
Anticoagulants from Hematophagous

Ana Marisa Chudzinski-Tavassi, Fernanda Faria and
Miryam Paola Alvarez Flores

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.78025>

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 IIb-IIIa 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 many 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 K_i 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 ($K_i = 12$ 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 cysteines 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 thrombin (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 K_i 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 K_i 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 (IC_{50} 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 (IC_{50} 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 K_i of 3.6 nM, and is able to inhibit the FXa also in the prothrombinase complex with IC_{50} 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 ($IC_{50} \sim 60\text{--}100$ 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 $IC_{50} \sim 125$ 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 administered 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 μ M) 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 saliva 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(genolytic) 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(genolytic) 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 (mDL-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 K_i of 0.588 for the native form, and K_i 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 ($K_i = 3.9 \mu\text{M}$) 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 variegatin. 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. Crystal 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 ambalin 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 K_i 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 K_i is comparable with that of hirudin and lower than 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 require 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 ($K_i = 4.89$ pM) [173], and monobin ($K_i = 7$ 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.

Acknowledgements

A.M.C.T. received financial support from São Paulo Research Foundation (CENTD, Grants No. 2015/50040-4; CeTICS 2013/07467-1) and from CNPq, Grant No. 305445/2023-8. F.F. received financial support from CNPq, Grant No. 480703/2013-2.

Conflict of interest

The authors declare that they have no competing interests.

Author details

Ana Marisa Chudzinski-Tavassi*, Fernanda Faria and Miryam Paola Alvarez Flores

*Address all correspondence to: ana.chudzinski@butantan.gov.br

Laboratory of Molecular Biology - Division of Innovation and Development – Instituto Butantan, São Paulo, Brazil

References

- [1] Bang NU. Leeches, snakes, ticks, and vampire bats in today's cardiovascular drug development. *Circulation*. 1991;**84**(1):436-438
- [2] Mans BJ, Neitz AW. Adaptation of ticks to a blood-feeding environment: Evolution from a functional perspective. *Insect Biochemistry and Molecular Biology*. 2004;**34**(1):1-17
- [3] Ribeiro JM. Blood-feeding arthropods: Live syringes or invertebrate pharmacologists? *Infectious Agents and Disease*. 1995;**4**(3):143-152
- [4] Ribeiro JM, Schneider M, Isaias T, Jurberg J, Galvao C, Guimaraes JA. Role of salivary antihemostatic components in blood feeding by triatomine bugs (Heteroptera). *Journal of Medical Entomology*. 1998;**35**(4):599-610
- [5] Sawyer RT. Thrombolytics and anti-coagulants from leeches. *Biotechnology (N.Y)*. 1991;**9**(6):513-515, 518
- [6] Sawyer RT. *Leech Biology and Behaviour: Anatomy, Physiology and Behaviour*. 11th ed. Oxford: Oxford University Press; 1986. 464 p
- [7] Cappello M, Vlasuk GP, Bergum PW, Huang S, Hotez PJ. Ancylostoma caninum anticoagulant peptide: A hookworm-derived inhibitor of human coagulation factor Xa. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;**92**(13):6152-6156

- [8] Ribeiro JM, Schneider M, Guimaraes JA. Purification and characterization of prolixin S (nitrophorin 2), the salivary anticoagulant of the blood-sucking bug *Rhodnius prolixus*. *The Biochemical Journal*. 1995;**308**(Pt 1):243-249
- [9] Waxman L, Smith DE, Arcuri KE, Vlasuk GP. Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. *Science*. 1990;**248**(4955):593-596
- [10] Gardell SJ, Ramjit DR, Stabilito II, Fujita T, Lynch JJ, Cuca GC, et al. Effective thrombolysis without marked plasminemia after bolus intravenous administration of vampire bat salivary plasminogen activator in rabbits. *Circulation*. 1991;**84**(1):244-253
- [11] Amorim AM, de Oliveira UC, Faria F, Pasqualoto KF, Junqueira-de-Azevedo Ide L, Chudzinski-Tavassi AM. Transcripts involved in hemostasis: Exploring salivary complexes from *Haementeria vizottoi* leeches through transcriptomics, phylogenetic studies and structural features. *Toxicon*. 2015;**106**:20-29. DOI: 10.1016/j.toxicon.2015.09.002
- [12] Faria F, Junqueira-de-Azevedo Ide L, Ho PL, Sampaio MU, Chudzinski-Tavassi AM. Gene expression in the salivary complexes from *Haementeria depressa* leech through the generation of expressed sequence tags. *Gene*. 2005;**349**:173-185. DOI: 10.1016/j.gene.2004.12.022
- [13] Siddall ME, Brugler MR, Kvist S. Comparative transcriptomic analyses of three species of Placobdella (Rhynchobdellida: Glossiphoniidae) confirms a single origin of blood feeding in leeches. *The Journal of Parasitology*. 2016;**102**(1):143-150. DOI: 10.1645/15-802
- [14] Abdulkader AM, Ghawi AM, Alaama M, Awang M, Merzouk A. Leech therapeutic applications. *Indian Journal of Pharmaceutical Sciences*. 2013;**75**(2):127-137
- [15] Tashiro K, Fujiki M, Arikawa M, Kagaya Y, Miyamoto S. Free flap salvage after recurrent venous thrombosis by means of large-scale treatment with medical leeches. *Plastic and Reconstructive Surgery Global Open*. 2016;**4**(12):e1157. DOI: 10.1097/GOX.000000-0000001157
- [16] Porshinsky BS, Saha S, Grossman MD, Beery Ii PR, Stawicki SP. Clinical uses of the medicinal leech: A practical review. *Journal of Postgraduate Medicine*. 2011;**57**(1):65-71. DOI: 10.4103/0022-3859.74297
- [17] Jha K, Garg A, Narang R, Das S. Hirudotherapy in medicine and dentistry. *Journal of Clinical and Diagnostic Research*. 2015;**9**(12):ZE05-ZE07. DOI: 10.7860/JCDR/2015/16670.6918
- [18] Knobloch K, Gohritz A, Busch K, Spies M, Vogt PM. *Hirudo medicinalis*-leech applications in plastic and reconstructive microsurgery – A literature review. *Handchirurgie, Mikrochirurgie, plastische Chirurgie: Organ der Deutschsprachigen Arbeitsgemeinschaft für Handchirurgie: Organ der Deutschsprachigen Arbeitsgemeinschaft für Mikrochirurgie der Peripheren Nerven und Gefässe*. 2007;**39**(2):103-107. DOI: 10.1055/s-2007-965138
- [19] Cornejo A, Shammas RL, Poveromo LP, Lee HJ, Hollenbeck ST. Institutional outcomes of leech therapy for venous congestion in 87 patients. *Journal of Reconstructive Microsurgery*. 2017;**33**(9):612-618. DOI: 10.1055/s-0037-1604082

