Exogenous Procoagulant Factors as Therapeutic and Biotechnological Tools

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Abstract

A diversity of animal venoms and secretions has been described to affect the hemostatic system with actions on blood coagulation and fibrinolytic pathways. These biological materials are rich sources of proteins and peptides with distinct biochemical properties, which have a biological function for the animal. Snake venoms are one of the richest sources of exogenous hemostatic factors, especially procoagulant proteins. Insects are another important source of proteins and peptides targeting the hemostatic system. Exogenous procoagulant factors have a large functional diversity and present potential applications in health and biotechnology. They have been important tools for the diagnosis and therapy of several blood coagulation disorders. Recently, many studies have pointed out that exogenous hemostatic factors can also display non-hemostatic functions, bringing new perspectives for the study of these molecules.

Keywords: Exogenous hemostatic factors; Coagulation; Fibrinolysis; Procoagulant; Anticoagulant; Toxins

Introduction

Many compounds affecting the hemostatic system have been described from several sources such as fungus, bacteria, plants, animal venoms, and animal fluids and secretions, playing roles on biological processes, such as feeding, digestion, self-defense, and also in the internal physiological process of the organism [1]. Especially the animal venoms and secretions are rich and complex mixtures of biologically active proteins and peptides that exhibit several functions on the hemostatic system.

Exogenous factors affecting hemostasis, especially toxins from animal venoms, have largely been studied with a purpose to understanding the pathophysiology of envenoming in human accidents involving snakes, spiders, caterpillars, and wasps, for instance [2]. Many of these toxins are proteins, triggering pro or anticoagulant activities, and they have been characterized in respect to their biochemical and pharmacological properties. They can be involved in various effects observed in the poisoning, such as bleeding, hemorrhage and disseminated intravascular coagulation [3].

On the other hand, efforts have been applied on the research of these proteins for cataloging and classifying them according to sequence analysis, structure and activity [4-6]. In this regard, new molecules have been discovered, opening new perspectives for basic and applied researches. These molecules can point out novel mechanisms of action, undiscovered molecular interactions and new classes of enzymes and inhibitors.

Exogenous hemostatic factors can also have a wide range of biotechnological and pharmacological applications [3]. Based on their specific activities, exogenous factors can be used as reagents in diagnostic kits to detect hemostatic disturbances and deficiencies of a clotting factor, or even be used as components for kits to monitor hemostatic parameters [7]. In addition, exogenous factors have been suggested as therapeutic agents for various disturbances that involve unbalanced hemostasis, such as thrombosis, atherosclerosis, stroke, clotting factor deficiencies, cancer and bleeding reverser [8,9].

The sources of exogenous hemostatic factors

Important sources of exogenous hemostatic factors are animal venoms mainly from snakes, which involve specialized structures for venom production, storage and injection. Toxins present in these venoms intended to act on the hemostatic system of the prey. On the other hand, there are venom toxins that can be obtained from animal tissue extracts, such as the caterpillar bristles, because they do not have well specialized structures devoted to venom production and injection, which means they are disperse over the caterpillar’s body [10]. In general, the exogenous action on hemostasis of these toxins has a defense purpose, protecting the poisoning animal against predators.

In the Insecta class there are a few members reported that produce toxins with direct activities on coagulation and fibrinolysis. Moth caterpillars from the genus *Lonomia* can cause a severe hemorrhagic syndrome after skin contact with their bristles, and they can be found mainly in Brazil and Venezuela [2]. In the South and Southeast regions of Brazil, the species *Lonomia obliqua* has been associated with human envenomation accidents since the 1980s, and it is considered as a public health problem [11]. The mechanism by which *Lonomia obliqua* induces the hemorrhagic syndrome is through a consumptive coagulopathy due to procoagulant toxins contained in its venom [12]. Wasps' venom has proteolytic enzymes that display anticoagulant function by hydrolyzing several clotting factors, such as FII, FVII, FVIII, FIX, FX and tissue factor (TF) [13].

White (2005) listed the snakes of medical importance that affect the hemostatic system. The venom of these reptiles can cause several types of coagulopathy including procoagulant and anticoagulant actions, fibrinogen clotting, fibrinolysis, platelet-activation, prothrombotic and hemorrhagic states [14]. A diversity of toxins from snake venoms has been described: hemorrhagins, clotting factor activators, clotting...
factor inhibitors, proteins affecting platelets and fibrinolysis [15]. Disturbances in the hemostatic system are not the main effects found for fish and spider venoms, except for the genus *Loxoceles* [16]. Envenomation by *Loxoceles* spiders causes an increase in the activated partial thromboplastin time and depletion of the clotting factors VIII, IX, XI, and XII, by a procoagulant activity causing disseminated intravascular coagulation [16]. However, some effects on hemostasis have been described for fish and other spider venoms [17,18].

