Chapter 3

Anticoagulants from Hematophagous

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Abstract

This chapter will focus on anticoagulant molecules described until now from hematophagous animals. The evolutionary scenario for hematophagous animals is convergent and has resulted on a wide diversity of saliva anticoagulants, substances with platelet anti-aggregation action, and also with vase-dilating action. Hematophagous animals such as bloodsuckers (leeches, mosquitoes, and ticks) have developed strategies that specifically target proteinases from the hemostatic system of the animals they feed, thus keeping the blood incoagulable. The saliva of those animals provides a large amount of molecules to modulate the innate immune response of the host and to inhibit blood coagulation in order to facilitate the feeding. Thus, anticoagulants from hematophagous animals represent a very interesting tool for studies ranging from basic research to applications in the therapeutic area, as anticoagulant medication. Several studies have pointed out that anticoagulants from hematophagous can also display non-hemostatic functions as anti-tumor, bringing new perspectives for the study of these molecules. The comprehension of the multi-faced physiological roles of those new anticoagulants from hematophagous opens new perspectives for therapeutic and biotechnological approaches.

Keywords: anticoagulant, hematophagous animals, blood coagulation, FXa inhibitor, thrombin inhibitor

1. Introduction

1.1. Hematophagous animals

On the search for niches to ensure the survival of their populations, some animal species adapted as parasites on other species, retrieving nutrients directly from the inner mediums

of their hosts. In this way, these animals acquired the capacity to mediate the host defenses in order to succeed on their search for food [1, 2].

Among the arthropods, there are more than 14,000 species which are classified in around 400 genders of blood sucking animals with specific need to suck the host's blood, mostly vertebrate. These types of ectoparasites are called hematophagous animals and their saliva feature a rich array of substances capable to keep the ingested blood unclotted in their digestive system [3, 4].

The hematophagous animals feature exquisite and highly specialized mouthparts and a saliva rich in anti-hemostatic components. These substances are able to interfere on different stages of the blood coagulation mechanism and fibrinolysis. There are different groups of hematophagous animals, such as annelids, like leeches [5, 6] and intestinal worms [7], including arthropods, like mosquitoes [8] and ticks (arachnids) [9], and even mammals such as vampire bats [10].

1.2. A little about hirudotherapy

The number of compounds that have been discovered with anticoagulant potential in leeches and other bloodsuckers has become increasingly larger with the advent of transcriptomic analysis [11–13]. Nevertheless, the use of the animal itself has being also an alternative. Hirudotherapy, after many time, re-emerged in the 1970s. Leeches have been used with success in some kinds of microsurgeries and surgeries, as for example, in repair of lost limbs or still in plastic surgeries, where the animal helps in the blood fluid reestablishment [14, 15]. Hirudotherapy has also been used to treat soft tissue swelling and hematomas in trauma [16]. In the literature, you can find many reviews that report the use of this animal in different applications [14, 16–18]. On the other hand, a study where the use of leeches as an adjunct for the management of venous congestion after reconstructive surgery, realized with 87 patients, demonstrated that the morbidity associated with it should be considered, particularly the need for a blood transfusion [19].

There are even studies that demonstrate the opinion of the population regarding the use of the animal in surgery, of course that is not very comfortable for the patient. However, in general social cognition, the acceptance of hirudotherapy may not be very welcome at first, but provided with proper information and explanations, overall compliance of patients and caregivers can be improved and consequently result in superior outcomes in flap salvage [20].

Actually, no international protocols on leech therapy instructions have been established; some reported that leech application for a week is sufficient to get good results [21, 22]. However, it already was published in the literature, a guide of procedure for the use of leeches in surgical interventions [23].

1.3. Hematophagous animals and role in hemostasis

In all animals, the circulatory system exists in a perfect balance between coagulation (clot formation) and fibrinolysis (clot dissolution); in other words, to maintain blood in a fluid state, minimizing blood loss when the vascular system is injured. In this mechanism, endothelial cells and platelets are extremely important to form the hemostatic plug helping to arrest

bleeding. First, after injury, circulating platelets bind to collagen in the exposed vessel wall and aggregate, and second, the clotting factors are activated in the coagulation cascade, resulting in the formation of a fibrin clot. Thus, hemostasis coagulation reactions and fibrinolysis are crucial for the proper functioning of the hemostatic system [24].

Hematophagous animals such as bloodsuckers (leeches, mosquitoes, and ticks) are rich sources of anticoagulant molecules; among them, clotting inhibitors, fibrinogenolytics, plasminogen activators, and platelet inhibitors, all are present in their fluids and secretions, with roles on physiological processes such as feeding, digestion, self-defense, etc. Bloodsuckers access the blood fluid through the wound made by specialized structures targeting blood coagulation components, specially thrombin, factor Xa and the prothrombinase and tissue factor/FVIIa complexes, as a strategy to maintain blood incoagulable over a relatively long period of time. These new anticoagulants from hematophagous animals have opened new perspectives in the scientific area for basic and applied researches, having applications in the therapeutic area, as anticoagulant medication. Therefore, hematophagous animals have been an excellent target for studies, including pharmacologists [3, 25, 26].

2. Anticoagulant from leeches

Leeches are annelids (Annelida, Hirudinea), and feature over 650 known species found on many parts of the planet, including the oceans. Some species do not suck blood, but prey on worms and other small animals. Many sensorial organs are deployed to detect feeding opportunities, such as receptors over the body sensitive to movements and vibrations of water and soil, and also the "ocelli," light-sensitive cells. Some receptors of these animals can detect very small quantities of some substances such as oils and blood [6].

