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Review Article

Modulation of the mammalian coagulation system by venoms and other proteins from snakes, arthropods, nematodes and insects

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ABSTRACT

The mammalian hemostatic system involves complex interactions between protein components of the coagulation cascade and platelets. The fibrinolytic system removes the hemostatic plug. Dysregulation of coagulation or fibrinolytic systems can induce bleeding or thrombosis. Animals, such as snakes, worms and insects, have evolved to express proteins that modulate the mammalian coagulation and fibrinolytic systems. Many of these proteins have been isolated and characterized. Understanding the mechanisms by which these exogenous factors from venoms and animal saliva modulate the mammalian coagulation and fibrinolytic systems has led to a better understanding of these systems. Furthermore, some of these exogenous proteins are used in diagnostic assays and as therapeutic drugs. This review summarizes our current knowledge of exogenous proteins from venom and saliva that either activate or inhibit the mammalian coagulation and fibrinolytic systems.

1. Overview of the mammalian coagulation and fibrinolytic systems

Hemostasis describes the process that occurs to prevent blood loss after vascular injury. It involves blood cells, mainly platelets, and a coagulation protease cascade. The fibrinolytic system removes hemostatic plugs.

Vascular injury leads to contact of circulating platelets with thrombogenic components within the injured vessel wall. Platelets rapidly adhere, spread and aggregate to seal the injury site. In parallel to the recruitment of platelets, the tissue factor (TF)-factor VII activated (FVIIa) complex activates the coagulation protease cascade (Fig. 1) [1]. This results in the formation of a fibrin net that stabilizes the platelet aggregate.

The coagulation system is comprised of a series of zymogens and cofactors that are activated by proteolytic cleavage that allows rapid amplification of the cascade. The TF-FVIIa complex activates FIX and FX [2]. In addition, the TF-FVIIa-FXa complex and FXa activate FVIII [3,4]. FXII activates FXI and FXIa activates FIX. The intrinsic tenase complex (FVIIIa-FIXa) also activates FX. The prothrombinase complex (FVa-FXa) activates prothrombin to thrombin, which is the terminal protease in the clotting cascade. Thrombin also activates other coagulation factors and co-factors, including FV, FVII, FVIII and FXI, – and activates platelets by cleavage of protease-activated receptors. An important role of thrombin is to cleave fibrinogen to fibrin monomers [1,5]. Fibrinogen is composed of three polypeptide chains (Aα, Bβ and γ), one central region (E) and two distal regions (D). Thrombin cleaves peptides of Aα and Bβ chains, named fibrinopeptides A and B, exposing the polymerization sites in the E region, which interact with complementary polymerization sites located in the D regions of other fibrin monomers [6]. Finally, thrombin activates the transglutaminase FXIII that cross-links fibrin polymers leading to the formation of an insoluble fibrin net [1,5]. FXIIa is not involved in hemostasis but can drive thrombosis by activating FXIa [7] (Fig. 1). FXIa can also be activated by thrombin in a positive feedback loop.

A number of anticoagulant pathways regulate the coagulation protease cascade. Tissue factor pathway inhibitor (TFPI) inhibits the TF-FVIIa complex in a FXa-dependent manner [8]. Activated protein C (APC) cleaves and inactivates cofactors FVa and FVIIIa [9]. Antithrombin inhibits a number of the coagulation proteases, including thrombin.

The fibrinolytic system removes hemostatic plugs by degrading fibrin. Conversion of plasminogen to plasmin is mediated by tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), and negatively regulated by plasminogen activator inhibitor type 1 (PAI-1). Plasmin is inhibited by α_2-antiplasmin and α_2-macroglobulin [5,10,11]. Carboxypeptidase B2 (also known as thrombin-activated fibrinolysis inhibitor) inhibits fibrinolysis by removing C-terminal lysines

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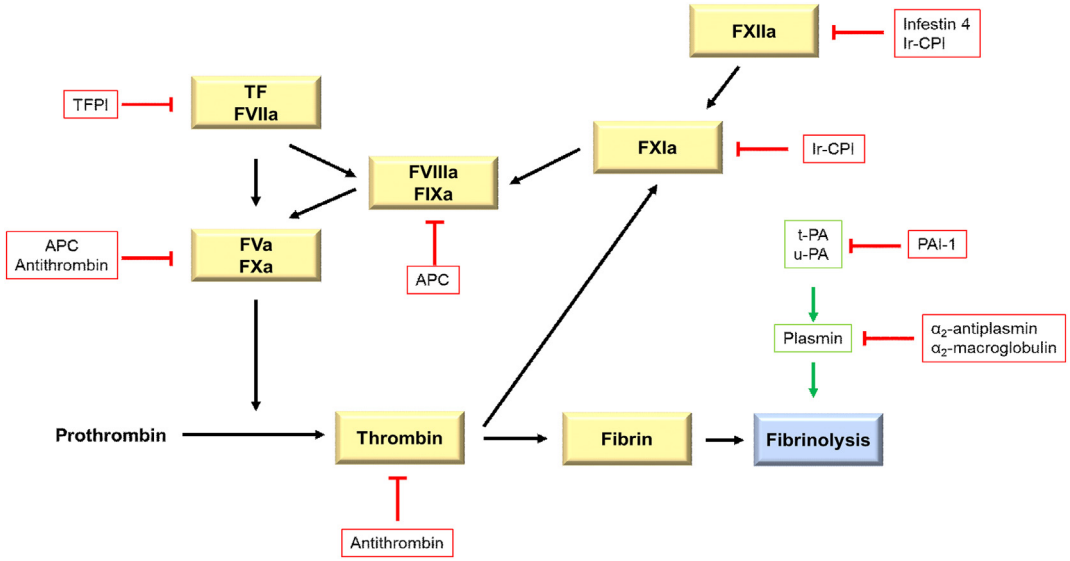

Fig. 1. Activation of the coagulation protease cascade induces the generation of thrombin and the formation of a cross-linked fibrin net. The coagulation cascade is regulated by anticoagulant pathways, such as TFPI, APC and antithrombin. Besides, the fibrinolytic system is activated and plasmin degrades fibrin. Tissue factor (TF); factor (F) VII, FVIII, FIX, FV, FX, FXI, FXII activated (a); tissue factor pathway inhibitor (TFPI); activated protein C (APC); tissue-type plasminogen activator (t-PA); urokinase plasminogen activator (u-PA); plasminogen activator inhibitor type 1 (PAI-1); *Ixodes ricinus* contact phase inhibitor (Ir-CPI).

from fibrin chains which are involved in binding and activation of plasminogen [12]. Dysregulation of coagulation and fibrinolytic systems can result in bleeding and thrombosis.