Apart from venoms, other animal fluids are the source of diverse proteins affecting hemostasis, such as hemolymph [19] and the bloodsucker’s saliva, which have actions over the coagulation system of another animal for feeding. In this case, exogenous factors act on the hemostasis of another animal to enhance the time of access to blood fluid through the wound made by specialized structures of the bloodsuckers [20]. Therefore, these animals have been an important source of new anticoagulants, among them, clotting inhibitors, fibrin(ogen)olytics, plasminogen activators and platelet inhibitors [21].

### The targets on blood coagulation and fibrinolytic pathways

According to the target and the kind of activity, exogenous factors can have pro or anticoagulant effects. Their target may virtually be any factor on the coagulation or fibrinolytic system. The coagulation system functions by intrinsic pathways, with the actions of distinct factors in a descending cascade that results in the activation of prothrombin (FII) into thrombin (FIIa), which finally converts fibrinogen into fibrin clots. Activation of the coagulation cascade can start with tissue factor and phospholipids, which form an enzymatic active complex with FVIIa, or with the formation of an enzymatic complex consisting of FXIia, prekallikrein and high molecular weight kininogen (HMWK), which had been previously distinguished as extrinsic and intrinsic pathways, respectively. Physiologically, there are intricate and complex interactions between each clotting factor, with regulation points through feedback mechanisms, endogenous inhibitors and cofactors [22]. Following the most recent concept, on a cell-based model, the pathways are not redundant, but operate in parallel [23].

The majority of clotting and fibrinolytic factors is synthesized and circulating in the blood in zymogen form. Exogenous factors can act through proteolytic activity by activating clotting factor zymogens (e.g. prothrombin activators, FX activators) or acting like a clotting factor (e.g. thrombin-like toxins), usually displaying procoagulant activity. Conversely, procoagulant proteins have been indicated as anticoagulant agents, because these enzymes can be administrated in therapeutic doses to deplete clotting factors, especially fibrinogen, without the bleeding risk commonly associated to therapeutic anticoagulants [24]. The thrombin-like enzymes have been highlighted as interesting therapeutic molecules for diseases associated to ischemic conditions, such as myocardial infarction and stroke, and in the prevention of thrombus formation and reduction of blood viscosity [25]. Exogenous inhibitors usually display anticoagulant activity by specifically inhibiting the activity of a clotting factor. Proteolytic enzymes that act on the fibrinolytic system have also been described, e.g. plasminogen activators, which have anticoagulant activity [26], and fibrinolytic enzymes, which are in general antithrombotic, because they can act on cross-linked fibrin [27,28].

### The distinct groups of exogenous hemostatic factors

Exogenous hemostatic factors identified in animal venoms or in the saliva of hematophagous animals can exert their effects on the hemostatic system by several mechanisms affecting coagulation, fibrinolysis, platelet function and vascular integrity. Larrieché et al. [29] classified them into four main groups: hemorrhagins; components affecting platelet function (which can also be referred as platelet activators/inhibitors); components affecting coagulation; and, components affecting fibrinolysis.

In general, the exogenous factors can be considered as two separated groups: procoagulants and anticoagulants. The procoagulants can include platelet activators, clotting factor activators, and thrombin-like enzymes, whereas the anticoagulants can include platelet inhibitors, fibrin(ogen)olytics, plasminogen activators, and clotting factor inhibitors, since they are able to prevent blood clotting and maintain blood incoagulable.

This paper is focused mainly on the exogenous procoagulant proteins from snakes and insects. The hemorrhage and components affecting platelet functions are out of the scope, but these groups of molecules were discussed by some authors [8]. For a review on the exogenous anticoagulants the reader is referred to Monteiro [30] and Kini [31,32].

Table 1 shows some proteins acting as procoagulants, which have been isolated from animal venoms or saliva of hematophagous animals and biochemically characterized.

### Exogenous procoagulant factors

In general, the exogenous procoagulant proteins are metalloproteinases or serine proteinase that display activity on a specific factor of the coagulation cascade. These proteins usually hydrolyze the zymogenic form of a clotting factor converting it in the active form. Snake venoms are the richest sources of procoagulant toxins among the animal venoms [15], but these enzymes can also be found in arthropod venoms [4,33].