Even before the medicinal principles of leeches were studied in depth, it was known that these animals had powerful anticlotting and antiprotease substances as the blood found in their intestines remained liquid for weeks [27]. The most studied substances come from the salivary glands of hematophagous leeches. As an example, we have the European leech *Hirudo medicinalis*, for over a century, reaching its highest popularity on the XIX century in Europe. These leeches feature three adapted jaws in their mouth system that perforate the host's skin [6].

Other well-studied leech is the *Haementeria ghilianii*, popularly named as Giant Leech, found mainly in the North of Brazil and in the French Guyana, reaching up to a 50 cm length. However, differently from the European ones, leeches of the *Haementeria* genus have a proboscis on the mouth system, which is introduced on the pores of the host animal to find peripheral blood vessels, from which they then feed [6].

The salivary secretions of leeches have different roles, which are more important for the sucking process than for digestion itself. Some of these functions are performed by substances that have not even been isolated and/or studied, such as an anesthetic agent that causes the "bite" of the mandibular leeches to be painless and also a vasodilator of the histamine type that prolongs the bleeding of the host [6]. Many other substances of pharmacological interest have been characterized and described [28].

2.1. Molecules with activity in the hemostatic system from leeches

Among different anticlotting molecules from leeches and involved in the coagulation cascade, fibrinolysis, or on the platelet aggregation process, three substances have been the main focus of investigation. They are hirudin (a thrombin inhibitor) [29], antistasin (factor Xa inhibitor) [30], and decorsin (an antagonist of the llb-llla glycoprotein of the platelet membrane) [31]. The amino acid sequences of these substances, together with studies of inhibitory activities from different molecules and designs of the three-dimension structure have been determined, and, then, the structural similarity of these molecules was observed, allowing for the design of a structure motif (L.A.P.: Cys-X6-12-Cys-X-Cys-X3-6-Cys-X3-6-Cys8-14) [32]. However, the mechanisms of action of these inhibitors and important epitopes for the connection to their respective targets are distinct [32], demonstrating the relevance of the many inhibition mechanisms on clotting processes, as well as the evolution of these processes. Many of these substances that come from leeches have been developed by the industry, as targets for different therapies and in different clinical trial stages.

2.1.1. Thrombin inhibitors

Thrombin is a key enzyme on the pathogenesis of coronary acute thrombosis. Therapies with heparin, an indirect thrombin inhibitor, have been used during the last four decades. Search for new alternatives has demonstrated that the development of direct thrombin inhibitors (DTIs) is a translational success story; an example in which the combination of scientific ingenuity, structure-based design (including leech molecules models), and rigorous clinical trials has created a new class of anticoagulants that has improved patient care [33].

Hirudin was discovered on the salivary glands of the *Hirudo medicinalis* leeches in 1884 [34], and its role as a powerful antithrombotic drug started to be investigated on the 1920s. Markwardt in 1957 started studies with hirudin as a direct agent on the inhibition of thrombin (DTI), and these studies have been progressing significantly [28, 29].

Hirudin is a natural peptide with a simple chain, featuring 65 with three disulfide bridges and one residue of sulfated tyrosine amino acid residues. Part of its N-terminal region is globular and very compact, due to the presence of three disulfide bridges. On the other hand, the C-terminal region is made up of a great number of negatively charged residues [35–38]. More than 100 years after its discovery, the cDNA of hirudin was cloned and the recombinant (rH) obtained in large scale on *Escherichia coli* [39], on *Saccharomyces cerevisiae* [40], and, more recently, on *Acremonium chrysogenum* [41]. Its way of action has been extensively compared to low-molecular-weight heparins. Hirudin is a strict thrombin inhibitor of the "tight binding" type [42], and cofactors are not needed for its activity. Preclinical evaluation and rH clinical selection of analog forms have been improved on the last years [43].

The complex formed between hirudin and thrombin involves the three amino acid residues from the N-terminal region, which link near to the active site, and the C-terminal tail is linked to the fibrinogen-linking site. Crystallographic studies have shown that 10 residues of amino acids of the C-terminal portion (residues 55–65) react with the anion present on the exosite of thrombin, an important region for linking to fibrinogen. The residues 1–48 of the N-terminal portion are also important for the action of hirudin over thrombin; they interact with the enzyme's catalytic site. These types of interaction explain why hirudin links only to thrombin and not to the blood semiproteases [44].

A significant advance was reached with the resolution of the tridimensional structure of hirudin, which allowed for the understanding and development of recombinants equivalent to this protein (rH). The increase of interest on protein inhibitors also was due to studies that demonstrated thrombocytopenia induced by heparin. These new agents produce a direct anticlotting response, having thrombin as target, and they also inhibit the activation of platelets and the increase of thrombin's activity on the coagulation cascade, as thrombin is a multifunctional enzyme responsible for the activation of manly factors, for example, factor V, VIII, and XI [45]. The use of rH has been promising in patients with unstable angina [46].

Lepirudin (Refludan) is an rH, and it was the first direct thrombin inhibitor (DTI) licensed for treatment of thrombosis complicating HIT and associated thromboembolic disease in order to prevent further thromboembolic complications [47]. It is given as an intravenous infusion with or without a bolus, and its dosing is dependent on body weight. It is renally excreted and dose adjustments are required in patients with renal impairment [48]. Significant limitations to its use are its narrow therapeutic window and potential for increased bleeding events [49]. Besides, it is a drug that forms immunogenic complexes and causes a delay in renal excretion causing its accumulation [50, 51]. Therefore, during the treatment, the dose adjustment based on aPTT is recommended. Although not common, anaphylaxis can also occur in patients with hirudin-induced antibodies during the re-exposition to drug [52]. To date, there are no reports of antidotes that reverse these effects of DTIs [53]. There are recent reports that lepirudin has been discontinued from the market [54, 55].