2. Venoms affecting the mammalian coagulation and fibrinolytic systems

In the course of evolution, various animals, including snakes, worms and insects, have developed proteins capable of modulating the mammalian coagulation and fibrinolytic systems. Hematophagous insects and nematodes possess constituents in their saliva that are essential to prevent mammalian blood coagulation, allowing them to obtain a blood meal. Other animals, such as snakes and arthropods, produce venoms that modulate the mammalian coagulation and fibrinolytic systems as defensive mechanisms, as well as a killing mechanism. Studies on the interaction of exogenous proteins and the coagulation and fibrinolytic systems have increased our understanding of the formation and degradation of clots and have led to the development of therapeutic drugs and diagnostic assays that are used widely in modern medicine. Herein, we describe exogenous activators and inhibitors of the mammalian coagulation and fibrinolytic systems (Fig. 2).

2.1. Activators of coagulation

Animal venoms contain a number of proteins that activate the mammalian coagulation system (Table 1). It has been known for many years that snake venoms contain pro-coagulant components that rapidly activate the mammalian coagulation system [13,14]. However, the amount of venom injected and the size of the prey or victim may influence the outcome of the envenomation. A slow and constant activation of coagulation by the venom can induce disseminated activation of coagulation and consumption of coagulation factors that can result in bleeding, whereas a rapid activation of coagulation can lead to thrombosis.

2.1.1. Activators of FX

Many studies have analyzed components of the venoms that are capable of directly activating mammalian coagulation factors. One of these components is the serine protease *Lonomia obliqua* Stuart factor activator (Losac). Losac was isolated from the bristle of the *Lonomia obliqua* caterpillar and activates FX. However, it does not require the presence of calcium ions for the proteolysis of FX. A synthetic recombinant form of the protease (rLosac) also cleaves FX in the same way as the native protease [15,16].

In addition, venoms of several genus of snakes contain proteins that activate FX. For instance, it was shown that the coagulant activity of *Bothrops jararaca* venom is partially dependent of its FX-activating proteins [17]. The serine protease Bojaractivase X was shown to activate FX in the presence of calcium ions [18]. However, the most well studied FX activator is Russell's viper venom FX activator (RVV-X), which is a metalloproteinase isolated from *Daboia russelli* venom over 80 years ago [19,20]. RVV-X cleaves FX at Arg^{194}-Ile^{195}, which is the same site cleaved by the endogenous activators and RVV-X requires calcium ions for its activity (Fig. 3a) [21–23].

2.1.2. Activators of FV

Thrombin activation of FV requires cleavage at three different sites – Arg^{709}-Ser^{710}, Arg^{1018}-Thr^{1019} and Arg^{1545}-Ser^{1546} – with cleavage at Arg^{1545} being required for full activation (Fig. 3b) [24]. Exogenous proteins are capable of activating FV in a similar way to thrombin. For instance, the metalloproteinase Lonomin VI:a, which was isolated from the hemolymph of *Lonomia achelous* caterpillar [25,26], activates FV.

Snake venoms also activate FV, such as the Russell's viper venom FV activator (RVV-V, from *Daboia russelli* venom) and *Vipera lebetina* factor V activator (VLFVA, from *Vipera lebetina* venom). These two serine proteases differ from thrombin because they are able to specifically cleave FV directly at Arg^{1545}-Ser^{1546} to activate FV [21,27,28].

2.1.3. Activators of prothrombin

The activation of prothrombin to α-thrombin is a key step in the coagulation cascade. The activation of prothrombin [29] occurs via two different pathways, depending of the initial cleavage site (Fig. 3c). The cleavage of Arg^{271}-Thr^{272} site generates inactive prethrombin-2 and requires cleavage at Arg^{320}-Ile^{321} to be converted to α-thrombin. Alternatively, the initial cleavage at Arg^{320}-Ile^{321} forms meizothrombin that is followed by the cleavage at Arg^{271}-Thr^{272} to generate α-thrombin.

Several exogenous proteins have been identified as prothrombin activators, such as the toxin *Lonomia obliqua* prothrombin activator protease (Lopap) isolated from the caterpillar *Lonomia obliqua* venom.