#### Prothrombin activators

FXa is the physiological activator of prothrombin. The hydrolysis of prothrombin in thrombin by FXa is enhanced up to 300,000 times in the presence of phospholipids, FVα and calcium ions, which form with FXα, the prothrombinase complex [34]. Meizothrombin is formed as an intermediate product, by consecutive cleavages at Arg323-Ile324 and Arg274-Thr275. The intermediate products generated in the hydrolysis of prothrombin by FXα in absence of prothrombinase complex are Fragment 1.2 and Prethrombin 2 by cleavage at Arg274-Thr275 followed by cleavage at Arg121-Ile124 [35]. The exogenous prothrombin activators can differ in the specific cleavage site on prothrombin, the end products formed (meizothrombin or α-thrombin), the cofactor requirements (calcium ions, phospholipids and FVα) and the susceptibility to proteinase inhibitors. Based on these properties, the exogenous prothrombin activators, especially those from snake venoms, can be classify into four main groups [36]. Group A and B prothrombin activators are metalloproteinases that hydrolyze prothrombin into meizothrombin. Prothrombin activators from groups C and D are serine proteinase, which are able to generate active thrombin (α-thrombin) [28,36]. These toxins have a FX-like domain, and are found in Australian Elapid snake venoms. Also, they have been well distinguished by molecular phylogenetic analysis [37]. Group C prothrombin activators also show a FV-like domain and resemble the prothrombinase complex [38]. Both domains are highly conserved in the elapid family and present high similarity with mammalian FXα, e.g. Hopsarin D from *Hoplocephalus stephensi*, Trocarin D from *Tropidechis carinatus* [37] and FXα-FVa complex, e.g. Pseutarin C [39]. Kini [36] has reported examples and the biochemical properties of prothrombin activators from snake venoms belonging to the distinct
classes. However, there are prothrombin activators that present particular properties and do not fit properly into any of the four classes, e.g. the *Lononia obliqua* prothrombin activator protease (Lopap) [4].

Insularinase A is a single-chain 23 kDa proteinase purified from *Bothrops insularis* venom. Insularinase A converts prothrombin into meizothrombin independent of the prothrombinase complex, and can also activate FX and hydrolyze fibrinogen and fibrin. CDNA sequence analysis revealed that the disintegrin domain of the precursor protein is post-translationally processed, producing mature Insularinase A [40].

Berythreactivase is a non-hemorrhagic prothrombin activator from *Bothrops erythromelas* snake venom, belonging to group A. It is a single-chain metalloproteinase of 78 kDa, which is also capable of hydrolyzing fibrinogen A2-chain and triggering endothelial proinflammatory and procoagulant cell responses. This toxin also induces the release of von Willebrand factor (vWF) and expression of α-chain and triggering endothelial of hydrolyzing fibrinogen A chain that consists of a serine proteinase domain. Its complete amino acid sequence has been previously reported [42].

Berythreactivase contains three distinct domains: metalloproteinase, desintegrin-like and cysteine-rich domains, and it is similar to other snake venom metalloproteinases [41].

Trocarin D is a highly expressed toxin in the venom of the snake *Tropidechis carinatus* [42]. This protein belongs to the group D prothrombin activator, structurally similar to mammalian FXA, and the structure was characterized from its gene by Reza [43]. It is a 47-kDa glycoprotein that has a light chain with N-terminal gamma-carboxyglutamic (Gla) domain, two EGF-like domains, and a heavy chain that consists of a serine proteinase domain. Its complete amino acid sequence has been previously reported [42].

Lopap is a prothrombin activator from the venom of *Lononia obliqua* moth caterpillar with serine proteinase-like activity [44], which does not fit in the current classification of snake venom prothrombin activators [36]. It is able to activate prothrombin in the absence of the prothrombinase complex. It has its activity enhanced by calcium ions and generates α-thrombin without the intermediate meizothrombin [45]. Lopap monomer has a molecular mass of 20 kDa.

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**Table 1:** Biochemical properties of procoagulant proteins from snake and arthropod venoms.