Desirudin (Iprivask) is also an rH, with very similar characteristics as lepirudin. Both rH are structurally identical except for their N-terminus sequences, which are Leu1-Tyr2 in lepirudin and Val1-Val2 in desirudin. It reversibly binds to the active thrombin site of free and clot-associated thrombin. Desirudin is able to inhibit different actions of thrombin as fibrin formation, activation of coagulation factors V, VII, and XIII, and platelet aggregation, resulting in a dose-dependent prolongation of aPTT. It is the only fixed-dose subcutaneously administered DTI approved by FDA for postoperative prevention of VTE in patients undergoing elective hip replacement surgery [56]. Eriksson and collaborators published two clinical studies comparing the efficacy and safety of desirudin (15 mg s.c. twice daily injections) with unfractionated heparin (5000 units s.c. three times daily) and enoxaparin (40 mg s.c. daily), for the prophylaxis of DVT in patients undergoing major orthopedic surgeries. After 8–12 days of treatment, desirudin proved to be superior to both heparin anticoagulants, while showing a similar safety profile [57, 58]. Recently, desirudin was also under investigation as a potential anticoagulant for patients with heparin induced-thrombocytopenia (HIT) with or without thrombosis. Desirudin was also compared with argatroban in PREVENT-HIT study. This is a small, randomized, open-label trial comparing the clinical efficacy, safety, and economic utility of fixed-dose s.c. of drugs. However, just as lepirudin, desirudin is also renally excreted; there is still a risk of accumulation if the renal function is impaired [59].

Bivalirudin, formerly named Hirulog, is not properly a molecule from leech, but is a synthetic peptide (20 amino acids) [60] and bivalent analog of hirudin with a thrombin inhibition activity nearly 800 times weaker than that of hirudin [61]. Unlike the rH, the binding of bivalirudin to thrombin is reversible, and after the binding, the inhibitor is slowly cleaved by thrombin. Then, thrombin activity is only transiently inhibited and its enzymatic activity is restored. This reversible relationship between bivalirudin and thrombin can be seen as a benefit, once may contribute

to its decreased bleeding risk when compared with rHs [62, 63]. Another advantage of bivalirudin was demonstrated in animal studies, where bivalirudin presented a wider therapeutic index than rHs, and an additional advantage of bivalirudin was its lack of immunogenicity [64].

There are many studies with bivalirudin as an alternative to heparin or the combination of heparin and a GpIIb/IIIa inhibitor in patients with acute coronary syndromes and those undergoing a PCI [65–67]. These trials demonstrated that bivalirudin was not significantly different from other tested inhibitors in relation to reduction in major bleeding; on the other hand, bivalirudin, unlike heparin and GpIIb/IIIa inhibitors, does not cause thrombocytopenia. In this study, it also was demonstrated that bivalirudin reduced cardiac mortality and all-cause mortality among patients undergoing primary PCI for ST-elevation-myocardial infarction in the HORIZONS-AMI trial [66]. Accordingly, bivalirudin (Angiomax, The Medicines Company, Parsippany, NJ, USA) has become one of most widely used antithrombotics in the United States for PCI. Bivalirudin has been further studied in other kind of surgeries, but has not been further developed for these indications. Some examples of clinical studies with bivalirudin were as an alternative to heparin in coronary artery bypass [68, 69] in a dose-finding study for VTE prevention in patients after hip or knee surgery [70] and for the treatment of calf vein thrombosis [71]. Finally, the FDA expanded its approval of bivalirudin to include its use as an alternative to heparin in HIT patients with or without thrombosis undergoing PCI [72].

2.1.2. Other thrombin inhibitors

Besides hirudin, other thrombin inhibitors less studied have been isolated from leeches. Among them are a granuline-similar peptide [73], bufrudin [74], theromin [75], and haemadin [76]. Haemadin and theromin are inhibitors and do not present homology in their sequences with the other inhibitors described up to now in all animal kingdom. Haemadin was isolated from the *Haemadipsa sylvestris*, leech, and it is a 5 kDa peptide with a Ki of 100 fM, kinetically less efficient than hirudin (21 fM) [76, 77]. In addition, in literature, we can find only studies about crystal of haemadin and formation of haemadin-thrombin complex, nothing more besides [78, 79].

Theromin is a potent inhibitor (Ki = 12 fM) which was isolated from the intestines of *Theromyzon tessulatum* leeches [75]. It is homodimer 67 amino acid residues, with 16 cysteines that share 8 disulfide bridges. Just like hirudin, the N-terminal sequence of theromin is highly negatively charged and its C-terminal portion is very compact, due to 10 residues of cysteine present on the sequence. Around 24% of the residues of the molecule be cysteins and this approaches it, in sequence similarity, to protease inhibitors of the antistasin family (more detailed below). Hence, considering the low identity on the general sequence between theromin and the peptides of this family, it is difficult to include theromin as a new member of the mentioned family. However, comparisons of sequences have been made between theromin and four different serine-protease inhibitors isolated from *T. tessulatum* leeches: cytin, therin, therostasin, and tessulin [80–83]. These comparisons revealed that in the case of therostasin [82] and tessulin [83], there was a high degree of sequence identity with theromin (70 and 52%, respectively).

It can also be added that among the leeches from the *Theromyzon* genus, three other thrombin inhibitors were also described [84]. In fact, Merck Company, in 1994, deposited patents for different applications observing three thrombin inhibitors with masses of 3, 9, and 14 kDa

[28]. The N-terminal of the 9 kDa inhibitor, EDDNPGPPRACPGE, presented homology with theromin (ECENTECPRACPGE), factor Xa inhibitor (DCENTECPRACPGE) [82], and trypsin inhibitor tessulin (MCENTECPRACPGE) [83]. This 9 kDa inhibitor features a pI of 4.9 and a specific activity at the end of the purification process of 25 IU for inhibition of thrombin and of 0.2 IU for factor Xa inhibition.