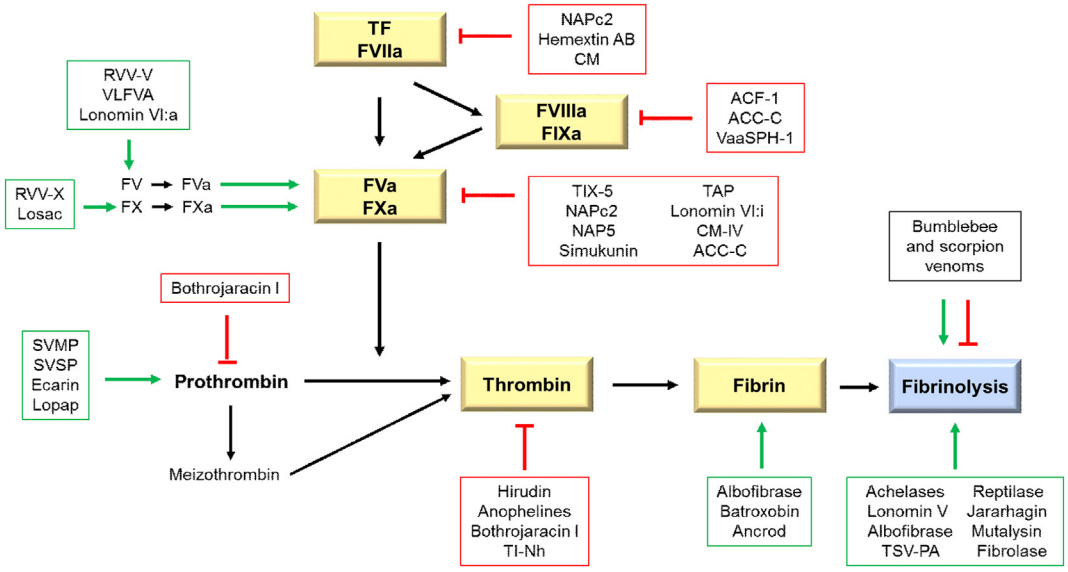

Fig. 2. Exogenous activators and inhibitors of the mammalian coagulation system and fibrinolytic system. Snake venom metaloproteinases (SVMP); snake venom serine proteases (SVSP); tissue factor (TF); factor (F) VII, FVIII, FIX, FV, FX activated (a); tissue factor pathway inhibitor (TFPI); activated protein C (APC); tissue-type plasminogen activator (t-PA); urokinase plasminogen activator (u-PA); plasminogen activator inhibitor type 1 (PAI-1).

Lopap differs from prothrombin activators from snake venoms because it does not require prothrombinase components and shows a FXa-like activity that is increased by the presence of calcium ions. Lopap leads to the generation of α-thrombin, but not meizothrombin and is inhibited by serine proteases inhibitors, such as phenylmethylsulfonyl fluoride (PMSF) [30–32].

Snake venoms contain various proteins that can activate prothrombin to thrombin. Snake venom metalloproteinases (SVMP) from saw-scaled vipers (*Echis* genus) and lance-headed vipers (*Bothrops* genus) cleave prothrombin at Arg^{320}-Ile^{321}, generating meizothrombin that needs autolysis at the Arg^{271}-Thr^{272} site to be converted to α-thrombin. Some SVMP, such as carinactivase-1 from *Echis carinatus* venom, require calcium ions to cleave prothrombin. However, most SVMPs, such as the toxin bothrojaractivase in *Bothrops* venoms and those isolated from *Bothrops jararaca* venom do not require cofactors [27,33,34].

Snake venom serine proteases (SVSP) present in venoms of Australian elapids also activate prothrombin. However, these proteases

Table 1
Activators of the mammalian coagulation system from exogenous sources.

Component	Exogenous source	Activity Diagnostic/clinical use	Ref.
Activators of factor X			
RVV-X	Snake venom (*Daboia russelli*)	Cleaves FX at the same site as endogenous activators dRVVT assay: diagnostic tool for coagulation deficiencies and lupus anticoagulants	[21–23,78]
Losac	Caterpillar bristle (*Lonomia obliqua*)	Cleaves FX similar to endogenous activators	[15,16]
Activators of factor V			
RVV-V	Snake venom (*Daboia russelli*)	Cleaves FV directly at the site required for its full activity Assessments of FV activation and FV Leiden mutation	[21,28,79,84]
VLFVA	Snake venom (*Vipera lebetina*)	Cleaves FV directly at the site required for its full activity	[21,27,28]
Lonomin VI:a	Caterpillar (*Lonomia achelous*)	Cleaves FV in a similar way as plasmin and thrombin	[25,26]
Activators of prothrombin			
Bothrojaractivase and other SVMP	Snake venom (*Echis carinatus* and *Bothrops*)	Cleaves prothrombin, generating meizothrombin that is converted to α-thrombin	[27,33,34]
Oscutarin, trocarin D and hopsarin D	Snake venom (Australian elapids)	Activates prothrombin at the same sites as FXa	[27,35,36]
Pseutarin C	Snake venom (*Pseudonaja textilis*)	Activates prothrombin at the same sites as FXa	[27,35,36]
Ecarin	Snake venom (*Echis carinatus*)	Activates prothrombin at the same sites as FXa Ecarin clotting time: analysis of anticoagulant therapies and lupus anticoagulants	[35,82–85]
Textarin®	Snake venom (*Pseudonaja textilis*)	Activates prothrombin at the same sites as FXa Ecarin/Textarin times: diagnostic tool for lupus anticoagulants	[27,35,83]
Lopap	Caterpillar (*Lonomia obliqua*)	FXa-like activity, direct generation of α-thrombin	[30–32]
Thrombin-like enzymes			
Albofibrase	Snake venom (*Thimeresurus albolabris*)	Thrombin-like activity	[40]
Batroxobin/Reptilase®	Snake venom (*Bothrops atrox* and *Bothrops moojeni*)	Thrombin-like activity Anticoagulant drug	[21,82]
Reptilase®	Snake venom (*Bothrops atrox* and *Bothrops moojeni*)	Thrombin-like activity Reptilase Time assay: analysis of fibrinogen and contamination with anticoagulants	[21,22,82]
Ancrod	Snake venom (*Calloselasma rhodostoma*)	Thrombin-like activity Anticoagulant drug	[21,41,82]