- [20] Kim KS, Sim HS, Shin JH, Hwang JH, Lee SY. The relationship between explanation and patient compliance in hirudotherapy. *Archives of Craniofacial Surgery*. 2017;**18**(3):179-185. DOI: 10.7181/acfs.2017.18.3.179
- [21] Whitaker IS, Cheung CK, Chahal CA, Karoo RO, Gulati A, Foo IT. By what mechanism do leeches help to salvage ischaemic tissues? A review. *The British Journal of Oral & Maxillofacial Surgery*. 2005;**43**(2):155-160. DOI: 10.1016/j.bjoms.2004.09.012
- [22] Knobloch K. Leeches in microsurgery – An evidence-based approach. In: Kini RM, Clemetson KJ, Markland FS, McLane MA, Morita T, editors. *Toxins and Hemostasis*. Netherlands: Springer Science; 2011. pp. 735-745
- [23] Bennett-Marsden M. Hirudotherapy: A guide to using leeches to drain blood from tissue. *Clinical Pharmacist*. 2014;**6**(3):69. DOI: 10.1211/CP.2014.11136626
- [24] Hoffman M, Monroe DM. Coagulation 2006: A modern view of hemostasis. *Hematology/Oncology Clinics of North America*. 2007;**21**(1):1-11. DOI: 10.1016/j.hoc.2006.11.004
- [25] Chmelar J, Calvo E, Pedra JH, Francischetti IM, Kotsyfakis M. Tick salivary secretion as a source of antihemostatics. *Journal of Proteomics*. 2012;**75**(13):3842-3854. DOI: 10.1016/j.jprot.2012.04.026
- [26] Champagne DE. Antihemostatic strategies of blood-feeding arthropods. *Current Drug Targets Cardiovascular & Haematological Disorders*. 2004;**4**(4):375-396
- [27] Bagdy D, Barabas E, Graf L, Petersen TE, Magnusson S. Hirudin. *Methods in Enzymology*. 1976;**45**:669-678
- [28] Salzet M. Leech thrombin inhibitors. *Current Pharmaceutical Design*. 2002;**8**(7):493-503
- [29] Markwardt F. Isolation and chemical characterization of hirudin. *Hoppe-Seyler's Zeitschrift fur physiologische Chemie*. 1957;**308**(2-4):147-156
- [30] Tuszynski GP, Gasic TB, Gasic GJ. Isolation and characterization of antistasin. An inhibitor of metastasis and coagulation. *The Journal of Biological Chemistry*. 1987;**262**(20):9718-9723
- [31] Seymour JL, Henzel WJ, Nevins B, Stults JT, Lazarus RA. Decorsin. A potent glycoprotein IIb-IIIa antagonist and platelet aggregation inhibitor from the leech *Macrobdella decora*. *The Journal of Biological Chemistry*. 1990;**265**(17):10143-10147
- [32] Krezel AM, Wagner G, Seymour-Ulmer J, Lazarus RA. Structure of the RGD protein decorsin: Conserved motif and distinct function in leech proteins that affect blood clotting. *Science*. 1994;**264**(5167):1944-1947
- [33] Coppens M, Eikelboom JW, Gustafsson D, Weitz JI, Hirsh J. Translational success stories: Development of direct thrombin inhibitors. *Circulation Research*. 2012;**111**(7):920-929. DOI: 10.1161/CIRCRESAHA.112.264903
- [34] Haycraft JB. Ueber die Einwirkung eines Secretes des officinellen Blutegels auf die Gerinnbarkeit des Blutes. *Arch exp Path Pharmak*. 1884;**18**:209-217
- [35] Markwardt F. The hirudin tolerance test. *Klinische Wochenschrift*. 1959;**37**:1142-1143

- [36] Markwardt F, Kaiser B, Nowak G. Studies on antithrombotic effects of recombinant hirudin. *Thrombosis Research*. 1989;**54**(5):377-388
- [37] Markwardt F, Nowak G, Sturzebecher U, Walsmann P. Studies on the pharmacokinetics of hirudin. *Biomedica Biochimica Acta*. 1987;**46**(4):237-244
- [38] Markwardt F, Schulz E. On the mechanism of the inhibitory effect of tabanine on blood coagulation. *Naunyn-Schmiedebergs Archiv für Experimentelle Pathologie und Pharmakologie*. 1960;**238**:320-328
- [39] Fortkamp E, Rieger M, Heisterberg-Moutsjes G, Schweitzer S, Sommer R. Cloning and expression in *Escherichia coli* of a synthetic DNA for hirudin, the blood coagulation inhibitor in the leech. *DNA Journal*. 1986;**5**(6):511-517
- [40] Courtney M, Loison G, Lemoine Y, Riehl-Bellon N, Degryse E, Brown SW, et al. Production and evaluation of recombinant hirudin. *Seminars in Thrombosis and Hemostasis*. 1989;**15**(3):288-292. DOI: 10.1055/s-2007-1002721
- [41] Radzio R, Kuck U. Efficient synthesis of the blood-coagulation inhibitor hirudin in the filamentous fungus *Acremonium chrysogenum*. *Applied Microbiology and Biotechnology*. 1997;**48**(1):58-65
- [42] Talbot MD, Ambler J, Butler KD, Findlay VS, Mitchell KA, Peters RF, et al. Recombinant desulphatohirudin (CGP 39393) anticoagulant and antithrombotic properties in vivo. *Thrombosis and Haemostasis*. 1989;**61**(1):77-80
- [43] Verstraete M, Zoldhelyi P. Novel antithrombotic drugs in development. *Drugs*. 1995;**49**(6):856-884
- [44] De Filippis V, Vindigni A, Altichieri L, Fontana A. Core domain of hirudin from the leech *Hirudinaria manillensis*: Chemical synthesis, purification, and characterization of a Trp3 analog of fragment 1-47. *Biochemistry*. 1995;**34**(29):9552-9564
- [45] Narayanan S. Multifunctional roles of thrombin. *Annals of Clinical and Laboratory Science*. 1999;**29**(4):275-280
- [46] Merlini PA, Ardissino D, Rosenberg RD, Colombi E, Agricola P, Oltrona L, et al. In vivo thrombin generation and activity during and after intravenous infusion of heparin or recombinant hirudin in patients with unstable angina pectoris. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2000;**20**(9):2162-2166
- [47] Gajra A, Husain J, Smith A. Lepirudin in the management of heparin-induced thrombocytopenia. *Expert Opinion on Drug Metabolism & Toxicology*. 2008;**4**(8):1131-1141. DOI: 10.1517/17425255.4.8.1131
- [48] Hirsh J, Bauer KA, Donati MB, Gould M, Samama MM, Weitz JI. Parenteral anticoagulants: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th ed.). *Chest*. 2008;**133**(Suppl 6):141S-159S. DOI: 10.1378/chest.08-0689
- [49] Alban S. Pharmacological strategies for inhibition of thrombin activity. *Current Pharmaceutical Design*. 2008;**14**(12):1152-1175