2.1.3. Factor Xa inhibitors

While FXa inhibition has emerged as a convenient pathway for management of VTE, currently three FXa inhibitors are available for anticoagulation management—rivaroxaban, apixaban, and edoxaban [85]. New researches about FXa inhibitors of hematophagous animals constantly have been sought.

Antistasin was the first factor Xa inhibitor described that originates from leeches. It is a 15 kDa protein isolated from the salivary glands of the Mexican leech *H. officinalis* [86, 87]. Soon after, a homologous protein, ghilanten, was isolated from the *H. ghiliani* leech [88]. Antistasin features 119 residues of amino acids with the domain I (residues 1–55) being 56% similar to the domain II (residues 56–110). Of the nine residues of the C-terminal (111–119), domain portion four was positively charged [86], and their active site was located on domain I [88–90]. The cDNA of antistasin was cloned [89] and the recombinant protein expressed in system of baculovirus vector in insect cells [90]. Pharmacological studies were carried out, and data showed that the protein remains active after 30 h of injection in animals. Besides this, when tested in different thrombosis models, antistasin proved superior to heparin [91].

Administration of recombinant antistasin in rabbits with atherosclerosis in the femoral artery, as an example, demonstrated reduction of restenosis after balloon angioplasty [91]. Besides this, chimeric peptides corresponding only to domain I were also tested, and it was checked that domains II and III do not feature any intrinsic inhibitory activity over factor Xa, and also do not contribute to activity of domain I [86]. The most powerful synthetic peptide derived from antistasin corresponds to amino acids 27–49, with a disulfide bridge (ATS29–47); this peptide was able to inhibit factor Xa with a Ki of 35 nM. The DRCRVHCP peptide, in micromolar concentrations, featured anticlotting activity and was able to prolong the coagulation time in 50%, when compared with the control [92].

2.1.4. Other inhibitors of factor Xa

Therostasin is a powerful inhibitor for FXa of the "tight binding" type, isolated from *T. tessulatum*, featuring a Ki of 34 pM [82]. The cDNA (825 bp) encodes 82 amino acids polypeptide (with 16 of them being cysteines) preceded by 19 residues representing the signal peptide. Therefore, just as other inhibitors, therostasin is expressed and kept in cells from the salivary glands of leeches [82].

Vizottin is a FXa inhibitor from the salivary complex of the leech *Haementeria vizottoi*. It has shown anticoagulant effects in human plasma, prolonging the recalcification time in a dose-dependent manner (IC50 40 nM). Vizottin was able to induce blood incoagulability in FX-deficient plasma, whereas in normal and reconstituted plasma, vizottin doubled the prothrombin time at 160 nM. At high concentrations, vizottin inhibited the amidolytic activity of factor VIIa/tissue factor (IC50 96.4 nM). It is a compound which is also able to inhibit FXa in

the prothrombinase complex and Gla-domain less FXa. The authors demonstrated that the inhibition of FXa by vizottin is through binding to the active site rather than an exosite. The structure of this molecule still need to be better studied [93].

A FXa inhibitor has been described in leech that are proven not part of the antistasin-family, the Lefaxin. This inhibitor was obtained from the salivary glands of the Brazilian leech, *Haementeria depressa*. It is a competitive inhibitor of FXa with a Ki of 3.6 nM, and is able to inhibit the FXa also in the prothrombinase complex with IC50 of 10 nM. It has a simple chain with 30 kDa and pI of 5.7 [94].

Among the FXa inhibitors from leeches, antistasin was the one that came closest to drug development; however, it did not get there. Even if these natural substances, as antistasin, are not being directly used in the human medical clinic, it was through the study of them that synthetic molecules focused on FXa were and are still being designed. This has provided potent and selective tools for evaluating the potential role of FXa in various diseases. In addition, these advances have been instrumental in defining the biology of FXa and have aided in the discovery of specific receptors and intracellular signaling pathways for FXa that may be important in the progression of, or the response to, various diseases [95].

2.1.5. Antiplatelet agents

2.1.5.1. Collagen-binding proteins

Leech antiplatelet protein (LAPP) is a specific inhibitor by collagen pathway from *Haementeria officinalis* leech salivary glands. It has around 13 kDa and pI 4.0. Recombinant LAPP (rLAPP) is able to inhibit collagen-mediated platelet aggregation under test-tube stirring conditions (IC50 ~ 60–100 nM) and, also, it is able to block platelet adhesion to soluble collagen under static conditions, a step mediated by integrin $\alpha 2\beta 1$ [96, 97]. There are reports demonstrating that this recombinant prevents integrin α-I domain binding to collagen with IC50 ~ 125 nM [98]. The platelet adhesion to collagen type I is inhibited by rLAPP at high shear rate (1600 s^{-1}) and this inhibitor is also able to prevent the binding of vWF to collagen type III [99]. In spite of this, rLAPP inhibits platelet deposition to cross sections of human atherosclerotic coronary arteries [99], and studies in baboons proved that rLAPP did not block collagen graft thrombosis, suggesting that inhibition of collagen alone is not enough to prevent thrombosis, possibly because TF exposure plays an important role in the model [100]. The crystal structure of LAPP has been determined and consists of a C-terminal domain which is very compact and a disordered N-terminal region [101].

