Lessons from snake venom: new insights into the structural and functional aspects of factor V and factor X

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Chapter 1

General introduction

The coagulation cascade

Initiation of coagulation

On a molecular level, hemostasis is characterized by the formation of a key regulatory enzyme in blood coagulation: thrombin. This clotting enzyme is typically generated upon vascular damage in a timely and localized manner through an intricate network of enzymatic reactions governed by (pro)enzymes, (pro)cofactors, and inhibitors, collectively known as the coagulation cascade (**Figure 1**). Thrombin converts, among others, soluble fibrinogen to insoluble fibrin strands that serve to stabilize the primary platelet-based blood clot [1]. Insufficient thrombin generation therefore results in poor clot stability, which is at the basis of recurrent bleeding in patients suffering from bleeding disorders such as hemophilia [2]. On the other hand, thrombin formation at the wrong time and place can lead to a pathologic condition known as thrombosis. Thrombin is generated through a complex series of enzymatic reactions. Under normal circumstances, vascular damage results in exposure of membrane bound tissue factor (TF) to the bloodstream where it encounters its ligand, coagulation factor VIIa (FVIIa), to form the 'extrinsic' tenase complex (**Figure 1**) [3]. The interaction between TF and FVIIa occurs exclusively on membranes that present negatively charged phosphatidylserine on their outer membrane leaflet, such as activated platelets or damaged vascular endothelium, and requires the presence of calcium ions. Once the extrinsic tenase complex has been assembled, it converts the zymogen form of coagulation factor X (FX) into the protease FXa (the suffix "a" indicates the activated form) by proteolytic removal of the FX activation peptide. The newly formed N-terminus subsequently folds back into the serine protease (SP)-domain of FX. This results in a molecular rearrangement of the serine protease domain that primes the protease active site and enables FXa to cleave peptide bonds in other proteins. Once activated, FXa performs a central role in hemostasis as it is the only endogenous enzyme capable of converting prothrombin (factor II) into thrombin (factor IIa). However, efficient prothrombin conversion is only achieved upon assembly of FXa into the prothrombinase complex together with its cofactor Va (FVa). Once thrombin has been generated it is able to further propagate coagulation as detailed in the next section.

Propagation of coagulation

Hemophilia is a hereditary bleeding disorder that is characterized by varying degrees of hemorrhagic tendencies that usually require immediate specialized medical attention, especially when combined with trauma. On a molecular level, hemophilia is characterized by a (functional) deficiency in coagulation factor VIII (FVIII) or factor IX (FIX). These two 'anti-hemophilic' clotting factors are essential components of the intrinsic coagulation system, which participates both in the so-called contact activation pathway, as well as the TF-FVIIa-mediated pathway