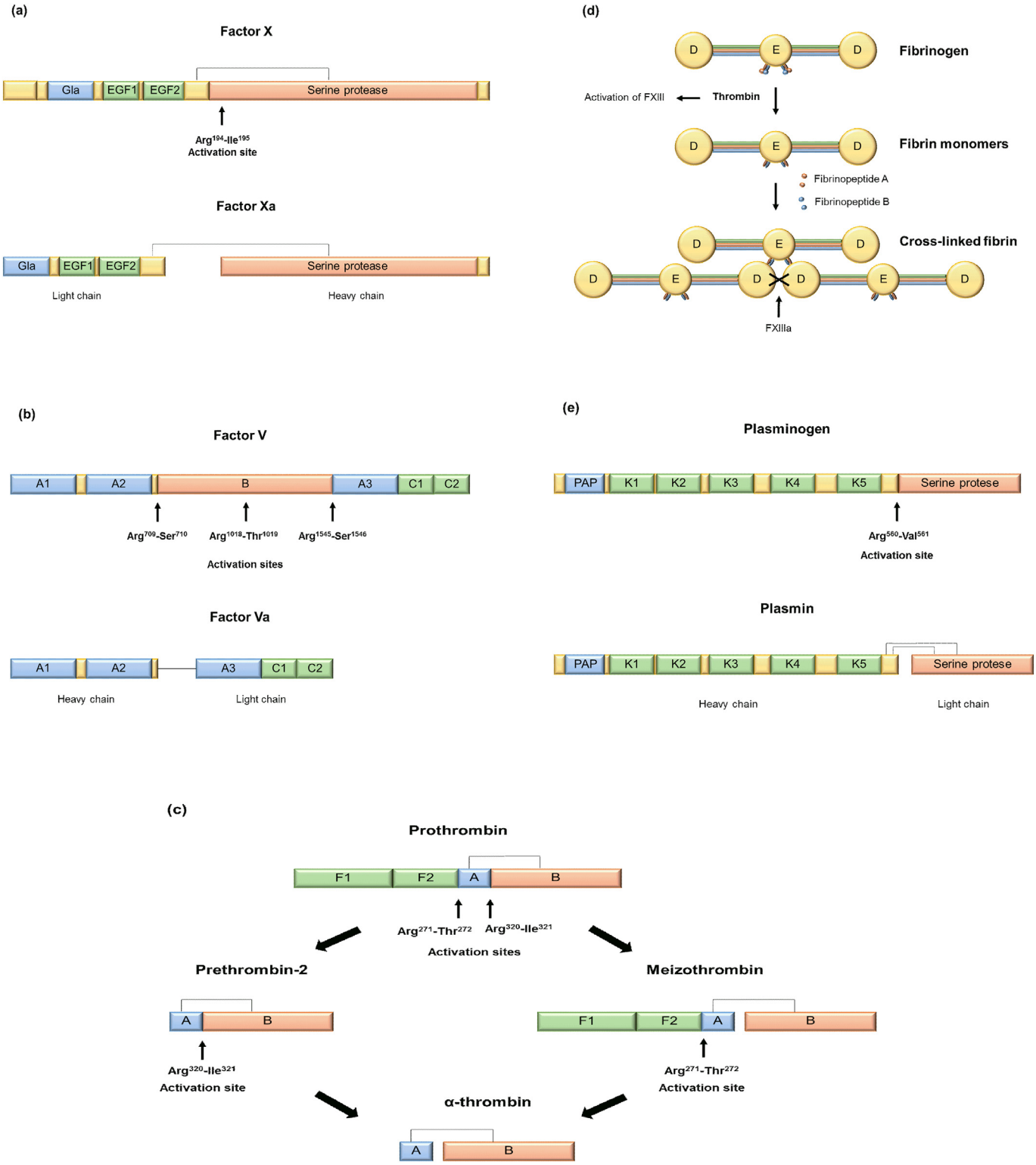

Fig. 3. Activation of coagulation factors, such as (**a**) factor X (FX), (**b**) factor V (FV) and (**c**) prothrombin is required for thrombin generation. (**d**) Thrombin cleaves fibrinogen to fibrin and factor XIII activated (FXIIIa) allows the cross-linking of fibrin. (**e**) Plasminogen is activated to plasmin, which degrades the fibrin net.

are FXa-like proteins that cleave prothrombin at both sites of activation (Arg^{271}-Thr^{272} and Arg^{320}-Ile^{321}) resulting in the generation of α-thrombin. The prothrombin activators trocarin D, hopsarin D, oscutarin, ecarin and textarin require calcium ions, negatively-charged phospholipids or FVa. Pseutarin C is a prothrombin activator that is present in common brown snake (*Pseudonaja textilis*) venom. Pseutarin C is composed of FXa-like and FV-like subunits and snake venom FV-like proteins have significant differences compared to mammalian FV. Venom FV-like proteins are proteases capable of assembling a complex with snake venom FXa-like proteins without binding to membranes. Moreover, these proteins are not inactivated by APC [27,35,36].

2.1.4. *Thrombin-like proteases*

Thrombin-like serine proteases that cleave fibrinogen to fibrin have

Table 2
Inhibitors of the mammalian coagulation system from exogenous sources.