Calin is isolated from the salivary secretion of the European leech *H. medicinalis,* as well as the rLAPP; it is able to inhibit the vWF-binding and platelet adhesion to collagen both under static and flow conditions [102]. Similarly, Saratin, from *Haementeria ghilianii* leeches, has been described as a platelet aggregation inhibitor that acts on collagen preventing the binding to integrin $\alpha 2\beta 1$ and vWF [103]. The recombinant Saratin was obtained in yeasts (*Hansenula polymorpha*) [104] and it is being commercialized by BioVascular which has developed this product to GMP standards and is evaluating the effects in clinical studies [105]. To date, in the literature, only a few animal studies have been published, where it has been given alone or together with other drugs in glaucoma rabbit models [106, 107]. Saratin, when administrated alone in rat carotid endarterectomy model, significantly decreased platelet adhesion,

intimal hyperplasia, luminal stenosis, and thrombosis. This inhibitor did not increase suture line bleeding or bleeding times, and did not decrease platelet counts. In this study, the authors also have concluded that Saratin may serve as a topical agent to be used for the site-specific inhibition of thrombosis and intimal hyperplasia after vascular manipulation [108].

2.1.5.2. Disintegrins

Disintegrins were first discovered in snake venoms where they are very well studied, and were instrumental in our understanding of integrin function and also for the development of antithrombotic drugs [109]. However, this molecule class also has been found in bloodsucker animals. In leeches, there are two more studied molecules with this profile, decorsin and ornatin.

Decorsin is a 39 amino acids protein purified from *Macrobdella decora* leech salivary glands that acts as an antagonist of glycoprotein GPIIb-IIIa. This disintegrin, like snake family of inhibitors, has six cysteines and an RGD motif near its C-terminus. It completely inhibits platelet aggregation ADP induced at high concentrations (1 μM) and is able to inhibit the interaction of GPIIb-IIIa with fibrinogen in ELISA assays (IC50 ~ 1.5 nM). The secretion of decorsin in the salive of this animal probably is one of its strategy to keep host blood flowing or to keep ingested blood from clotting, as leeches store ingested blood for long periods of time [31]. The structure of decorsin was determined by nuclear magnetic resonance (NMR) and it is interestingly similar to that of hirudin from *Hirudo medicinalis* leech [32].

Ornatin is a disintegrin described on *Placobdella ornate* leech that is 40% similar to decorsin. Studies with ornatin demonstrated that it is able to inhibit fibrinogen binding to GPIIb-IIIa (IC50 ~ 5 nM); on the other hand, it inhibits platelet aggregation at higher concentrations (IC50 ~ 300 nM) [110]. Studies with the recombinant protein demonstrated that the native disulfide bonds are required for the optimal GPIIb-IIIa antagonist activity of the ornatin [111].

2.1.6. Regulators of fibrinogenolysis

As described in this chapter, various thrombin inhibitors from hematophagous animals together with other kind of anticoagulant as FXa inhibitor and anti-platelets not only maintain anticoagulant potential of the salivary gland secretions but also play a role of blood preservatives in the gut channel of the bloodsuckers. On the other hand, little is known on the degradation of fibrinogen and fibrin by secretions of bloodsuckers. However, we relate here some data obtained about molecules from some leeches of *Haementeria* genus and from specie *Hirudo medicinalis* that act as regulators of fibrinogenolysis and/or fibrinolysis.

2.1.6.1. FXIIIa inhibitors

Factor XIIIa promotes the covalent crosslinking of fibrin polymers and incorporation of proteins into the fibrin network and thus the thrombus can be stable and relative resistance to plasmin-mediated degradation. Besides, FXIIIa is involved in other processes such as wound healing and arteriosclerosis. Therefore, selective FXIIIa inhibitors may be a valuable tool for evaluation of the various functions of FXIIIa and their pharmacological control [112]. In this field, a potent FXIIIa inhibitor was found in leeches. Tridegin was discovered in salivary glands of blood-sucking leech, *Haementeria ghilianii*. It is a highly specific inhibitor of factor

XIIIa with about 7 kDa, this inhibitor works with effective concentrations in the nanomolar range [113]. It was also related the presence of transcripts similar to tridegin in some transcriptome analysis of other leeches specie [12], but the obtaining of new molecules from leeches with this function was not yet published. Some tridegin analog peptides have been synthesized and analyzed for their action improvement, but so far, nothing very relevant has been exposed [114]. Although not used in clinical trials on its recombinant form (T087), a derivative of tridegin is being marketed by more than one company for use in laboratory research.

2.1.6.2. Fibrino(geno)lytics molecules

Hementin is responsible for proteolysis of blood fibrinogen with formation of products which block conversion of fibrinogen into fibrin catalyzed by thrombin; this molecule was discovered in salivary gland from *Haementeria ghilianii* [115]. Since fibrinogen is involved in the formation of platelet clot, hementin is able to prevent the platelet aggregation induced by ADP and collagen; on the other hand, it can also induce disaggregation of platelet aggregation induced by ADP, but not collagen [116]. Hementin can lyse fibrin clots; but its fibrinolytic activity is less potent than the fibrinogenolytic one. It does not influence the activity of other plasma proteins [117].

It was also demonstrated that plasma clots formed in the presence of tridegin are more sensitive to lyses by hementin (time required for 50% lysis in the presence and absence of hementin was 16 and about 22 h, respectively) [118]. Study of lysis of clots formed from PRP revealed that in the presence of tridegin the effect of fibrinolytic enzymes was the same as in PPP, whereas lysis of platelet-containing clots occurred slower. Thus, the importance of the platelets in the resistance of plasma clots to fibrinolytic enzymes and also the importance of cross-linking in this process [119].

Considering that both molecules are obtained in the same leech species, it was suggested that hementin and tridegin have a synergic action in feeding process of *Haementeria ghilianii*. They may be considered as promising thrombolytic agents.