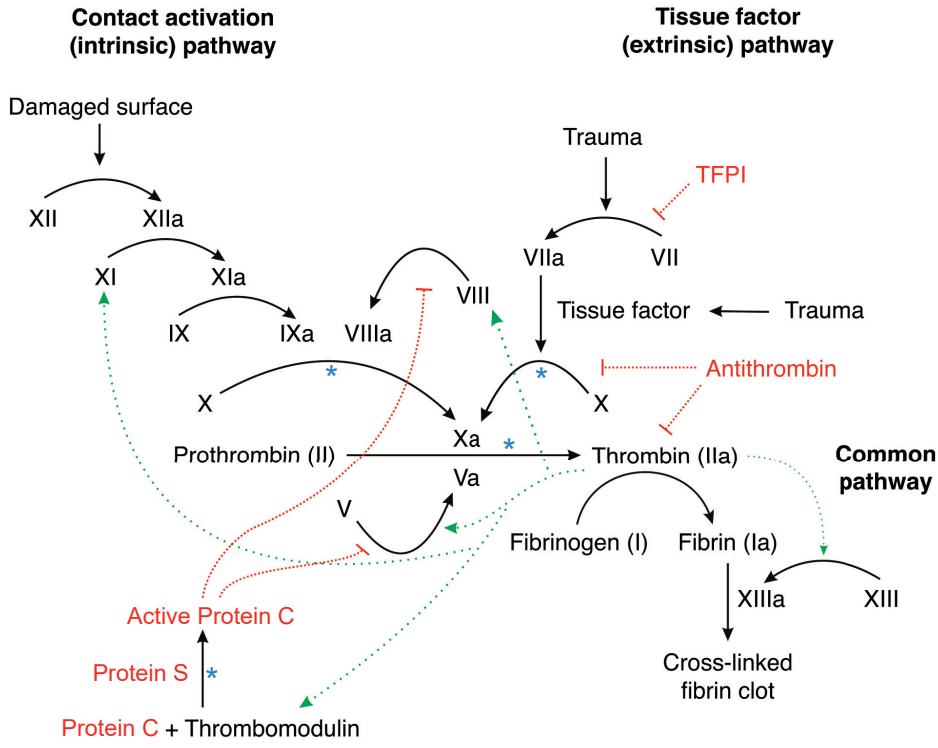

Figure 1. The coagulation cascade. Trauma is the major initiator of the coagulation cascade, resulting in activation of thrombin via tissue factor/factor VIIa and factor Xa/Va. Thrombin is then able to further propagate coagulation by additional activation of factor V and by enhancing factor X activation via the contact activation pathway through factors VIII and XI. Thrombin also mediates downregulation of the coagulation cascade through activation of protein C. Major inhibitors of the tissue factor pathway are TFPI (Tissue Factor Pathway Inhibitor) and antithrombin (which are highlighted in red). Roman numerals are used as numbering system for coagulation factors. Arrows denote negative (red) and positive (green) feedback loops. Blue asterisks indicate phospholipid-dependent reactions. Image scourced from Wikimedia [4].

[5] (**Figure 1**). The intrinsic system is traditionally regarded as a positive feedback loop to thrombin generation and is directly engaged by thrombin itself through proteolytic activation of the procofactor FVIII and zymogen FXI. Following activation, the serine protease FXIa directly converts FIX to FIXa. In addition, FIX may also be activated by FVIIa on activated platelets [6]. Once FVIII and FIX have been activated, these are able to interact on a negatively charged phospholipid surface in the presence of calcium ions to form the intrinsic tenase complex and to cooperatively activate FX. Intriguingly, the contact pathway may also be engaged upon exposure of the blood to negatively charged surfaces, such as by polyphosphates from invading pathogens [7]. This results in the generation of the serine protease FXIIa, which in turn leads to activation of FXI and FIX. However, the physiological relevance of this activation route in normal hemostasis remains contentious [8]. Accumulating evidence indicates that FXIIa-mediated FXI activation may be important in pathologic thrombus formation [9, 10]. In addition, a deficiency in FXI (hemophilia C) is not commonly associated with bleeding and is only linked to excessive hemorrhage upon trauma or surgery [11]. On the other hand, deficiencies in either FVIII (hemophilia A) or FIX (hemophilia B) are most commonly associated with recurrent bleeding episodes and treatment typically requires factor replacement therapy. The societal impact of these forms of hemophilia is extensive. For example, in the US alone, annual treatment costs may be as high as 300.000 dollar per individual, with up to 94% of the costs related to factor replacement products [12].

The prothrombinase complex

While activation of FX is prerequisite to the propagation of thrombin generation, physiologically relevant rates of thrombin generation are only achieved in the presence of activated coagulation factor V (FVa), which is an essential cofactor to FXa. FV circulates in blood as an inactive procofactor, and its domain organization (A1-A2-B-A3-C1-C2) is homologous to that of blood coagulation FVIII [13]. Activation of FV proceeds via limited proteolysis by either thrombin or FXa, which results in removal of its central B-domain that contains auto-inhibitory sequences [14-17]. Like the extrinsic and intrinsic tenase complexes, FVa and FXa exclusively associate on anionic membrane surfaces and in the presence of calcium ions, thereby forming 'the prothrombinase complex'. In FVa, membrane binding is governed by its lipid-binding C-domains, while the three A domains are involved in interactions with the protease FXa and physiological substrate prothrombin [18]. In FXa (domain organization: GLA-EGF1-EGF2-SP), membrane binding is governed by its N-terminal GLA-domain. This domain binds calcium ions which enables FX to interact with phosphatidylserine in the membrane via ionic interactions. Membrane binding GLA-domains are also found in homologous clotting factors such as prothrombin, FVII, FIX, protein C (PC), and protein S. Suggestions as to how the membrane surface propagates the coagulation reactions include increased local

reactant concentrations, induced conformational changes, or restricted protein movement. However, molecular details adequately describing this process are incomplete. Once FXa and FVa have assembled into the prothrombinase complex on a membrane surface, FVa is able to radically increase the catalytic rate of FXa towards prothrombin by serving as a platform to which FXa and prothrombin are able to bind [18, 19]. The prothrombinase complex forms the starting point of the so-called 'common pathway' which governs all thrombin-mediated clotting processes including: fibrin deposition, platelet and cell activation, and the activation of FV, FVIII, FXI, FXIII, and protein C (**Figure 1**).