Component	Exogenous source	Activity Diagnostic/clinical use	Ref.
Inhibitors of the intrinsic pathway			
Infestin 4	Hematophagus insect (*Triatoma infestans*)	Inhibits FXIIa	[7,42]
Ir-CPI	Tick (*Ixodes ricinus*)	Inhibits FXIIa and FXIa	[7,43]
Inhibitors of TF-FVII			
NAPc2	Hematophagous hookworm (*Ancylostoma caninum*)	Inactivation of TF-FVIIa by binding to FX or FXa On clinical trials for treatment of ischemia and thrombosis	[44,93,94]
Hemextin AB	Snake venom (*Hemachatus haemachatus*)	Direct inactivation of FVIIa	[45,46]
CM-I and CM-II	Snake venom (*Naja nigricollis*)	Enzymatic: hydrolysis of negatively charged phospholipids	[27,47]
CM-IV	Snake venom (*Naja nigricollis*)	Enzymatic and non-enzymatic	[27,47]
Inhibitors of FV-FX or FVIII-FIX			
TIX-5	Tick saliva (*Ixodes scapularis*)	Delay the FXa-mediated FV activation	[48,49]
NAP5	Hookworm saliva (*Ancylostoma caninum*)	Inhibition by binding to FX	[50,51]
Simukunin	Black fly saliva (*Simulium vittatum*)	Inhibition by binding to FX	[52]
TAP	Tick extract (*Ornithodoros moubata*)	Inhibits FX	[53]
Lonomin VI:i	Caterpillar hemolymph (*Lonomia achelous*)	Inhibits the activity of FV	[25]
CM-IV	Snake venom (*Vipera a. ammodytes*)	Blocks the formation of FXa-FVa by binding to FXa	[47]
ACF-1	Snake venom (*Agkistrodon acutus*)	Inhibition by binding to FIX and FX	[54,55]
VaaSPH-1	Snake venom (*Vipera a. ammodytes*)	Inhibition by binding to FVIII	[56]
Inhibitors of thrombin			
Hirudin, bivalirudin, lepirudin, desirudin	Leech saliva (*Hirudo medicinalis*)	Binding to active site and exosite of α-thrombin Anticoagulant drugs	[57,86–90]
Anophelines	Malaria vectors saliva (*Anopheles* genus)	Binding to active site and exosite of α-thrombin	[58,59,91,92]
Bothrojaracin I	Snake venom (*Bothrops jararaca*)	Inhibition by binding to exosites of prothrombin and thrombin	[61,62,95]
TI-Nh	Snake venom (*Naje haje*)	Specific and direct inhibition of thrombin	[63]
Protein C activator			
ACC-C/Protac®	Snake venom (*Agkistrodon contortix*)	Activates protein C independently of thrombomodulin ProC Global Assay, analysis of anticoagulant deficiencies and FV Leiden	[21,22,64,65,84]

been identified in more than 100 different snake venoms. Snake venom thrombin-like enzymes (SVTLEs) show only partial similarity to thrombin and have several differences regarding their activities. In addition, SVTLEs are not inhibited by endogenous or exogenous thrombin inhibitors, such as antithrombin, heparin and hirudin [37]. SVTLEs normally only cleave one of the fibrinopeptides (A or B) of fibrinogen (Fig. 3d) and do not activate FXIII. Therefore, the fibrin network formed with SVTLEs has a loose structure and is more easily degraded by plasmin [21,27,38,39]. Among the several different SVTLEs, albofibrase, isolated from green pit viper venom (*Thimeresurus albolabris*) shows a thrombin-like activity and an alpha fibrinogenase activity (cleaves the Aα-chain of fibrinogen) [40]. Other well-known SVTLEs are Batroxobin/Reptilase® from *Bothrops atrox* and *Bothrops moojeni* venom and ancrod, purified from Malayan pit viper venom (*Calloselasma rhodostoma*) [38,41].

2.2. Inhibitors of coagulation

Exogenous proteins also inhibit the mammalian coagulation system by several mechanisms (Table 2). These mechanisms include the binding of exogenous proteins to active sites or exosites of the coagulation factors, which may inhibit the activity of coagulation factors or prevent the assembly of coagulation factor-cofactor complexes. In addition, assembly of some coagulation factor-cofactor complexes requires the presence of a negatively-charged phospholipid layer. Snake venoms contain proteins, such as phospholipases A_2, which hydrolyze phospholipid surfaces and thus, prevent or delay the assembly of coagulation-cofactor complexes on cells. The activation of the different mammalian anticoagulant pathways by exogenous proteins is also another mechanism to limit coagulation.

Proteins from exogenous sources are also capable of inhibiting the intrinsic pathway of coagulation. These inhibitors were recently reviewed [7]. For instance, infestin 4 is a protein which inhibits FXIIa and was isolated from the hematophagus insect *Triatoma infestans* [42]. Another protein, named *Ixodes ricinus* contact phase inhibitor (Ir-CPI) inhibits both FXIIa and FXIa and was isolated from the tick *Ixodes ricinus* [43]. Ir-CPI has a Kunitz domain that is similar to the domains present in TFPI.

2.2.1. Inhibitors of TF-FVIIa

The TF-FVIIa complex is the major physiological initiator of the coagulation cascade [1]. Nematode anticoagulant protein c2 (NAPc2) inhibits the TF-FVIIa complex [44]. This protein was isolated from the hematophagous hookworm *Ancylostoma caninum*. In contrast to TFPI that only binds to the active site of FXa, NAPc2 inactivates TF-FVIIa by binding to an exosite in FX or FXa prior to the binding to the TF-FVIIa complex [44].

The anticoagulant protein complex hemextin AB, isolated from the African ringhals cobra venom (*Hemachatus haemachatus*), inhibits the TF-FVIIa complex independently of FX. Hemextin AB was the first natural component reported to inactivate FVIIa in a non-competitive and FX-independent way [45,46].

Snake venom phospholipases A_2 ($SVPLA_2$) inhibit the TF-FVIIa complex by an enzymatic mechanism that involves hydrolysis of negatively-charged membrane phospholipids. In addition, some $SVPLA_2$ also inhibit the TF-FVIIa complex by non-enzymatic mechanisms that are not well understood [47]. Some $SVPLA_2$ have both activities (enzymatic and non-enzymatic), such as CM-IV (*Naja nigricollis* venom), and are more potent anticoagulants. $SVPLA_2$ with only enzymatic activity include CM-I and CM-II (*Naja nigricollis* venom) [27,47]. It is not known how these phospholipases recognize the TF-FVIIa complex. Interestingly, the region of CM-IV that contains the anticoagulant activity has homology to TF, which could be important for targeting the TF-FVIIa complex [48].