- [50] Nutescu EA, Shapiro NL, Chevalier A. New anticoagulant agents: Direct thrombin inhibitors. *Cardiology Clinics*. 2008;**26**(2):169-187, v-vi. DOI: 10.1016/j.ccl.2007.12.005
- [51] Greinacher A, Warkentin TE. The direct thrombin inhibitor hirudin. *Thrombosis and Haemostasis*. 2008;**99**(5):819-829. DOI: 10.1160/TH07-11-0693
- [52] Warkentin TE, Greinacher A, Koster A. Bivalirudin. *Thrombosis and Haemostasis*. 2008;**99**(5):830-839. DOI: 10.1160/TH07-10-0644
- [53] Hirsh J, O'Donnell M, Weitz JI. New anticoagulants. *Blood*. 2005;**105**(2):453-463. DOI: 10.1182/blood-2003-12-4195
- [54] Schindewolf M, Steindl J, Beyer-Westendorf J, Schellong S, Dohmen PM, Brachmann J, et al. Use of Fondaparinux off-label or approved anticoagulants for management of heparin-induced thrombocytopenia. *Journal of the American College of Cardiology*. 2017;**70**(21):2636-2648. DOI: 10.1016/j.jacc.2017.09.1099
- [55] Lepirudin [Internet]. 2017. Available from: <https://www.drugs.com/pro/refludan.html> [Accessed: Dec 19, 2017]
- [56] Desirudin [Internet]. 2017. Available from <https://www.drugs.com/pro/refludan.html> [Accessed: Nov 29, 2017]
- [57] Eriksson BI, Wille-Jorgensen P, Kalebo P, Mouret P, Rosencher N, Bosch P, et al. A comparison of recombinant hirudin with a low-molecular-weight heparin to prevent thromboembolic complications after total hip replacement. *The New England Journal of Medicine*. 1997;**337**(19):1329-1335. DOI: 10.1056/NEJM199711063371901
- [58] Eriksson BI, Ekman S, Lindbratt S, Baur M, Bach D, Torholm C, et al. Prevention of thromboembolism with use of recombinant hirudin. Results of a double-blind, multi-center trial comparing the efficacy of desirudin (Revasc) with that of unfractionated heparin in patients having a total hip replacement. *The Journal of Bone and Joint Surgery. American Volume*. 1997;**79**(3):326-333
- [59] Frame JN, Rice L, Bartholomew JR, Whelton A. Rationale and design of the PREVENT-HIT study: A randomized, open-label pilot study to compare desirudin and argatroban in patients with suspected heparin-induced thrombocytopenia with or without thrombosis. *Clinical Therapeutics*. 2010;**32**(4):626-636. DOI: 10.1016/j.clinthera.2010.04.012
- [60] Maraganore JM, Chao B, Joseph ML, Jablonski J, Ramachandran KL. Anticoagulant activity of synthetic hirudin peptides. *The Journal of Biological Chemistry*. 1989;**264**(15):8692-8698
- [61] Steinmetzer T, Sturzebecher J. Progress in the development of synthetic thrombin inhibitors as new orally active anticoagulants. *Current Medicinal Chemistry*. 2004;**11**(17):2297-2321
- [62] Bates SM, Weitz JI. The mechanism of action of thrombin inhibitors. *The Journal of Invasive Cardiology*. 2000;**12**(Suppl F):27F-232F
- [63] Reed MD, Bell D. Clinical pharmacology of bivalirudin. *Pharmacotherapy*. 2002;**22**(6 Pt 2):105S-111S