Inhibition of coagulation

The progressive activation of clotting factors in the coagulation cascade creates a potent positive feedback loop that can rapidly produce a sustained burst of thrombin activity. In order to prevent runaway coagulation, several molecular mechanisms are in place that contain the thrombin burst to the site of injury. First and foremost, formation of the FX- and prothrombin-activating complexes each require the presence of negatively charged membrane surfaces, which effectively localizes the conversion of FX and prothrombin to activated platelets and damaged endothelium. In addition, naturally occurring inhibitors of coagulation present in blood tightly control the proteolytic activity of procoagulant serine proteases such as thrombin and FXa. The largest group of inhibitors are the serine protease inhibitors (serpins) [20]. These inhibitors prevent clotting enzymes from cleaving peptide bonds by occupying the region (active site) where substrates would normally bind and undergo proteolysis. Antithrombin (AT) is the main active site inhibitor of FXa, FIXa, and thrombin [21]. AT binds irreversibly and traps the enzyme in an inactive conformation, which is then cleared from the circulation and degraded. Another important anticoagulant protein is tissue factor pathway inhibitor (TFPI), which reversibly inhibits the active site of FXa. In addition, TFPI is able to inhibit the extrinsic tenase complex, both independently and when complexed with FXa. Furthermore, it is able to suppress FV activation and can associate with FV to suppress prothrombinase activity [22, 23]. Finally, thrombin also mediates downregulation of prothrombinase and intrinsic tenase through proteolytic activation of protein C, which is mediated by the cofactor thrombomodulin. Together with its cofactor protein S, activated protein C can proteolytically degrade FVa and FVIIIa, thereby limiting their cofactor activities [24] (**Figure 1**).

Thrombosis and anticoagulant drugs

Thrombosis is defined as unwanted clot formation (thrombus) within the vasculature that may obstruct the flow of blood through the vessel, or become dislodged and enter the circulation as an embolus, resulting in thromboembolism [25]. Thrombotic disorders are generally sub-defined by the type of blood vessel

that is affected (arterial or venous) and the exact location of the blood vessel or the organ it supplies. Common risk factors for arterial and venous thrombosis include (among others): advanced age, cardiovascular health (atherosclerosis, smoking, hypertension), atrial fibrillation, cancer, surgery, fractures, prolonged immobilization and oral contraceptive use [26, 27]. In addition, many genetic predispositions to venous thrombosis have been documented relating to mutations in genes encoding coagulation factors [28].

Thrombosis represents a major public health concern; it is estimated that one out of ten individuals world-wide will suffer from a thrombotic disorder during his or her lifetime. For example, in the United States up to 600.000 individuals per year are affected by venous thromboembolism alone [29]. Millions of patients worldwide therefore require anticoagulant drugs for the prophylactic management of thrombotic disorders. Over the past decades, warfarin and other vitamin K antagonists have been prescribed as anticoagulants. These anticoagulant drugs inhibit the turn-over of vitamin K (K=Koagulation) during post-translational ɣ-carboxylation of glutamic acid residues (Glu) to Gla residues. Clotting factors that comprise a GLA-domain, such as FX and prothrombin, typically require this post-translational modification in order to bind calcium ions and engage negatively charged membranes. While the effectiveness of vitamin K antagonists has been well established, their use has been implicated in many bleeding-related adverse events [30, 31].