2.2.2. Inhibitors of FVa-FXa or FVIIIa-FIXa

Proteins from animals also inhibit other coagulation factor-cofactor complexes, as the FVa-FXa and FVIIIa-FIXa complexes. Among these inhibitors, the tick inhibitor of factor Xa towards factor V (TIX-5) is a protein isolated from tick saliva (*Ixodes scapularis*) that is required for tick feeding. Recombinant TIX-5 delays FXa-mediated FV activation in a FV B-domain-dependent manner. This reveals the importance of FXa

Table 3
Activators of the mammalian fibrinolytic system from exogenous sources.

Component	Exogenous source	Activity Diagnostic/clinical use	Ref.
Profibrinolytic			
Achelases I and II	Caterpillar (*Lonomia achelous*)	Plasmin-like activity	[26,66,67]
Lonomin V	Caterpillar (*Lonomia achelous*)	u-PA-like activity	[26,66,67]
Serine protease	Jellyfish venom (*Nemopilema nomurai*)	Fibrinolytic	[68]
Albofibrase	Snake venom (*Thimeresurus albolabris*)	Activates fibrinogen	[40]
TSV-PA	Snake venom (*Trimeresurus stejnegeri*)	t-PA and u-PA-like activity	[69,70]
Reptilase®	Snake venom (*Bothrops atrox* and *Bothrops moojeni*)	Inhibition of PAI-1 and α_2-antiplasmin Reptilase Time assay	[71]
Jararhagin	Snake venom (*Bothrops jararaca*)	Cleaves fibrinogen, increases t-PA activity and inactivates α_2-antiplasmin inhibitor	[72,73]
Mutalysin II	Snake venom (*Lachesis muta muta*)	Fibrogenase	[75]
Fibrolase (alfimeprase)	Snake venom (*Agkistrodon contortrix*)	Fibrogenase	[99,100]
Pro- and antifibrinolytic			
Venom	Bumblebee venom (*Bombus ignitu*)	Fibrin(ogen)olytic activity and inhibition of plasmin	[76]
Venom	Scorpion venom (*Tityus discrepans*)	t-PA-like, fibrin(ogen)olytic and inhibition of plasmin	[77]

activation of FV in the activation of the coagulation system, and is also an important mechanism used by ticks to inhibit the mammalian coagulation system [49,50].

Proteins isolated from hematophagous animals also have anticoagulant activity by inhibition of FXa. As mentioned above, NAPc2 inactivates FXa by binding to the active site of FXa. Similarly, the nematode anticoagulant protein 5 (NAP5), produced by the hookworm *Ancylostoma caninum* also inhibits FXa [51]. NAP5 binds to the active site of FXa and also to an exosite and is a potent inhibitor of FXa activity. The saliva of the black fly *Simulium vittatum* contains the anticoagulant protein Simukunin [52]. Simukunin inhibits FXa activity by binding to its active site. The tick anticoagulant peptide (TAP) was purified from the soft tick *Ornithodoros moubata* and possesses homology to the Kunitz-type inhibitors. TAP acts as an anticoagulant in different clotting assays and specifically inhibits FXa [53].

The FVa-FXa complex is also inhibited by venoms. The hemolymph of the caterpillar *Lonomia achelous* contains the protein Lonomin VI:i, which inhibits the activity of FV [25]. Snake venom proteins, such as CM-IV and ammodytoxins from *Vipera ammodytes ammodytes* venom, inhibit the FVa-FXa complex in a phospholipid-independent and non-enzymatic way. CM-IV blocks the formation of the FVa-FXa complex by binding to FXa. Interestingly, the anticoagulant activity site of CM-IV is positively-charged and has homology with the light chain of FVa [48].

Snake C-type lectins bind to FIXa, FXa or both factors in the presence of calcium ions. These include anticoagulant factor I (ACF I), which was isolated from the *Agkistrodon acutus* snake venom, that inhibits both FIXa and FXa [54,55].

Recently, a new glycoprotein was isolated from nose-horned viper (*Vipera ammodytes ammodytes*) venom and named Vaa serine proteinase homolog 1 (VaaSPH-1). VaaSPH-1 has a structural that is similar to serine protease but has no protease activity. VaaSPH-1 interacts with factors and cofactors of the coagulation cascade, acting as an inhibitory protein. For instance, VaaSPH-1 binds to FVIII and prevents its interaction with FIX, showing potential as a template for the development of new anticoagulants [56].

2.2.3. Inhibitors of thrombin

The saliva of hematophagous arthropods contains several proteins that inhibit thrombin. For instance, hirudin was isolated from saliva of the leech *Hirudo medicinalis* and binds to the active site and exosite 1 of α-thrombin [57]. In addition, the anopheline protein family isolated from the saliva of the malaria vectors (*Anopheles* genus) binds to the active site and to an exosite of α-thrombin and inhibits its activity [58–60].

Several snake venom C-type lectins inhibit thrombin, such as bothrojaracin I. This protein was purified from *Bothrops jararaca* venom and inhibits α-thrombin by the binding to exosites 1 and 2. Bothrojaracin also decreases the binding of α-thrombin to fibrinogen and thrombomodulin and decreases the activation of protein C and prothrombin [61,62].

The thrombin inhibitor from *Naja haje* (TI-Nh) is a constituent of Egyptian cobra *Naja haje* venom that was the first phospholipase to be described as an inhibitor of thrombin. TI-Nh was characterized as a selective mixed-type thrombin inhibitor and it decreased fibrin clot formation in human plasma [63].

2.2.4. Protein C activators

Protein C is activated by thrombin bound to thrombomodulin. APC inhibits coagulation by inactivating the cofactors FVa and FVIIIa. Protein C activators are found in various snake venoms. For instance, the serine protease ACC-C from venom of the Southern copperhead (*Agkistrodon contortix*) activates protein C in the absence of thrombomodulin [21,27,64,65].