| Molecule function | Molecule name | Animal source (sp) | Molecular mass (Da) | Additional targets | Functional characteristic | Reference |
|-------------------|--------------|--------------------|---------------------|--------------------|--------------------------|-----------|
| **Factor X activators** | RRV-X | Vipera russelli (Daboia russelli) | 92,880 | FIX | metalloproteinase, Ca²⁺ dependent | Kisiel et al. [52]; Takeya et al. [50] |
| | VLFXA | Vipera lebetina | 89,400 | FIX | metalloproteinase, Ca²⁺ dependent | Siigur et al. [51,54] |
| | - Bungarus fasciatus | 70,000 | S-2266 and S-2302 (kalikrein substrates) | serino proteinase, Ca²⁺ dependent | Zhang et al. [49] |
| | Losac | Lononia obliqua | 45,000 | - | serino proteinase, Ca²⁺ independent | Alvarez Flores et al. [33] |
| | Ecarin | Echis carinatus | 56,000 or 72,000 | - | metalloproteinase, Ca²⁺ independent group A | Yamada et al. [71]; Moore [72] |
| | Insularinase A | Bothrops insularis | 22,639 | FX, fibrinogen, fibrin | metalloproteinase, group A | Modesto et al. [40] |
| | Berythreactivase | Bothrops erythromelas | 78,000 | - | metalloproteinase, group A | Silva et al. [41] |
| | Carinactivase-1 | Echis carinatus | 87,000 | - | metalloproteinase Ca²⁺ dependent, group B | Yamada et al. [71] |
| | Pseutanin C | Pseudonaja textilis | ~250,000 | - | serino proteinase, dependent on Ca²⁺ and phospholipids, group C, structurally and functionally similar to the mammalian FXa-FVa complex | Rao and Kini [39] |
| | Trocarin D | Tropidechis carnatus | 46,515 | - | serino proteinase, dependent on Ca²⁺, phospholipids and FVa group D, structurally similar to the mammalian FXa | Joseph et al. [42] |
| | Textarin | Pseudonaja textilis | 53,000 | - | serino proteinase, dependent on Ca²⁺, phospholipids and FVa, group D | Stocker et al. [73] |
| | Lopap | Lononia obliqua | 69,000 or 20,800 | - | serino proteinase, activity is enhanced by Ca²⁺ ions, structurally similar to lipocalin family members | Reis et al. [4,44,45] |
| **Factor V activators** | RRV-V | Vipera russelli (Daboia russelli) | 29,000 | - | serino proteinase | Kisiel [1] |
| | LVV-V (VLFVA) | Vipera lebetina (Daboia lebetina) | 28,400 | - | serino proteinase | Siigur et al. [54] |
| | Lonomin Vl: a | Lononia aechelous | - | - | metalloproteinase | López et al. [59] |
| **Thrombin-like** | Ancrod (Arvin) | Calloselasma rhodostoma (Agkistrodon rhodostoma) | 48,000 (29,000³) | - | serine protease | Burkhart et al. [94]; Yu et al. [93] |
| | Batroxobin (Reptilase or Defibrinase) | Bothrops atrox (Bothrops moojeni) | 29,100 (25,503³) | - | serine protease | Marsh [78]; Vu et al. [86] |
| | Thrombocytilin | Bothrops atrox | 36,000 | prothrombin, FXIII, FVIII and platelets | serine protease | Niewiarowski et al. [61]; Giussa et al. [62] |

*Some molecules can display more than one function.

*According to the classification of Kini (2005)

*Based on amino acid composition without carbohydrate content
and its sequence is not similar to other known serine proteinase or prothrombin activators, but it is structurally similar to members of the lipocalin family. It is the first lipocalin presenting proteolytic activity [4]. This protein has been characterized in vitro and in vivo, and plays an important function in the consumptive coagulopathy caused by L. obtusa envenomation [44,45]. This toxin also triggers various responses in endothelial cells and displays antiapoptotic activity. Recently our research group have shown that N-Lopap is able to reverse the bleeding induced by LMWH in vivo [9,46,47].

Factor X activators: The physiological activators of FX are FIXa, in the intrinsic pathway, and FVIIa-TF, in the extrinsic pathway. FIXa can form a catalytic complex, called factor X-activating complex or tenase complex, in the presence of calcium ions, FVIIa and phospholipids. FX activation by FIXa in this complex is accelerated 24 million-fold [34]. Despite the fact that many FX activator enzymes have been reported in the venom of various snake species, only a limited number has been isolated and characterized, and most of them as being metalloproteinases from snakes of the Viperidae and Crotalidae families [48]. A few serine proteinase FX activators were described in Elapidae venom [49], and only one was reported from a lepidopter [33]. The complete amino acid sequence analysis was reported for two metalloproteinases, RVV-X (Russell’s viper venom factor X activator) [50] and VLFXA (Vipera lebetina factor X activator) [51]. Both proteins are glycosylated and show disintegrin-like, cystein-rich and metalloproteinase domain in the heavy chain. Also, they have C-type lectin domains in two light chains. The heavy and light chains are linked by disulfide bonds [50]. According to Tans and Rosing [48], the exogenous FX activators from snake venom can be classified according to the molecular mass, the number of subunits (polypeptide chains), and the susceptibility to proteinase inhibitors. Generally, there are two distinguished groups of FX activators, metalloproteinase and serine proteinase activators. The activators of the metalloproteinase are structurally and functionally similar to RVV-X. These RVV-like enzymes have three subunits held together by disulfide bonds and require calcium ions for their activity. On the other hand, the activators of the serine proteinase correspond to single-chain proteins, strongly dependent on calcium as the RVVX-like activators.

RVV-X was the first identified FX activator, which was purified from the venom of Vipera russelli (Daboia russelli) [52]. RVV-X is also capable to activate FIX. In 1997, the crystal structure of RVV-X was determined [53].

The VLFXA was purified from Vipera lebetina venom [54] and its amino acid sequence was deduced from the nucleotide sequences of cDNAs encoding the light and heavy chains, which are synthesized from different genes. VLFXA was the first FX activator who’s heavy and light chains were cloned [51]. Besides cleaving the Arg226-Val227 bond in the heavy chain of FX, VLFXA is able to cleave the Arg226-Val227 bond in human FIX precursor. VLFXA could not activate prothrombin, and did not have any effect on fibrinogen. It had no arginine esterase activity toward benzoylarginine ethyl ester [54].