Hementerin (HT) is a single-chain 80 kDa, Ca++-dependent metalloproteinase, which specifically degrades fibrin(ogen) through a plasminogen-independent pathway. The amino terminal sequence of 8 residues shows 80% similarity with hementin. However, their activities differ somewhat in terms of kinetics and with regard to the structure of the fibrin(ogen) fragments they may produce. Cleavage by HT of fibrinogen A-alpha, gamma, and B-beta chains, in that order, produces fragments differ from those produced by plasmin. HT was also able to degrade cross-linked fibrin although at a lower rate as compared to fibrinogen. HT is a plasminogen-independent fibrino(geno)lytic metalloproteinase that degrades fibrinogen faster than fibrin, prevents the coagulation and destroys fibrin clots *in vitro* [120]. The action of HT was also studied in different platelet assays and the studies have indicated that HT is an effective inhibitor of human platelet aggregation, presumably through activation of the platelet's nitridergic pathway [121].

Destabilase was discovered in salivary glands from *Hirudo medicinalis* and it was able to hydrolyze the epsilon-(gamma-glutamyl)-lysine bonds as a result of fibrin stabilization by FXIIIa in the presence of calcium ions [122]. It was characterized as a polyfunctional molecule

and is a unique representative of invertebrate lysozymes. This molecule combines the properties of endo-s-lysyl-y-glutamyl isopeptidase (D-dimer monomerase), lysozyme, and chitinase and simultaneously is also a non-enzymatic antimicrobial agent. Its ability to hydrolyze endoisopeptide bonds formed by transglutaminases, which are involved in many pathological conditions, including thrombosis, causes this enzyme to become a focus to seek its use in practice [123], on the other hand, none was presented after that.

The substrate of destabilase is the D-D-dimer, a protein of 190 kDa that contains fragments of all three chains of monomer fibrin (alpha, beta, and gamma) and there is a nonlinear dependence of the reaction rate on substrate concentration. The crosslinked fibrin is also a substrate of destabilase, which catalyzes hydrolysis of isopeptide bonds connecting gamma-gamma and alpha-alpha-chains of this protein [124, 125].

Recently, a study demonstrated an optimization procedures related to the expression, isolation, and purification of active destabilase isoforms (mlDL-Ds1, 2, 3) using an *Escherichia coli* expression system, where their muramidase, lytic, isopeptidase and antimicrobial activities were detected and compared. Analyses of the tested activities revealed that all isoforms had almost identical patterns of pH and ionic strength effects. It was determined that three isoforms possessed non-enzymatic antibacterial activity independent of their muramidase activity. It was also demonstrated, for the first time, the fibrinolytic activity of the recombinant destabilase and showed that only intact proteins possessed this activity, suggesting being an enzymatic property [126].

3. Anticoagulants from ticks

Most anticoagulants from ticks are produced for the salivary glands and play essential functions during feeding. Ticks inject the saliva into the skin of a wide range of terrestrial vertebrates and absorb it along with the blood of the animal. Faced with an injury inflicted by tick bite, the animal respond by activating blood coagulation, vasoconstriction, inflammation, and tissue remodeling related to wound healing. However, these ectoparasites have a complex and potent pharmacological mechanism to overcome the host defenses, blocking pain and itch and facilitating blood flow to allow the feeding [25, 127, 128].

Differences in the composition of tick saliva are reflected in the co-evolution between ticks and their host, the feeding strategies, the tick developmental stage, the process of penetration of the host skin, and the duration of the feeding. This can be observed between the two major families, Argasidae and Ixodidae. The first family (family Argasidae) is called soft ticks. They feed fast, less than 1 h, for multiple times causing profound damage to the host skin due the deep mouthparts penetration, while hard ticks (family Ixodidae) feed for a prolonged period (days to weeks) in each developmental stage. Hard ticks have strategies to firmly attach to its host, producing large amount of cement or glue to penetrate the host skin and cause a superficial damage (Metastriata ticks, e.g., *Dermacentor* or *Rhipicephalus* genera), or by attaching more deeply to the host skin by physical mechanisms using longer, barbed mouthparts. Females hard tick feed only once and may ingest more blood than 100-times their initial body weight to die later after oviposition (Prostriata, e.g., *Ixodes*, *Metastriata*, and *Amblyomma* genera) [127, 129–131].

3.1. Components affecting coagulation

Ticks saliva has other strategies besides inhibiting blood coagulation factors, in order to facilitate the feeding. After injury, subendothelial tissue get exposed, activated platelets bind to exposed von Willebrand factor and collagen through its surface receptors and platelets release soluble vasoconstrictor mediators (ADP, serotonin, and thromboxane A_2). Physiologically, there are three major mechanisms that regulate anticoagulation: TFPI, antithrombin III (ATIII), and protein C/thrombomodulin/activated protein C. Until now, there are no description of tick saliva components interfering with or imitating antithrombin, protein S, protein C, heparin, or thrombomodulin [24]. However, many ticks can inhibit thrombin-induced platelet aggregation. On the other hand, anticoagulant molecules from tick saliva also regulate hemostasis by inhibiting blood coagulation factors (FXa or thrombin) or tenase complexes (FVIIa/TF and FIXa) and/or platelet aggregation [132].

Anticoagulants from tick saliva can be classified according with their biochemical characteristics and structure, some of them belonging to the Kunitz-type domain inhibitors and Serpin domain inhibitors [127]. Members of those families can modulate coagulation, inflammation, or vasoconstriction. For example, the Serpin IRS-2 (*I ricinus* Serpin-2) from *I. ricinus* inhibits cathepsin G and chymase, both known as mediators of platelet aggregation and inflammation [133], as well as to mediate vascular permeability [25]. Besides, Kunitz domain inhibitors are widely expressed and characterized as anticoagulants, some of them having just one Kunitz domain being able to inhibit factor Xa [134] or thrombin, such as savigin from *Ornithodoros savignyi* [135].