- [64] Garcia DA, Baglin TP, Weitz JI, Samama MM. Parenteral anticoagulants: Antithrombotic therapy and prevention of thrombosis. 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;**141**(Suppl 2):e24S-e43S. DOI: 10.1378/chest.11-2291
- [65] Lincoff AM, Bittl JA, Harrington RA, Feit F, Kleiman NS, Jackman JD, et al. Bivalirudin and provisional glycoprotein IIb/IIIa blockade compared with heparin and planned glycoprotein IIb/IIIa blockade during percutaneous coronary intervention: REPLACE-2 randomized trial. *Journal of the American Medical Association*. 2003;**289**(7):853-863
- [66] Stone GW, Witzenbichler B, Guagliumi G, Peruga JZ, Brodie BR, Dudek D, et al. Heparin plus a glycoprotein IIb/IIIa inhibitor versus bivalirudin monotherapy and paclitaxel-eluting stents versus bare-metal stents in acute myocardial infarction (HORIZONS-AMI): Final 3-year results from a multicentre, randomised controlled trial. *Lancet*. 2011;**377**(9784):2193-2204. DOI: 10.1016/S0140-6736(11)60764-2
- [67] Stone GW, McLaurin BT, Cox DA, Bertrand ME, Lincoff AM, Moses JW, et al. Bivalirudin for patients with acute coronary syndromes. *The New England Journal of Medicine*. 2006;**355**(21):2203-2216. DOI: 10.1056/NEJMoa062437
- [68] Dyke CM, Smedira NG, Koster A, Aronson S, McCarthy 2nd HL, Kirshner R, et al. A comparison of bivalirudin to heparin with protamine reversal in patients undergoing cardiac surgery with cardiopulmonary bypass: The EVOLUTION-ON study. *The Journal of Thoracic and Cardiovascular Surgery*. 2006;**131**(3):533-539. DOI: 10.1016/j.jtcvs.2005.09.057
- [69] Smedira NG, Dyke CM, Koster A, Jurmann M, Bhatia DS, Hu T, et al. Anticoagulation with bivalirudin for off-pump coronary artery bypass grafting: The results of the EVOLUTION-OFF study. *The Journal of Thoracic and Cardiovascular Surgery*. 2006;**131**(3):686-692. DOI: 10.1016/j.jtcvs.2005.10.049
- [70] Ginsberg JS, Nurmohamed MT, Gent M, MacKinnon B, Sicurella J, Brill-Edwards P, et al. Use of Hirulog in the prevention of venous thrombosis after major hip or knee surgery. *Circulation*. 1994;**90**(5):2385-2389
- [71] Ginsberg JS, Nurmohamed MT, Gent M, MacKinnon B, Stevens P, Weitz J, et al. Effects on thrombin generation of single injections of Hirulog in patients with calf vein thrombosis. *Thrombosis and Haemostasis*. 1994;**72**(4):523-525
- [72] Lee CJ, Ansell JE. Direct thrombin inhibitors. *British Journal of Clinical Pharmacology*. 2011;**72**(4):581-592. DOI: 10.1111/j.1365-2125.2011.03916.x
- [73] Hong SJ, Kang KW. Purification of granulin-like polypeptide from the blood-sucking leech, *Hirudo nipponia*. *Protein Expression and Purification*. 1999;**16**(2):340-346. DOI: 10.1006/prev.1999.1077
- [74] Electricwala A, Sawyer RT, Jones CP, Atkinson T. Isolation of thrombin inhibitor from the leech *Hirudinaria manillensis*. *Blood Coagulation & Fibrinolysis*. 1991;**2**(1):83-89
- [75] Salzet M, Chopin V, Baert J, Matias I, Malecha J. Theromin, a novel leech thrombin inhibitor. *The Journal of Biological Chemistry*. 2000;**275**(40):30774-30780. DOI: 10.1074/jbc.M000787200

- [76] Strube KH, Kroger B, Bialojan S, Otte M, Dodt J. Isolation, sequence analysis, and cloning of haemadin. An anticoagulant peptide from the Indian leech. *The Journal of Biological Chemistry*. 1993;**268**(12):8590-8595
- [77] Strube KH, Bialojan S, Kroeger B, Friedrich T. Thrombin-inhibitory proteins from terrestrial leeches. Google Patents. 1995
- [78] Richardson JL, Fuentes-Prior P, Sadler JE, Huber R, Bode W. Characterization of the residues involved in the human alpha-thrombin-haemadin complex: An exosite II-binding inhibitor. *Biochemistry*. 2002;**41**(8):2535-2542
- [79] Richardson JL, Kroger B, Hoeffken W, Sadler JE, Pereira P, Huber R, et al. Crystal structure of the human alpha-thrombin-haemadin complex: An exosite II-binding inhibitor. *The EMBO Journal*. 2000;**19**(21):5650-5660. DOI: 10.1093/emboj/19.21.5650
- [80] Chopin V, Bilfinger TV, Stefano GB, Matias I, Salzet M. Amino-acid-sequence determination and biological activity of cytin, a naturally occurring specific chymotrypsin inhibitor from the leech *Theromyzon tessulatum*. *European Journal of Biochemistry*. 1997;**249**(3):733-738
- [81] Chopin V, Matias I, Stefano GB, Salzet M. Amino acid sequence determination and biological activity of therin, a naturally occurring specific trypsin inhibitor from the leech *Theromyzon tessulatum*. *European Journal of Biochemistry*. 1998;**254**(3):565-570
- [82] Chopin V, Salzet M, Baert J, Vandenbulcke F, Sautiere PE, Kerckaert JP, et al. Therostasin, a novel clotting factor Xa inhibitor from the rhynchobdellid leech, *Theromyzon tessulatum*. *The Journal of Biological Chemistry*. 2000;**275**(42):32701-32707. DOI: 10.1074/jbc.M90-9217199
- [83] Chopin V, Stefano GB, Salzet M. Amino-acid-sequence determination and biological activity of tessulin, a naturally occurring trypsin-chymotrypsin inhibitor isolated from the leech *Theromyzon tessulatum*. *European Journal of Biochemistry*. 1998;**258**(2):662-668
- [84] Hemberger J, Sawyer R, Wolf S, Dodt J. Thrombin-inhibitors. Google Patents. 2003
- [85] Rao PSS, Burkart T. Advances in oral anticoagulation therapy – What's in the pipeline? *Blood Reviews*. 2017;**31**(4):205-211. DOI: 10.1016/j.blre.2017.02.002
- [86] Nutt E, Gasic T, Rodkey J, Gasic GJ, Jacobs JW, Friedman PA, et al. The amino acid sequence of antistasin. A potent inhibitor of factor Xa reveals a repeated internal structure. *The Journal of Biological Chemistry*. 1988;**263**(21):10162-10167
- [87] Bott JA. Leech protein having anticoagulant and antimetastatic properties. Google Patents. 1993
- [88] Blankenship DT, Brankamp RG, Manley GD, Cardin AD. Amino acid sequence of ghilanten: Anticoagulant-antimetastatic principle of the South American leech, *Haementeria ghilianii*. *Biochemical and Biophysical Research Communications*. 1990;**166**(3):1384-1389