From lepidopterans, the first identified FX activator was named Losac (Lonomia obtiqua Stuart-factor activator). It consists of a 45-kDa serine proteinase purified from the bristles of the Lonomia obtiqua moth caterpillar, which is able to activate FX in absence of calcium [33], unlike the other snake venom FX activators, which require calcium ions for their activity [48]. Besides its procoagulant activity, this protein also functions as a growth stimulator of endothelial cells and is an inhibitor of apoptosis by inducing the liberation of nitric oxide and tPA [35,55,56].

Factor V activators: Thrombin is the physiological activator of FV, as well as FXa [57]. Exogenous FV activators can be found in the venom of snakes from the families Crotalidae (Bothrops atrox), Elapidae (Naja naja oxtiana) and Viperidae (Vipera russelli, Vipera lebetina, Vipera ursini), and in the hemolymph of Lonomia achelous caterpillar. All of them have serine proteinase-like activity [58], except for the activator from caterpillar, which is a metalloproteinase [59]. The FV activators from the venoms of Vipera russelli (RVV-V) and Vipera lebetina (LVV-V) are single-chain proteinases of 26 and 28 kDa, respectively. These toxins had their three-dimensional structure models predicted and their mechanism of activation of FV compared to human α-thrombin [60]. RVV-V and LVV-V activate FV by cleavage in a single peptide bond, in contrast to endogenous activation by thrombin, which cleavages three peptide bonds [58,60].

Thrombin-like enzymes: Thrombin is a multifunctional enzyme, which plays a key role in the coagulation system, because it directly converts circulating fibrinogen to an insoluble fibrin clot. Thrombin-like enzymes are a group of toxins with serine proteinase-like activity able to clot fibrinogen. They are functionally and structurally related to thrombin, and are present in the venoms of several species of snakes from the families Viperidae and Colubridae [25]. Like thrombin, thrombin-like toxins can be multifunctional enzymes. There are thrombin-like enzymes reported to have FV activator activity [58], such as Thrombocytin from Bothrops atrox venom, which can also hydrolyze prothrombin, activate FXIII, FVIII and platelets [61], and cause an endothelium-dependent relaxation on arteries [62]. Despite most snake venom thrombin-like enzymes that only hydrolyze fibrinogen and have no effect on the other clotting factors, some thrombin-like toxins have shown activity in nervous and complement systems [25]. Castro et al. [25] have reviewed the structural and functional features of thrombin-like enzymes. In addition, some reports have shown the molecular cloning, phylogeny, as well as structural, biochemical and biological characterization of thrombin-like toxins [63].

As previously discussed, thrombin-like enzymes display procoagulant activity by converting fibrinogen to fibrin, but in vivo they can induce an anticoagulant effect by causing fibrinogen depletion [64,65]. In contrast to fibrinogenolytics enzymes that usually cleave fibrinogen on the C-terminal portion hindering clot formation, thrombin-like enzymes hydrolyze fibrinogen at the N-terminal end of the Aa and/or Bβ chain, releasing relatively small portions of the fibrinogen molecule, called fibrinopeptides A and B, respectively [25]. These cleavages in the fibrinogen molecule allow its polymerization, with the formation of the fibrin clot [66]. However, in contrast to thrombin, the majority of the thrombin-like enzymes from snake venoms preferentially releases only fibrinopeptide A or B, resulting in the formation of abnormal fibrin clots, and some of them are not able to activate FXIII [67], necessary to form insoluble and cross-linked fibrin clots. Consequently, these enzymes form an instable fibrin clot, which is easily removed by the fibrinolytic system [68]. Two well-characterized thrombin-like enzymes, Ancrod and Batroxobin, are currently used therapeutically as defibrinogenating agents [64].

Thrombin-like enzymes are present on snake venoms; in contrast, at the moment it was not yet described thrombin-like enzymes from Lonomia venoms.

Exogenous Procoagulant Factors as Reagents for Diagnostic Tests
To date, the most important application for procoagulant toxins is in the area of diagnosis. Several toxins have been proved to be useful as reagents in laboratory tests for diagnosis, for example, to detect clotting factor deficiencies and to monitor patients undergoing anticoagulant therapy [7]. The diagnostic uses of snake venom toxins have been reviewed by different authors [7,69,70]. Prothrombin activators have a wide range of applications; they have been used for prothrombin assays, to detect dysprothrombinemias, disseminated intravascular coagulation and to assay PIVKA-II (protein induced by vitamin K absence or antagonist-II). PIVKA-II or des-gamma carboxyprothrombin is a non-functional prothrombin precursor, which accumulates during therapy with vitamin K antagonists, and is also a biomarker of hepatocellular carcinoma. Group A prothrombin activators, such as Ecarin, can cleave the descarboxy variety of prothrombin, because these enzymes act independently of calcium ions [71]. Therefore, these enzymes are not indicated to monitor patients’ anticoagulated with vitamin K antagonists.