Depending on the mechanism of action, they can include platelet inhibitors, factor Xa inhibitors and thrombin inhibitors, since they are able to prevent blood clotting and maintain blood incoagulable. Those blood coagulation inhibitors from tick are the major focus of this section.

3.1.1. Antiplatelet agents

The primary response to injury is the activation of circulating platelets, which bind to collagen in the exposed vessel wall and aggregate, arresting bleeding. In addition, thrombin, a multifunctional serine protease, activates platelets by cleaving platelet receptors [24]. Thus, saliva from ticks possess molecules to able to target platelet activation and aggregation in several ways, some of them inhibiting thrombin-induced platelet activation [136], other interfering with the adhesion of platelet to collagen or other ligands [136] or inhibiting the activation of protease-activated receptors (PARs). An example of the first group is the Serpin IRS-2 (*I ricinus* Serpin-2) from *Ixodes ricinus* which inhibits platelet aggregation induced by both thrombin and cathepsin G [133]. Another Serpin, IxscS from *I. scapularis*, was described to inhibit thrombin and to interfere with platelet aggregation induced by thrombin or ADP [137]. Also, in *I. scapularis*, the enzyme apyrase (an adenosine triphosphate (ATP) diphosphohydrolase) degrades active ATP and ADP into non-active AMP [138].

Some molecules can interfere with the adhesion of platelets to collagen, for example, the tick adhesion inhibitor (TAI) from *Ornithodoros moubata* [139, 140]. Other inhibitors act by binding competition through an integrin recognition motif RGD or KGD preventing the binding to

fibrinogen or other ligands to platelet receptors such as savignygrin from *O. savignyi* [141]. Variabilin is another anti-platelet RGD-containing peptide from *Dermacentor variabilis* [142]. Some inhibitors identified in *I. pacificus* and *I. scapularis*, known as ixodegrins, display some differences with variabilin by having cysteines flanking the RGD motif, and with savignygrin, which have a non-canonical RGD peptide inserted into a Kunitz fold [127, 143].

Other anti-platelet molecules from ticks were reported: monogrin from *Argas monolakensis* [144], moubatin, a lipocalin derived from *O. moubata* which inhibits collagen-induced platelet aggregation by scavenging thromboxane A2 [139, 140, 144], longicornin, isolated from the salivary gland of *Haemaphysalis longicornis*, which also inhibits collagen-mediated platelet aggregation [145].

3.1.2. Tenase complex inhibitors

To target blood coagulation, components from tick saliva have inhibitory activities on the extrinsic tenase complex in blood coagulation [132]. From the studies in *I. scapularis* tick (Acari: Ixodidae) [146], two classes of extrinsic tenase complex inhibitors were identified acting similarly, but not identically, to the physiological inhibitor, tissue factor pathway inhibitor (TFPI) [136]. The first group is represented by ixolaris [147], a 15.7 kDa molecule obtained from the cDNA library of the salivary glands of *I. scapularis* consisting of 140 amino acid residues containing 10 cysteine and two-Kunitz tandem domain which does not bind to FXa active site, in contrast TFPI. It was hypothesized that the second Kunitz domain of ixolaris binds first to FX/FXa (on a heparin binding proexosite/exosite) before binding to the FVIIa-TF complex via the first Kunitz domain. The native inhibitor has a molecular mass of 24 kDa, and both forms are equally effective as anticoagulants. Functionally, the Ixolaris is structurally distinct from human tissue factor pathway inhibitor (TFPI) [146]. The second group is represented by penthalaris [148], a five-Kunitz tandem domain which uses FX or FXa as scaffold to inhibit the FVIIa-TF complex.

3.1.3. Factor Xa inhibitors

One of the main classes of FXa inhibitors characterized from soft tick saliva is the atypical, non-canonical Kunitz-type inhibitors including the tick anticoagulant peptide (TAP), obtained from the *Ornithodoros moubata* tick [9] and FXa-inhibitor (FXaI) from *O. savignyi* tick [149] (Acari: Argasidae). Both inhibitors possess a single Kunitz domain, in contrast to the tandem Kunitz type thrombin inhibitors. Kinetically, both are slow, tight-binding, competitive inhibitors of FXa. The recombinant (rTAP) TAP has a single-chain acidic polypeptide composed of 60 amino acids including 6 cysteine residues, and is a competitive FXa inhibitor highly selective and reversible. Its molecular weight is 6.8 kDa, pI 4.5 and Ki of 0.588 for the native form, and Ki of 0.18 nM for the recombinant form, expressed in *Saccharomyces cerevisiae* [150–152].

Amblyomin-X is a FXa inhibitor identified molecule in the transcriptomics profile of the salivary glands by Expressed Sequence Tags (ESTs) from the hard tick *Amblyomma cajennense* (currently *Amblyomma sculptum*) [153], containing an unique structure with a N-terminal Kunitz-type domain of 60 amino acids and a C-terminal with 49 amino acids. Amblyomin-X is able to inhibit factor Xa, prothrombinase and tenase activities. As FXa inhibitor, Amblyomin-X

acts as a noncompetitive inhibitor (Ki = 3.9 μM) of factor Xa. It is a substrate for plasmin and trypsin, but not for factor Xa and thrombin. The prolongation of PT and aPTT is reversible [154]. Interestingly, several studies pointed out Amblyomin-X as an anti-cancer molecule *in vitro* and *in vivo* [154–161].

Other FXa inhibitors were reported in *I. scapularis* belonging to the salivary protein (Salp) family, which specifically inhibits the FXa active site [162]. Other inhibitors act on FXa through binding to prothrombinase complex [163].