- [89] Theunissen HJ, Dijkema R, Swinkels JC, de Poorter TL, Vink PM, van Dinther TG. Mutational analysis of antistasin, an inhibitor of blood coagulation factor Xa derived from the Mexican leech *Haementeria officinalis*. *Thrombosis Research*. 1994;**75**(1):41-50
- [90] Han JH, Law SW, Keller PM, Kniskern PJ, Silberklang M, Tung JS, et al. Cloning and expression of cDNA encoding antistasin, a leech-derived protein having anti-coagulant and anti-metastatic properties. *Gene*. 1989;**75**(1):47-57
- [91] Ragosta M, Gimple LW, Gertz SD, Dunwiddie CT, Vlasuk GP, Haber HL, et al. Specific factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation*. 1994;**89**(3):1262-1271
- [92] Ohta N, Brush M, Jacobs JW. Interaction of antistasin-related peptides with factor Xa: Identification of a core inhibitory sequence. *Thrombosis and Haemostasis*. 1994;**72**(6):825-830
- [93] Oliveira DG, Alvarez-Flores MP, Lopes AR, Chudzinski-Tavassi AM. Functional characterisation of vizottin, the first factor Xa inhibitor purified from the leech *Haementeria vizottoi*. *Thrombosis and Haemostasis*. 2012;**108**(3):570-578. DOI: 10.1160/TH12-04-0235
- [94] Faria F, Kelen EM, Sampaio CA, Bon C, Duval N, Chudzinski-Tavassi AM. A new factor Xa inhibitor (lefaxin) from the *Haementeria depressa* leech. *Thrombosis and Haemostasis*. 1999;**82**(5):1469-1473
- [95] Chudzinski-Tavassi AM, Carrijo-Carvalho LC, Faria F, Alvarez Flores MP, Simons SM. Exogenous factors affecting hemostasis: New therapeutic perspectives and biotechnological approaches. In: Lima ME, Pimenta AMC, Martin-Eauclaire MF, Zingali RB, Rochat H, editors. *Animal Toxins: State of the Art Perspectives in Health and Biotechnology*. Belo Horizonte: UFMG; 2009. pp. 495-523
- [96] Connolly TM, Jacobs JW, Condra C. An inhibitor of collagen-stimulated platelet activation from the salivary glands of the *Haementeria officinalis* leech. I. Identification, isolation, and characterization. *The Journal of Biological Chemistry*. 1992;**267**(10):6893-6898
- [97] Keller PM, Schultz LD, Condra C, Karczewski J, Connolly TM. An inhibitor of collagen-stimulated platelet activation from the salivary glands of the *Haementeria officinalis* leech. II. Cloning of the cDNA and expression. *The Journal of Biological Chemistry*. 1992;**267**(10):6899-6904
- [98] Depraetere H, Kerekes A, Deckmyn H. The collagen-binding leech products rLAPP and calin prevent both von Willebrand factor and alpha2beta1(GPIIb/IIIa)-I-domain binding to collagen in a different manner. *Thrombosis and Haemostasis*. 1999;**82**(3):1160-1163
- [99] van Zanten GH, Connolly TM, Schiphorst ME, de Graaf S, Slootweg PJ, Sixma JJ. Recombinant leech antiplatelet protein specifically blocks platelet deposition on collagen surfaces under flow conditions. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 1995;**15**(9):1424-1431

- [100] Schaffer LW, Davidson JT, Vlasuk GP, Dunwiddie CT, Siegl PK. Selective factor Xa inhibition by recombinant antistasin prevents vascular graft thrombosis in baboons. *Arteriosclerosis and Thrombosis : A Journal of Vascular Biology*. 1992;**12**(8):879-885
- [101] Huizinga EG, Schouten A, Connolly TM, Kroon J, Sixma JJ, Gros P. The structure of leech anti-platelet protein, an inhibitor of haemostasis. *Acta Crystallographica Section D, Biological Crystallography*. 2001;**57**(Pt 8):1071-1078
- [102] Harsfalvi J, Stassen JM, Hoylaerts MF, Van Houtte E, Sawyer RT, Vermynen J, et al. Calin from *Hirudo medicinalis*, an inhibitor of von Willebrand factor binding to collagen under static and flow conditions. *Blood*. 1995;**85**(3):705-711
- [103] White TC, Berny MA, Robinson DK, Yin H, DeGrado WF, Hanson SR, et al. The leech product saratin is a potent inhibitor of platelet integrin $\alpha 2\beta 1$ and von Willebrand factor binding to collagen. *The FEBS Journal*. 2007;**274**(6):1481-1491
- [104] Barnes CS, Krafft B, Frech M, Hofmann UR, Papendieck A, Dahlems U, et al. Production and characterization of saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion to collagen. *Seminars in Thrombosis and Hemostasis*. 2001;**27**(4):337-348. DOI: 10.1055/s-2001-16887
- [105] Saratin [Internet]. 2017. Available from: <http://www.biovascularinc.com/saratin.html> [Accessed: Dec 17, 2017]
- [106] Min J, Lukowski ZL, Levine MA, Meyers CA, Beattie AR, Schultz GS, et al. Prevention of ocular scarring post glaucoma filtration surgery using the inflammatory cell and platelet binding modulator saratin in a rabbit model. *PLoS One*. 2012;**7**(4):e35627. DOI: 10.1371/journal.pone.0035627
- [107] Martorana GM, Schaefer JL, Levine MA, Lukowski ZL, Min J, Meyers CA, et al. Sequential therapy with saratin, bevacizumab and ilomastat to prolong bleb function following glaucoma filtration surgery in a rabbit model. *PLoS One*. 2015;**10**(9):e0138054. DOI: 10.1371/journal.pone.0138054
- [108] Cruz CP, Eidt J, Drouilhet J, Brown AT, Wang Y, Barnes CS, et al. Saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion, decreases platelet aggregation and intimal hyperplasia in a rat carotid endarterectomy model. *Journal of Vascular Surgery*. 2001;**34**(4):724-729. DOI: 10.1067/mva.2001.116801
- [109] Assumpcao TC, Ribeiro JM, Francischetti IM. Disintegrins from hematophagous sources. *Toxins*. 2012;**4**(5):296-322. DOI: 10.3390/toxins4050296
- [110] Mazur P, Henzel WJ, Seymour JL, Lazarus RA. Ornatins: Potent glycoprotein IIb-IIIa antagonists and platelet aggregation inhibitors from the leech *Placobdella ornata*. *European Journal of Biochemistry*. 1991;**202**(3):1073-1082
- [111] Mazur P, Dennis MS, Seymour JL, Lazarus RA. Expression, purification, and characterization of recombinant ornatin E, a potent glycoprotein IIb-IIIa antagonist. *Protein Expression and Purification*. 1993;**4**(4):282-289. DOI: 10.1006/prep.1993.1036
- [112] Prasa D, Sturzebecher J. Inhibitors of factor XIIIa. *Hämostaseologie*. 2002;**22**(1):43-47