The procoagulant activity in plasma exerted by prothrombin activators can be affected by Hirudin, a thrombin inhibitor from leech saliva that is used as anticoagulant medication. In contrast, heparin and lupus anticoagulant cannot affect the procoagulant activity of these enzymes. Thus, prothrombin activators such as Ecarin are commonly used in laboratory tests (e.g. Ecarin clotting time) to monitor patients under treatment with Hirudin. In addition, Ecarin time has been proposed to be used in association with Taipan snake venom time to detect lupus anticoagulant in patients receiving oral anticoagulant therapy [72].

Textarin, a group D prothrombin activator has been also suggested as a reagent for the detection of lupus anticoagulant [73]. This enzyme is used in a test denominated Textarin time assay, to detect resistance to activated protein C (APC-resistance), which is frequently associated with a single point mutation in the FV gene, known as Factor V Leiden [74]. In this coagulation disorder, FVa is resistant to inactivation by APC, implicating in a thrombotic risk for the patient (thrombophilia) [75]. Therefore, the ratio of prolongation of clotting time in the presence of APC is less pronounced in the plasma from APC resistant patients [74]. There are a variety of prothrombin activators from the venoms of the Australian brown snake Pseudonaja textilis (Textarin), the saw-scaled viper Echis carinatus (Ecarin), the mainland Australian tiger snake Notechis scutatus scutatus (Noscarkin), and the Taipan snake Oxyuranus scutellatus that are commercially available [68].

Among the snake venom FV activators commercially available, the RVV-X, a toxin from Russell’s viper venom is one of the most used. RVV-X is the key reagent of different diagnostic kits. One of them is a clotting test based on the prothrombinase complex, and is used to monitor patients undergoing anticoagulant therapy, except for vitamin K antagonists. In presence of RVV-X the prothrombinase complex is formed very quickly, generating active thrombin that cleaves fibrinogen to fibrin. Another kit using RVV-X is applied to detect Factor V Leiden mutation genotype. This test consists of two steps, one involving the activation of FV by RVV-X followed by inactivation of FVa by the addition of APC. In the second step, the group D prothrombin activator Noscarin is added to generate active thrombin and finally the fibrin clot. Since Noscarin activity is dependent of FVa as a cofactor, patients with Factor V Leiden mutation have short clotting times in comparison with normal individuals [7]. Another toxin from Russell’s viper venom, RVV-X is a FX activator also used for diagnostic tests, for detection of factor X deficiency [76] and lupus anticoagulant [77], which is another important risk factor for thrombophilia. A commonly used assay to detect lupus anticoagulant is the dilute Russell’s viper venom time dRVVT, which is based on the activity of RVV-X [70,77]. There are various kits commercially available to assay dRVVT, which contain purified RVV-X or the whole venom from Russell’s viper [70,77].

Thrombin-like enzymes from snake venoms are not inhibited by heparin, but they are used for detecting dysfibrinogenemias and to remove fibrinogen for different assays [78]. Batroxobin, a thrombin-like enzyme from Bothrops moojeni or Bothrops atrox, is used in the diagnostic procedures and test kits, such as the Reptilase time [7].

**Exogenous procoagulant factors as therapeutic agents**

Unlike thrombin, which cleaves both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from fibrinogen, many thrombin-like enzymes usually only cleave FPA and do not activate FXIII. This aspect makes the thrombin-like enzymes interesting tools to remove fibrinogen from plasma (defibrinogenation) without the risk of thrombosis, because the fibrin clot formed is very unstable in contrast to cross-linked fibrin [68]. Therefore, the fibrin is rapidly removed by the fibrinolytic system. Otherwise, administration of a thrombin-like enzyme as a defibrinogenating agent has a low bleeding risk in comparison to other anticoagulants that have been used [24]. Among the procoagulant proteins from animal venoms currently in use as therapeutic tools, the most relevant are the thrombin-like enzymes. Ancrod is a serine proteinase toxin from a Malayan pit viper snake Agkistrodon rhodostoma (Calloselasma rhodostoma) [79] that cause reduction in plasma fibrinogen concentration in vivo by formation of soluble fibrin complexes, which are degraded by plasmin. Also, it induces plasminogen activation and leads to a fibrinolytic response [80].

Ancrod and Batroxobin, which are also commercially named Arvin and Defibrase, respectively, are indicated as defibrinogenating drugs to patients with stroke, deep vein thrombosis, myocardial infarction, peripheral arterial thrombosis, priapism, and sickle-cell crisis [64,81,82].