3.1.4. Thrombin inhibitors

The main effector blood coagulation factor is thrombin, which is the enzyme involved in the final (common pathway of the blood coagulation, responsible for the conversion of fibrinogen in fibrin and also regulates the activity of other coagulation factor with great specificity. Thrombin is a multifunctional molecule acting in cell signaling, fibrinolysis, and inflammation system [164]. Thrombin has three domains, the active site and two regulator sites, named exosites. Exosite I is the site that links the enzyme with fibrinogen, the platelet receptor and protease activated receptors (PARs), as well as the endothelial receptor, thrombomodulin. Exosite II recognizes glycosaminoglycans such as heparin, platelet receptor GP Ib-IX-V and fibrin (for a recent review on the role of thrombin exosites, see Ref. [165]. Thus, the choice of thrombin as a target for new anticoagulants seems logical, since its inhibition not only attenuates fibrin formation, but also blocks thrombin-mediated feedback amplification of clotting [166].

Kunitz-type thrombin inhibitors from ticks were identified in hard (Ixodidae family) and soft (Argasidae family) ticks, and have differences that place them in two different protein subclasses, based on their sequences, probably as an adaptation of their different blood-feeding behaviors [2]. Avathrin is a recombinant thrombin inhibitor from the salivary glands of the ixodid tick, *Amblyomma variegatum*. It shares 31–34% of identity with variegin. Kinetically, avathrin is a fast, tight binding competitive inhibitor (545 pM) with high affinity for thrombin rather than other serine proteases of the coagulation system. Cristal structure of avathrin and thrombin reveal an interaction through the active site and exosite-I of thrombin. Moreover, cleavage products continue to exert prolonged inhibition in a murine carotid artery thrombosis model [167]. From hard ticks, other thrombin inhibitor was isolated including amblin from *Amblyomma hebraeum* [168], boophilin from the cattle tick *Boophilus microplus* [169], and hemalin from *Haemaphysalis longicornis* [170].

Boophilin has been cloned and overexpressed in *E. coli*, which potently inhibits additional trypsin-like serine proteases, including trypsin and plasmin and displays an apparent molecular mass of ~23 kDa. This inhibitor binds bovine thrombin with tight-binding kinetics, and was determined an apparent Ki of 1.8 nM. The crystal structure of the bovine α-thrombin boophilin complex reveals a non-canonical binding mode to the protease. The N-terminal region of the mature inhibitor binds in a parallel manner across the active site of the protease, while the C-terminal Kunitz domain is negatively charged and docks into the basic exosite I of thrombin [169].

Recently, a new thrombin inhibitor from *Amblyomma sculptum* was identified in the transcriptomics analysis of tick's salivary glands [171]. Scupltin was cloned and expressed in

E. coli as a 20 kDa protein sharing only few similarities with hirudin and more similarity with serine protease inhibitors of the antistasin family. Sculptin is a novel class of competitive, reversible, and specific inhibitor of thrombin because its mechanism of inhibition is slightly different than hirudin. The Ki is comparable with that of hirudin and lower then hirulogs. Interestingly, sculptin phylogenetically diverges from hirudin. Sculptin has not inhibitory activity on FXa, trypsin and plasmin. However, it is degraded by serine proteases including thrombin, thus would not requires antidotes. The sculptin fragments produced by thrombin have not thrombin inhibitory activity, while sculptin fragments produced by FXa can inhibit thrombin independently. Sculptin increases blood coagulation parameter in concentration dependent manner. Sculptin has been filed for patenting in Brazil [171].

From soft ticks, Kunitz-type thrombin inhibitors include ornithodorin from *Ornithodoros moubata* [172], savignin from *Ornithodoros savignyi* [135, 173] and monobin from *Argas monolakensis* [174]. Kinetically, they are slow, tight-binding, competitive inhibitors of thrombin: savignin (Ki = 4.89 pM) [173], and monobin (Ki = 7 pM) [174].

As mentioned above, most Ixodidae ticks produce a cement or glue to attach to the host skin to facilitate the penetration of mouthparts for feeding. Interestingly, in *Amblyomma americanum,* the compositions of this cement revealed by the presence of glycine-rich proteins, lipids, and certain carbohydrates, besides serine protease inhibitors and metalloproteases. Some molecules from tick cement were considered promising candidates for an anti-tick vaccine because of their antigenic properties [136, 175].

3.2. Components affecting fibrinolysis

Fibrinolytic enzyme with metalloprotease activity has been described in the hard tick *I. scapularis* [176]. On the other hand, an activator of plasminogen, called longistatin from *H. longicornis*, was found to cause hydrolysis of fibrinogen and delay formation of the fibrin clot as comparable to that of tissue-type plasminogen activator (t-PA) [177]. The recombinant form of longistatins is able to inhibit inflammation associated to tick feeding [178].

4. Conclusions

To conclude, hematophagous animals have evolved effective means of inhibiting thrombosis, thereby facilitating the acquisition and digestion of a blood meal. To date, specific inhibitors of coagulation, platelet function and fibrinolysis regulators have been identified from numerous invertebrate species, mainly leeches, ticks, and mosquitoes, representing an impressive array of convergent functional strategies. These parasites may serve as potentially useful therapeutic agents for the treatment of a variety of conditions associated with activation of thrombosis. A number of anticoagulants and platelet inhibitors from bloodsuckers have been evaluated *in vivo*, with some currently in varying stages of preclinical and clinical development. Because of the unique specificity and potency of anticoagulants from hematophagous, these kinds of products hold great promise for improving the treatment of a variety of human illnesses, as heart disease and stroke.

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Conflict of interest

The authors declare that they have no competing interests.

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