- [113] Finney S, Seale L, Sawyer RT, Wallis RB. Tridegin, a new peptidic inhibitor of factor XIIIa, from the blood-sucking leech *Haementeria ghilianii*. *The Biochemical Journal*. 1997;**324**(Pt 3):797-805
- [114] Bohm M, Kuhl T, Harges K, Coch R, Arkona C, Schlott B, et al. Synthesis and functional characterization of tridegin and its analogues: Inhibitors and substrates of factor XIIIa. *ChemMedChem*. 2012;**7**(2):326-333. DOI: 10.1002/cmdc.201100405
- [115] Budzynski AZ, Olexa SA, Sawyer RT. Composition of salivary gland extracts from the leech *Haementeria ghilianii*. *Proceedings of the Society for Experimental Biology and Medicine*. 1981;**168**(2):259-265
- [116] Munro R, Jones CP, Sawyer RT. Effects of hementin on fibrinogen mediated platelet aggregation/deaggregation in vitro. *Platelets*. 1991;**2**(1):55-56. DOI: 10.3109/09537109-109005505
- [117] Budzynski AZ. Interaction of hementin with fibrinogen and fibrin. *Blood Coagulation & Fibrinolysis*. 1991;**2**(1):149-152
- [118] Wallis RB, Seale L, Finney S, Sawyer RT, Bennett GM, Ross-Murphy SB. Reduction of plasma clot stability by a novel factor XIIIa inhibitor from the Giant Amazon leech, *Haementeria ghilianii*. *Blood Coagulation & Fibrinolysis*. 1997;**8**(5):291-295
- [119] Seale L, Finney S, Sawyer RT, Wallis RB. Tridegin, a novel peptidic inhibitor of factor XIIIa from the leech, *Haementeria ghilianii*, enhances fibrinolysis in vitro. *Thrombosis and Haemostasis*. 1997;**77**(5):959-963
- [120] Chudzinski-Tavassi AM, Kelen EM, de Paula Rosa AP, Loyau S, Sampaio CA, Bon C, et al. Fibrino(gen)olytic properties of purified hementerin, a metalloproteinase from the leech *Haementeria depressa*. *Thrombosis and Haemostasis*. 1998;**80**(1):155-160
- [121] Chudzinski-Tavassi AM, Bermej E, Rosenstein RE, Faria F, Sarmiento MI, Alberto F, et al. Nitridergic platelet pathway activation by hementerin, a metalloprotease from the leech *Haementeria depressa*. *Biological Chemistry*. 2003;**384**(9):1333-1339. DOI: 10.1515/BC.2003.150
- [122] Baskova IP, Nikonov GI. Destabilase: An enzyme of medicinal leech salivary gland secretion hydrolyzes the isopeptide bonds in stabilized fibrin. *Biokhimiia*. 1985;**50**(3):424-431
- [123] Baskova IP, Zavalova LL. Polyfunctionality of destabilase, a lysozyme from a medicinal leech. *Bioorganicheskaia Khimiia*. 2008;**34**(3):337-343
- [124] Zavalova LL, Nikonov GI, Kuzina EV, Popova G, Baskova IP. A dimer of fragment D from stabilized fibrin – A substrate for the destabilase enzyme (gamma-glutamyl-epsilon-lysyl-isopeptidase). *Biokhimiia*. 1991;**56**(1):115-124
- [125] Baskova IP, Zavalova LL, Basanova AV, Grigor'eva OV. The hydrolysis of Glu(gamma-epsilon)Lys-amide bonds in D-dimer fragment of fibrin by destabilase from medicinal leech. *Bioorganicheskaia Khimiia*. 1999;**25**(6):435-438
- [126] Kurdyumov AS, Manuvera VA, Baskova IP, Lazarev VN. A comparison of the enzymatic properties of three recombinant isoforms of thrombolytic and antibacterial

- protein – Destabilase-lysozyme from medicinal leech. *BMC Biochemistry*. 2015;**16**:27. DOI: 10.1186/s12858-015-0056-3
- [127] Francischetti IM, Sa-Nunes A, Mans BJ, Santos IM, Ribeiro JM. The role of saliva in tick feeding. *Frontiers in Bioscience (Landmark Ed)*. 2009;**14**:2051-2088
- [128] Kazimirova M, Stibraniova I. Tick salivary compounds: Their role in modulation of host defences and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*. 2013;**3**:43. DOI: 10.3389/fcimb.2013.00043
- [129] Binnington KC, Kemp DH. Role of tick salivary glands in feeding and disease transmission. *Advances in Parasitology*. 1980;**18**:315-339
- [130] Bowman AS, Coons LB, Needham GR, Sauer JR. Tick saliva: Recent advances and implications for vector competence. *Medical and Veterinary Entomology*. 1997;**11**(3):277-285
- [131] Sonenshine DE. *Biology of Ticks*. New York: Oxford University Press; 1991
- [132] Koh CY, Kini RM. Anticoagulants from hematophagous animals. *Expert Review of Hematology*. 2008;**1**(2):135-139. DOI: 10.1586/17474086.1.2.135
- [133] Chmelar J, Oliveira CJ, Rezacova P, Francischetti IM, Kovarova Z, Pejler G, et al. A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation. *Blood*. 2011;**117**(2):736-744. DOI: 10.1182/blood-2010-06-293241
- [134] Joubert AM, Louw AI, Joubert F, Neitz AW. Cloning, nucleotide sequence and expression of the gene encoding factor Xa inhibitor from the salivary glands of the tick, *Ornithodoros savignyi*. *Experimental & Applied Acarology*. 1998;**22**(10):603-619
- [135] Mans BJ, Louw AI, Neitz AW. Amino acid sequence and structure modeling of savignin, a thrombin inhibitor from the tick, *Ornithodoros savignyi*. *Insect Biochemistry and Molecular Biology*. 2002;**32**(7):821-828
- [136] Simo L, Kazimirova M, Richardson J, Bonnet SI. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*. 2017;**7**:281. DOI: 10.3389/fcimb.2017.00281
- [137] Ibelli AM, Kim TK, Hill CC, Lewis LA, Bakshi M, Miller S, et al. A blood meal-induced *Ixodes scapularis* tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. *International Journal for Parasitology*. 2014;**44**(6):369-379. DOI: 10.1016/j.ijpara.2014.01.010
- [138] Mans BJ, Coetzee J, Louw AI, Gaspar AR, Neitz AW. Disaggregation of aggregated platelets by apyrase from the tick, *Ornithodoros savignyi* (Acari: Argasidae). *Experimental & Applied Acarology*. 2000;**24**(4):271-282
- [139] Waxman L, Connolly TM. Isolation of an inhibitor selective for collagen-stimulated platelet aggregation from the soft tick *Ornithodoros moubata*. *The Journal of Biological Chemistry*. 1993;**268**(8):5445-5449
- [140] Karczewski J, Waxman L, Endris RG, Connolly TM. An inhibitor from the argasid tick *Ornithodoros moubata* of cell adhesion to collagen. *Biochemical and Biophysical Research Communications*. 1995;**208**(2):532-541. DOI: 10.1006/bbrc.1995.1371