In a large-scale trial utilizing Ancrod (Arvin), for example, a higher proportion of patients achieved good functional outcomes when the drug was given within 3 h of stroke onset and continued for five days compared to placebo [83]. However, the phase III trials were terminated because they showed that giving the drug to patients within 6 h of stroke onset was ineffective [84]. Therefore, the dosing regimen appears to be an important criterion for successful outcomes in the use of these defibrinogenating agents.

The thrombin-like enzymes can be used as procoagulants for hemorrhage management. A mixture of two enzymes from the venom of B. atrox, a thrombin-like enzyme and a thromboplastin-like enzyme, form a clot-promoting product called Haemocoagulase®. These enzymes cooperate, targeting different points at the coagulation cascade to form blood clots. The thrombin-like enzyme directly cleaves fibrinogen into fibrin monomers, and the thromboplastin-like enzyme activates FX, which in turn converts prothrombin into thrombin [69]. Batroxobin is a component present on venom from B. atrox moojeni that acts as thrombin-like enzyme [81,82,85]. This serine proteinase only releases fibrinopeptide A by specific cleavage of Arg-Glu bond in the Aa-chain of fibrinogen, and it is not inhibited by antithrombin or heparin cofactor II [82,86].

In another therapeutic field, defibrinogenating agents may be used as a hemostatic agent to arrest bleeding, for example, during surgical procedures. The hemostatic reagents, which have been available for
over the last fifty years and are still currently used for these purposes, include absorbable gelatin sponge, oxidized cellulose, microfibrillar collagen and thrombin, which act by forming an artificial clot or by producing a mechanical matrix that facilitate clotting when applied directly to denuded or bleeding surfaces [87]. A fibrinogen-thrombin-collagen-based material has been demonstrated to be advantageous because it is quickly available and easily applicable, but there are disadvantages inherent to exogenous fibrinogen and thrombin sources, which might be potentially infectious, and a rigorous control of several types of contamination would become necessary [88]. Therefore, development of hemostatic agents based on exogenous procoagulant factors would be an interesting approach. In addition, research on possible antidotes for the currently available anticoagulants should also be an interesting issue, especially in the cases of bleeding risk and surgical procedures. Recent studies have showed the recombiant form of the prothrombin activator isolated from Lononion obliqua (rLopap), [4] as a first exogenous prothrombin activator capable of reversing bleeding induced by LMWH [9].

Table 2 shows some exogenous procoagulant factors as diagnostic test reagents and therapeutic drugs.

### The non-hemostatic effects of exogenous hemostatic factors

Endogenous coagulation and fibrinolytic factors can trigger effects not directly related to blood coagulation and fibrinolysis, such as effects related to inflammation, homeostasis and cell responses. Accordingly, an increasing number of studies have demonstrated non-hemostatic actions displayed by molecules characterized as exogenous hemostatic factors.

The involvement of endogenous hemostatic factors was demonstrated in the cell regulation, cancer, angiogenesis, nervous system [89]. They can display various biological activities, hemostatic and non-hemostatic [32]. Exogenous hemostatic factors can modulate the endothelial cell responses, the release and synthesis of bioactive substances, the gene expression, the cell signaling, the cell adhesion, apoptosis, the proliferation and inflammatory reactions [90-92]. Two procoagulant proteins, Insularinase A and Lopap, have increased the levels of nitric oxide and prostacyclin released by endothelial cells, for instance [40,47]. In addition, Losac and Lopap have shown antiapoptotic activities [33,47]. Trocarin D, a well-characterized prothrombin activator from snake venom, can also trigger non-hemostatic roles [90].

### Diagnostic tests

| Procoagulant factor | Main target | Application | Commercial names | Characteristic | Reference |
|---------------------|-------------|-------------|------------------|---------------|-----------|
| Ecarin              | prothrombin | Clotting time assay | Tirofiban | Prothrombin activator | Moore [72] |
| Texarin             | prothrombin | Lupus anticoagulant Factor V Leiden Disease | Prothrombin activator | Stocker [73] |
| Noscarin            | prothrombin | Factor V Leiden Disease | Prothrombin activator | Marsh and Williams [68] |
| RVV-X               | FX          | Factor X deficiency Lupus anticoagulant | RVV-X | Factor X activator | Bezeaud et al. [76]; Triplett [77] |
| RVV-V               | FV          | Clotting time assay Factor V Leiden Disease | RVV-V | Factor V activator | Schöni [7] |
| Ancrod              | Fibrinogen | Delfibrinogenating agents | Viprinx | Thrombin-like | Bell [64] |
| Batroxobin          | Fibrinogen | Delfibrinogenating agents | Repliase; Defibrase | Thrombin-like | Bell [64]; Qin et al. [81]; Serrano [82] |
| rLopap              | Prothrombin | Bleeding reversor | Prothrombin activator | Andrade et al. [9] |
| Fibrinogen and Factor X | Procoagulant for hemorrhage | Haemocoagulase | Mixture; Thrombin-like and thromboplastin like enzyme | McCleary and Kini [69] |

