on the platelets. Tridegin, a factor XIIIa inhibitor, has also been isolated from *H. ghilianii* [58].

Leeches have also developed molecules able either to cleave fibrin cross-links, such as destabilase [59] or the fibrinogenolytic-enzyme-like hementin [60]. Hementin inhibits platelet aggregation by destroying the fibrinogen necessary to aggregate them. Moreover, this enzyme is also able to disaggregate platelets once they have aggregated by destroying the fibrinogen that links the platelets together. By contrast, hementerin [61] is able to activate the fibrinolytic human plasma system in presence of a serum co-factor and calcium. This molecule displays similarity with streptokinase.

3. Conclusions

Taken together, this overview demonstrates that leeches have developed a panoply of molecules that interfere with the coagulation cascade and platelet activation. Leeches are a valuable reservoir of new powerful natural peptides to treat inflammation, coagulation, thrombosis, emphysema, and cancer, which are all diseases of aging.

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