- [141] Mans BJ, Louw AI, Neitz AW. Disaggregation of aggregated platelets by savignygrin, a α IIb β 3 antagonist from *Ornithodoros savignyi*. *Experimental & Applied Acarology*. 2002;**27**(3):231-239
- [142] Wang X, Coons LB, Taylor DB, Stevens SE Jr, Gartner TK. Variabilin, a novel RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor from the hard tick *Dermacentor variabilis*. *The Journal of Biological Chemistry*. 1996;**271**(30):17785-17790
- [143] Francischetti IM. Platelet aggregation inhibitors from hematophagous animals. *Toxicon*. 2010;**56**(7):1130-1144. DOI: 10.1016/j.toxicon.2009.12.003
- [144] Mans BJ, Ribeiro JM. Function, mechanism and evolution of the moubatin-clade of soft tick lipocalins. *Insect Biochemistry and Molecular Biology*. 2008;**38**(9):841-852. DOI: 10.1016/j.ibmb.2008.06.007
- [145] Cheng Y, Wu H, Li D. An inhibitor selective for collagen-stimulated platelet aggregation from the salivary glands of hard tick *Haemaphysalis longicornis* and its mechanism of action. *Science in China Series C, Life Sciences*. 1999;**42**(5):457-464. DOI: 10.1007/BF02881768
- [146] Francischetti IM, Valenzuela JG, Andersen JF, Mather TN, Ribeiro JM. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: Identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. *Blood*. 2002;**99**(10):3602-3612. DOI: 10.1182/blood-2001-12-0237
- [147] Francischetti IM, Valenzuela JG, Pham VM, Garfield MK, Ribeiro JM. Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*. *The Journal of Experimental Biology*. 2002;**205**(Pt 16):2429-2451
- [148] Francischetti IM, Mather TN, Ribeiro JM. Tick saliva is a potent inhibitor of endothelial cell proliferation and angiogenesis. *Thrombosis and Haemostasis*. 2005;**94**(1):167-174. DOI: 10.1160/TH04-09-0566
- [149] Gaspar AR, Joubert AM, Crause JC, Neitz AW. Isolation and characterization of an anticoagulant from the salivary glands of the tick, *Ornithodoros savignyi* (Acari: Argasidae). *Experimental & Applied Acarology*. 1996;**20**(10):583-598
- [150] Neeper MP, Waxman L, Smith DE, Schulman CA, Sardana M, Ellis RW, et al. Characterization of recombinant tick anticoagulant peptide. A highly selective inhibitor of blood coagulation factor Xa. *The Journal of Biological Chemistry*. 1990;**265**(29):17746-17752
- [151] Vlasuk GP, Ramjit D, Fujita T, Dunwiddie CT, Nutt EM, Smith DE, et al. Comparison of the in vivo anticoagulant properties of standard heparin and the highly selective factor Xa inhibitors antistasin and tick anticoagulant peptide (TAP) in a rabbit model of venous thrombosis. *Thrombosis and Haemostasis*. 1991;**65**(3):257-262
- [152] Koh CY, Kini RM. Molecular diversity of anticoagulants from haematophagous animals. *Thrombosis and Haemostasis*. 2009;**102**(3):437-453. DOI: 10.1160/TH09-04-0221

- [153] Batista IF, Chudzinski-Tavassi AM, Faria F, Simons SM, Barros-Batesti DM, Labruna MB, et al. Expressed sequence tags (ESTs) from the salivary glands of the tick *Amblyomma cajennense* (Acari: Ixodidae). *Toxicon*. 2008;**51**(5):823-834. DOI: 10.1016/j.toxicon.2007.12.011
- [154] Branco VG, Iqbal A, Alvarez-Flores MP, Sciani JM, de Andrade SA, Iwai LK, et al. Amblyomin-X having a Kunitz-type homologous domain, is a noncompetitive inhibitor of FXa and induces anticoagulation in vitro and in vivo. *Biochimica et Biophysica Acta*. 2016;**1864**(10):1428-1435. DOI: 10.1016/j.bbapap.2016.07.011
- [155] de Souza JG, Morais KL, Angles-Cano E, Bouffleur P, de Mello ES, Maria DA, et al. Promising pharmacological profile of a Kunitz-type inhibitor in murine renal cell carcinoma model. *Oncotarget*. 2016;**7**(38):62255-62266. DOI: 10.18632/oncotarget.11555
- [156] Morais KL, Pacheco MT, Berra CM, Bosch RV, Sciani JM, Chammas R, et al. Amblyomin-X induces ER stress, mitochondrial dysfunction, and caspase activation in human melanoma and pancreatic tumor cell. *Molecular and Cellular Biochemistry*. 2016;**415**(1-2):119-131. DOI: 10.1007/s11010-016-2683-4
- [157] Chudzinski-Tavassi AM, Morais KL, Pacheco MT, Pasqualoto KF, de Souza JG. Tick salivary gland as potential natural source for the discovery of promising antitumor drug candidates. *Biomedicine & Pharmacotherapy*. 2016;**77**:14-19. DOI: 10.1016/j.biopha.2015.11.003
- [158] Pacheco MT, Morais KL, Berra CM, Demasi M, Sciani JM, Branco VG, et al. Specific role of cytoplasmic dynein in the mechanism of action of an antitumor molecule, Amblyomin-X. *Experimental Cell Research*. 2016;**340**(2):248-258. DOI: 10.1016/j.yexcr.2015.12.016
- [159] Pacheco MT, Berra CM, Morais KL, Sciani JM, Branco VG, Bosch RV, et al. Dynein function and protein clearance changes in tumor cells induced by a Kunitz-type molecule, Amblyomin-X. *PLoS One*. 2014;**9**(12):e111907. DOI: 10.1371/journal.pone.0111907
- [160] Ventura JS, Faria F, Batista IF, Simons SM, Oliveira DG, Morais KL, et al. A Kunitz-type FXa inhibitor affects tumor progression, hypercoagulable state and triggers apoptosis. *Biomedicine & Pharmacotherapy*. 2013;**67**(3):192-196. DOI: 10.1016/j.biopha.2012.11.009
- [161] Maria DA, de Souza JG, Morais KL, Berra CM, Zampolli Hde C, Demasi M, et al. A novel proteasome inhibitor acting in mitochondrial dysfunction, ER stress and ROS production. *Investigational New Drugs*. 2013;**31**(3):493-505. DOI: 10.1007/s10637-012-9871-1
- [162] Narasimhan S, Koski RA, Beaulieu B, Anderson JF, Ramamoorthi N, Kantor F, et al. A novel family of anticoagulants from the saliva of *Ixodes scapularis*. *Insect Molecular Biology*. 2002;**11**(6):641-650
- [163] Limo MK, Voigt WP, Tumbo-Oeri AG, Njogu RM, Ole-MoiYoi OK. Purification and characterization of an anticoagulant from the salivary glands of the ixodid tick *Rhipicephalus appendiculatus*. *Experimental Parasitology*. 1991;**72**(4):418-429