*The given references may not be of the first author to describe the respective molecules

### Clinical therapy

Table 2: Exogenous procoagulant factors as diagnostic test reagents and therapeutic drugs.
Cerebral microvascular occlusion [112]. Unlike Ancrod, Bathroxobin levels of t-PA in microvascular endothelium that could result in a hydrogel capsule based on polyethylene glycol (PEG) [109]. Antigenic effects such as, oral administration of Ancrod delivered but repeated intravenous or subcutaneous administration of Ancrod treatment effectively reduce blood viscosity by inducing defibrination, is the management and unpredictable effects of its enzymatic activity associated to research, development and production, can direct the preferences for the use of exogenous hemostatic factors as diagnostic instead of therapeutic tools. It has to be taken into consideration that biochemical, biophysical and pharmacological properties of a recombinant protein may not completely be the same as their native form, reflecting on the biological activity. Numerous procoagulant proteins have been isolated in their native form from animal venoms, mainly from snakes. They have been sequenced and cloned, but the majority has not been functionally expressed as recombinant molecules. However, many efforts have been currently applied in this sense.

Identification of new targets and new effects for the exogenous factors already described can open new perspectives, or the modification of the molecule based on functional and structural regions, for example the removal of the enzymatic activity when undesired to avoid a specific effect or the design and synthesis of short peptides to minimize toxic and side effects, all these are interesting task.

**Concluding Remarks and New Perspectives**

To date, the largest applications of procoagulant snake venom toxins have been used as reagents in laboratory tests and diagnostic kits [7]. Probably, this is due to potential risks associated to toxicity and immunogenicity of the clinical use of these proteins as therapeutic agents. In addition, the need for several regulatory procedures and approvals for the development of therapeutic drugs, and the high costs associated to research, development and production, can direct the preferences for the use of exogenous hemostatic factors as diagnostic instead of therapeutic tools. It has to be taken into consideration that biochemical, biophysical and pharmacological properties of a recombinant protein might not be completely the same as its native form, reflecting on the biological activity. Numerous procoagulant proteins have been isolated in their native form from animal venoms, mainly from snakes. They have been sequenced and cloned, but the majority has not been functionally expressed as recombinant molecules. However, many efforts have been currently applied in this sense.

Opportunistically, the number of studies dedicated to the production of recombinant proteins as tool for health care and biotechnology has risen over the last few years. For example, there are publications reporting the production of recombinant Batroxobin [114] and Ancrod [115-116] in *Pichia pastoris*, a yeast vector. *Pichia pastoris* is now being elected for the expression of recombinant proteins with therapeutic purposes due to advantages as the presence of a post translational modification machinery, which is absent in prokaryotic expression systems, and the cost-effectiveness and easy-to-assembly cultivation system suitable for up scaling in comparison to *E. coli* and *Saccharomyces* [117]. Other exogenous procoagulant proteins that have been functionally expressed as recombinant molecules are the prothrombin activators from the snake *Pseudonaja textilis* [118] and from the *Lonomia obliqua* caterpillar (Lopap) [4], respectively.

The research and development involving the use of procoagulant proteins are still incipient in comparison to the use of exogenous inhibitors in the therapeutic field. One of the reasons could be the management and unpredictable effects of its enzymatic activity in the human organism, in addition to its immunogenic potential. Although several toxins that act as activators or inhibitors in the hemostatic system have been described and well characterized, much have to be investigated regarding their other possible roles in the organism. These biological effects are currently under investigation for various toxins from different poisoning species, and can provide additional data related to the understanding of the complex reactions involved in poisoning. The multi-faced physiological action of endogenous and exogenous factors can be explained by a converging evolution of distinct activities in a same molecule, or a common molecular evolutive origin [25]. In the case of exogenous factors, the structure-function relationship suggests that a molecule with intrinsic roles for the animal could have acquired another function, which would provide advantages for animal defense, attack or feeding purposes, for instance [119].

The knowledge about the non-hemostatic effects of the exogenous factors can point out new targets for disease control and therapy, and
concomitantly bring new perspectives for the use of these proteins in the study and treatment of a wide range of dysfunctions. In this regard, studies of site-directed mutagenesis and sequence mapping can be considered as an interesting tool, allowing the removal of undesired enzymatic activities. In addition, the diversity of known exogenous factors and the innumerous recently identified also suggest that there are many other molecules to be discovered, which could belong to the established classes or even present new biochemical properties.

Acknowledgement

The authors were supported by fellowships from Brazilian Agencies, São Paulo Research Foundation - FAPESP (to L.C.C.C., grant 2005/59739-9, to A.M.C.T., grant 2013/07467-1 and 2013/06892-0) and CNPq (to A.M.C.T.).

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