2.3. Activators of fibrinolysis

The fibrinolytic system lyses clots as the first step in wound healing [10]. Exogenous proteins from animal sources are capable of activating fibrinolysis in different ways (Table 3). The venom of the caterpillar *Lonomia achelous* has proteins with plasmin-like and u-PA-like activities called Achelase (I and II) and Lonomin V, respectively. These proteins lyse blood clots. The fibrinolytic activities of Achelases and Lonomin V are not inhibited by endogenous mammalian anti-fibrinolytic proteins. In addition, Achelases generate different fibrin/fibrinogen degradation products from the ones generated by plasmin, which indicates that they cleave fibrinogen at different sites [26,66,67]. Although not well-characterized yet, the venom of the jellyfish *Nemopilema nomurai* also contains a serine protease with fibrinolytic activity [68].

A few plasminogen activators have been isolated from snake venoms. The SVTLE albofibrase (*Thimeresurus albolabris*) activates plasminogen but has 4-fold lower activity when compared to u-PA [40]. Another of these activators is the serine protease *Trimeresurus stejnegeri* snake venom plasminogen activator (TSV-PA). TSV-PA cleaves plasminogen at the same site as u-PA and t-PA (Arg^{560}-Val^{561}) to generate plasmin (Fig. 3e). In addition, TSV-PA is not efficiently inactivated by endogenous fibrinolysis inhibitors, such as PAI-1 and α_2-antiplasmin [69,70]. The thrombin-like protein reptilase promotes fibrinolysis by inhibiting the anti-fibrinolytic proteins PAI-1 and α_2-antiplasmin [71].

Jararhagin is a metalloproteinase isolated from *Bothrops jararaca* venom and degrades fibrinogen. It also increases t-PA activity by inducing the disassociation of the inhibitory t-PA-PAI-1 complex and promotes fibrinolysis by inactivating α_2-antiplasmin [72,73].

Several enzymes that degrade fibrin or fibrinogen have been isolated from snake venoms. These serine proteases and

metalloproteinases are able to degrade fibrin-rich clots and are not inhibited by mammalian endogenous inhibitors. This group of proteins has potential use for the treatment of thrombotic disorders [27,74]. In fact, the fibrinogenase mutalysin II isolated from the venom of bushmaster snake (*Lachesis muta muta*) was able to lyse thrombi in the microcirculation in a mice model and restore blood flow in a similar manner as u-PA [75].

Some venoms contain both activators and inhibitors of fibrinolysis. For instance, the venom of the bumblebee (*Bombus ignitu*) contains a serine protease with fibrin(ogen)olytic activities called Bi-VSP and also a protein that inhibits plasmin [76]. The venom of the scorpion *Tityus discrepans* also has dual activities, such as a t-PA-like, fibrino(geno)lytic activity and an inhibitor of plasmin. One of these isolated proteins is called discreplasmin and inhibits plasmin and t-PA with a similar potency to aprotinin, which is a well-known serine protease inhibitor used to decrease hyperfibrinolysis [77].

3. Use of venom proteins in diagnostic assays

Venom proteins are used in various diagnostic assays. One of the most well-known venom proteins used in a diagnostic assay is RVV-X (from the venom of *Daboia russelli* snake). It is used in several assays that analyze levels of FX or FXa, detect coagulation factor deficiencies and detect lupus anticoagulants. RVV-X activates FX in plasma. The FXa concentration is subsequently measured using a clotting assay or using chromogenic substrates (such as Pefachrome® FXa) [21]. Lupus anticoagulants (LA) bind to phospholipids and interfere with coagulation assays, such as the activated partial thromboplastin time (aPTT) and the prothrombin time (PT). The dilute Russell's viper venom time (dRVVT) is used to detect LA [21–23]. For the dRVVT assay, the venom of *Daboia russelli*, calcium ions and a low concentration of phospholipids are added to the patient plasma with or without normal plasma and clotting times measured. The results allow the identification of plasmas with coagulation factors deficiency or LA, since these plasmas have longer clotting times. The test is performed again with the addition of an equal volume of normal plasma to the patient plasma. If the clotting time is normalized, the result indicates a deficiency of coagulation factors (factors X, V, prothrombin or fibrinogen). However, if the clotting time is still prolonged, it indicates the presence of LA. To confirm the diagnosis, the test may be performed with the addition of an excess of phospholipids to normalize the clotting time of plasmas with LA. [78].

The venom of the *Daboia russelli* snake also contains RVV-V, a protein used to assay the activation of FV [21,79]. RVV-V is also used to detect FV Leiden, which is resistant to inactivation by APC. One of the APC resistance tests is based on a clotting time assay in which RVV-V is used to activate FV. A shortened clotting times with exogenous APC indicates the presence of FV Leiden [21,79–81].

Ecarin, a prothrombin activator from snake venom, has several different clinical applications, as well as being used in the diagnostic assay called the ecarin clotting time assay. Ecarin is used to analyze levels of thrombin inhibitors, anticoagulant therapies using hirudin, protein-induced by vitamin K absence (PIVKA) syndrome in liver disease, lupus anticoagulants and disseminated intravascular coagulation (DIC) [82,83]. Briefly, ecarin is added to plasma and the clotting time is recorded. Ecarin cleaves prothrombin in meizothrombin in a manner independent of calcium ions, phospholipids and FV. Using different standard curves, ecarin can be used to determine prothrombin levels and monitoring hirudin levels (hirudin interacts with meizothrombin) [85]. Ecarin is also used to determine the ratio of Ecarin/Textarin times. Textarin® (isolated from the eastern brown snake *Pseudonaja textilis*) activates prothrombin to α-thrombin and is dependent on calcium ions, phospholipids and FV. LA prolong the Textarin clotting time but not the Ecarin clotting time so an increased ratio of Textarin/Ecarin times is indicative of a LA, which is confirmed with the addition of an excess of phospholipids to the test [83].