- [164] Crawley JT, Zanardelli S, Chion CK, Lane DA. The central role of thrombin in hemostasis. *Journal of Thrombosis and Haemostasis*. 2007;**5**(Suppl 1):95-101. DOI: 10.1111/j.1538-7836.2007.02500.x
- [165] Bock PE, Panizzi P, Verhamme IM. Exosites in the substrate specificity of blood coagulation reactions. *Journal of Thrombosis and Haemostasis*. 2007;**5**(Suppl 1):81-94. DOI: 10.1111/j.1538-7836.2007.02496.x
- [166] Weitz JI. Factor Xa or thrombin: Is thrombin a better target? *Journal of Thrombosis and Haemostasis*. 2007;**5**(Suppl 1):65-67. DOI: 10.1111/j.1538-7836.2007.02552.x
- [167] Iyer JK, Koh CY, Kazimirova M, Roller L, Jobichen C, Swaminathan K, et al. Avathrin: A novel thrombin inhibitor derived from a multicopy precursor in the salivary glands of the ixodid tick, *Amblyomma variegatum*. *The FASEB Journal*. 2017;**31**(7):2981-2995. DOI: 10.1096/fj.201601216R
- [168] Lai R, Takeuchi H, Jonczy J, Rees HH, Turner PC. A thrombin inhibitor from the ixodid tick, *Amblyomma hebraeum*. *Gene*. 2004;**342**(2):243-249. DOI: 10.1016/j.gene.2004.07.012
- [169] Macedo-Ribeiro S, Almeida C, Calisto BM, Friedrich T, Mentele R, Sturzebecher J, et al. Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLoS One*. 2008;**3**(2):e1624. DOI: 10.1371/journal.pone.0001624
- [170] Liao M, Zhou J, Gong H, Boldbaatar D, Shirafuji R, Battur B, et al. Hemalin, a thrombin inhibitor isolated from a midgut cDNA library from the hard tick *Haemaphysalis longicornis*. *Journal of Insect Physiology*. 2009;**55**(2):164-173. DOI: 10.1016/j.jinsphys.2008.11.004
- [171] Iqbal A, Goldfeder MB, Marques-Porto R, Asif H, Souza JG, Faria F, et al. Revisiting antithrombotic therapeutics; sculptin, a novel specific, competitive, reversible, scissile and tight binding inhibitor of thrombin. *Scientific Reports*. 2017;**7**(1):1431. DOI: 10.1038/s41598-017-01486-w
- [172] van de Locht A, Stubbs MT, Bode W, Friedrich T, Bollschweiler C, Hoffken W, et al. The ornithodorin-thrombin crystal structure, a key to the TAP enigma? *The EMBO Journal*. 1996;**15**(22):6011-6017
- [173] Nienaber J, Gaspar AR, Neitz AW. Savignin, a potent thrombin inhibitor isolated from the salivary glands of the tick *Ornithodoros savignyi* (Acari: Argasidae). *Experimental Parasitology*. 1999;**93**(2):82-91. DOI: 10.1006/expr.1999.4448
- [174] Mans BJ, Andersen JF, Schwan TG, Ribeiro JM. Characterization of anti-hemostatic factors in the argasid, *Argas monolakensis*: Implications for the evolution of blood-feeding in the soft tick family. *Insect Biochemistry and Molecular Biology*. 2008;**38**(1):22-41. DOI: 10.1016/j.ibmb.2007.09.002
- [175] Bullard R, Allen P, Chao CC, Douglas J, Das P, Morgan SE, et al. Structural characterization of tick cement cones collected from in vivo and artificial membrane blood-fed lone

star ticks (*Amblyomma americanum*). Ticks and Tick-borne Diseases. 2016;7(5):880-892. DOI: 10.1016/j.ttbdis.2016.04.006

- [176] Francischetti IM, Mather TN, Ribeiro JM. Cloning of a salivary gland metalloprotease and characterization of gelatinase and fibrin(ogen)lytic activities in the saliva of the Lyme disease tick vector *Ixodes scapularis*. Biochemical and Biophysical Research Communications. 2003;305(4):869-875
- [177] Anisuzzaman IMK, Alim MA, Miyoshi T, Hatta T, Yamaji K, et al. Longistatin, a plasminogen activator, is key to the availability of blood-meals for ixodid ticks. PLoS Pathogens. 2011;7(3):e1001312. DOI: 10.1371/journal.ppat.1001312
- [178] Anisuzzaman HT, Miyoshi T, Matsubayashi M, Islam MK, Alim MA, et al. Longistatin in tick saliva blocks advanced glycation end-product receptor activation. The Journal of Clinical Investigation. 2014;124(10):4429-4444

IntechOpen