Snake venom thrombin-like enzymes that lead to the formation of fibrin are used in diagnostic assays. For instance, Reptilase® is used in the Reptilase Time (RT) assay. The assay is performed by adding Reptilase® to plasma and recording the clotting time. The thrombin time test is assayed in parallel to the RT assay. The test detects decreases in fibrinogen levels, dysfunctional fibrinogen and also contamination of plasma with anticoagulants as heparin [21,22,82].

Protac® (Pentapharm) was developed using the anticoagulant protein ACC-C from snake venom. Protac® is used to analyze the levels of protein C and as a functional assay for the protein C anticoagulant pathway. In the ProC Global Assay, Protac® is added to plasma and an aPTT-based clotting test is performed. Protac® also may be added to plasma and levels of APC determined using a specific chromogenic substrate (Pefachrome® PCa, Pentapharm). These assays are used to identify patients with protein C and protein S deficiencies, and also to identify patients with Factor V Leiden [22,65].

4. Clinical applications of venoms

Venoms have been developed as therapeutics. Importantly, Captopril was the first synthetic drug based on a protein present in the venom of the Brazilian pit viper *Bothrops jararaca*. It has been used extensively as a hypertensive drug since its approval in the 1980's [82]. Since then, numerous exogenous activators and inhibitors of the mammalian coagulation and fibrinolytic systems have been identified, isolated, produced in recombinant forms and applied as therapeutics.

A few anticoagulating drugs were developed based on exogenous proteins. Hirudin, isolated from leech saliva, was the first exogenous inhibitor of thrombin to be used in clinical trials. Bivalirudin (Angiomax®) is a synthetic peptide derived from hirudin and is used as a second line anticoagulant agent. Bivalirudin is used also used as an alternative anticoagulant in patients with heparin-induced thrombocytopenia [86,87]. The synthetic recombinant forms of hirudin – lepirudin and desirudin – are used as alternative preoperative and postoperative anticoagulants, such as in cardiac surgeries [88,89]. Lepirudin and desirudin also showed promising outcomes in clinical trial on thromboprophylaxis, disseminated intravascular coagulation, coronary syndromes and heparin-induced thrombocytopenia [57,90–92]. The recombinant NAPc2 (rNAPc2) showed promising results in phase I and II clinical trials for reducing the activation of coagulation, thrombin formation, ischemia and deep vein thrombosis. rNAPc2 also had a half-life of approximately 50 h with good tolerance [93,94].

Proteins isolated from snake venom were also studied for therapeutic approaches and applied to the clinical practice. For instance, Bothrojaracin was shown to protect mice from thrombin-induced fatal thromboembolism. In addition, Bothrojaracin reduced venous thrombosis induced by hypercoagulability and stasis in rats [95].

Batroxobin is a snake venom thrombin-like enzyme which has been used for the treatment of thrombotic disorders, ischemic stroke and myocardial infraction. Batroxobin induces the cleavage of fibrinogen to a loose, easily degraded fibrin. Batroxobin decreases levels of fibrinogen and increases fibrinolytic activity [21,82]. Similarly, Ancrod was developed as an anticoagulant drug for the treatment of deep-vein thrombosis, myocardial infarction, ischemic stroke, thrombosis syndrome and other thrombosis-associated conditions [21,41]. However, Ancrod produced mixed results in clinical trials [82]. Recently, an international collaborative study was developed in the attempt to calibrate the World Health Organization International Standard for Ancrod and the Reference Reagent for Batroxobin [96].

Other thrombin-like enzyme from snake venom used for clinical applications is the hemocogulase agkistrodon (HCA), isolated from the Chinese moccasin snake venom (*Deinagkistrodon acutus*). HCA was successfull in a phase III clinical trial for clinical practice to improve hemostasis after abdominal surgeries. However, more studies are required regarding its mechanisms of action and other clinical applications [22,97,98].

The metalloproteinase fibrolase was isolated from the venom of the Southern copperhead snake (*Agkistrodon contortrix*). Recombinant alfimeprase was shown to be six times more potent than plasminogen activators, but failed in phase III clinical trials for not meeting the trials endpoints [99,100].

5. Future directions

Besides the exogenous molecules currently used in the clinical practice, there are other substances currently being studied or undergoing clinical trials. Textilinin-1 is an anti-fibrinolytic protein isolated from the venom of the Eastern brown snake (*Pseudonaja textilis*). Textilinin-1 inhibits plasmin and the recombinant form of textilinin is associated with decreased blood loss in cardiothoracic surgeries [22,101]. Two other proteins have been isolated from *Pseudonaja textilis* venoms, named Haempatch™ and CoVase™. Haempatch™ is a FXa-like protein that has been assessed for controlling blood loss after a surgery or trauma. Haempatch™ was tested in different rat surgical models, including liver, spleen, tail tip, kidney and dermal injury models. Haempatch™ showed promising results and was more effective in reducing blood loss than thrombin. CoVase™ is a FVa-like protein which is not inhibited by APC and is being evaluated in the treatment of non-compressible hemorrhage. Textilinin-1, Haempatch™ and CoVase™ are currently undergoing pre-clinical trials [22,82,101].

Desmoteplase is a protein found in the saliva of the common vampire bat (*Desmodus rotundus*). Desmoteplase is a plasminogen activator that shares similarities with t-PA. Based on its fibrinolytic activities, desmoteplase is being tested in phase III clinical trials for acute ischemic stroke [102,103].

Although there is great potential of proteins in venoms and saliva of hematophagous animals as sources of bioactive molecules, only a small portion of these molecules are well-characterized and used for therapeutic or diagnostic purposes. The recent development of sensitive methods that generate large-scale data is an important tool to increase the discovery of new molecules. Approaches such as transcriptomics and proteomics allow for the discovery of new peptides [102,104–106]. Understanding the effect of the exogenous proteins on the coagulation and fibrinolytic systems may provide new insights into the regulation of these critical and complex systems.

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