Since 2013, new oral anticoagulant therapies have become available which directly inhibit either thrombin (dabigatran) or FXa (apixaban, rivaroxaban, edoxaban). The advantage of these so-called direct oral anticoagulants (DOACs) over the traditional vitamin K antagonists is their rapid therapeutic effectiveness, ease of dosing, and lack of monitoring requirements. Importantly, a worldwide-approved specific reversal strategy to prevent and stop potential life-threatening bleeding complications associated with anticoagulant therapy should be available. This is particularly important considering that annually 1-3% of the patients treated with DOACs suffer an adverse severe bleeding event, of which up to 1 in 5 are fatal [32]. For dabigatran, idarucizumab was succesfully introduced as specific reversal agent [33]. Nevertheless, specific reversal strategies are not available for all patients taking the FXa-inhibiting DOACs. Prothrombin complex concentrates (PCCs) are nonetheless regularly used in clinic in case of bleeding or acute surgery [34, 35]. Even so, recent studies have shown that PCCs are not able to fully restore hemostasis in all patients [36]. In addition, a recombinant modified factor Xa protein known as Andexanet-alfa has recently been approved in both the US and EU as antidote for FXa-DOACs [37]. However, the use of Andexanet-alpha carries an elevated thrombogenic risk due to sequestering of TFPI by the FXa-like molecule [38].

Factors V and X from snake venom

Over the past decade, significant scientific effort has been put into studying venom-derived toxins in order to harness their potential as drug discovery platform [39-41]. Snake venoms are widely recognized as an exceptional source of biologically active proteins and peptides that interact with the hemostatic system [42]. This is best exemplified by a family of Australian Elapid snakes, in which the blood coagulation proteins FV and FX have been converted into potent biological weapons to support envenomation of prey [43]. Pseutarin C, a powerful prothrombin activator that is found in the venom of the Australian elapid *Pseudonaja textilis*, or common brown snake, has been studied most extensively so far [44-46]. This unique prothrombin activator consists of a FVa-FXa complex and can efficiently generate thrombin in the absence of a negatively charged phospholipid surface [47], unlike any other FVa-FXa complex known to date. In addition, several unique gain-of-function modifications have been identified in both *P. textilis* venom FV and FX that serve to enhance their procoagulant potential. For example, venom FV is constitutively active due to the absence of a regulatory B-domain sequence [44, 47]. Moreover, it is functionally resistant to proteolytic inactivation by the enzymatic regulator of FV, APC [47]. Venom FX is hallmarked by a significantly shortened activation peptide, which possibly reflects the need for a more protease-like venom protein [48]. Additional modifications can be identified in the antithrombin binding site, which were shown to result in an impaired inhibition of venom FXa by antithrombin [49]. The venom FXa substrate binding pocket has also been structurally modified through an extended 99-loop (His91-Asp102), which is a surface-exposed loop that borders the active site in serine proteases [48, 50, 51]. These combined alterations enable the *P. textilis* 'venom prothrombinase' to escape hemostatic regulation and initiate indiscriminate clotting throughout the vasculature. The venom FVa-FXa complex is therefore an attractive model for prospective protein engineering strategies.

Outline of the thesis

The aim of this thesis was to uncover the unique structural and functional relationships that govern venom FXa-FVa complex assembly and function, with the intention to harness its potent procoagulant potential. In addition, by studying these venom-derived clotting factors we intend to better our understanding of the molecular mechanisms that drive prothrombinase assembly and function in humans.

In the first part of this thesis, we studied the genomic, structural, and functional implications of several molecular modifications found in the *P. textilis* venom FV molecule. In **chapter 2**, we have aimed to shed more light on the absence of the regulatory B-domain in the venom FV molecule by studying liver-expressed FV transcripts in snakes from the Elapid family and in various other snakes. In **chapter 3**, we have studied the functional implications of a disulfide bond that is exclusively found in the venom FV molecule with respect to APC inactivation of FV and lipid-independent cofactor function. In **chapter 4** of this thesis we investigated the functional relationship between cofactor stability and phospholipid binding in FV by exchanging the C-domains between human FV and *P. textilis* venom FV.

The second part of this thesis is focused on the structural and functional aspects of venom FX. In **chapter 5**, we studied the functional implications of the extended 99-loop that is unique to venom FXa. This study was partly carried out in silico through analysis of the molecular dynamics that goven venom FXa, and partly through biochemical characterization of human-snake FXa chimeras in which the snake 99-loop element was introduced. In **chapter 6**, we aimed to identify a suitable non-human plasma model system for the preclinical development of our new FX molecule. Using routine and specialty coagulation assays we established global clotting parameters in mouse, rat, rabbit, porcine, and goat plasma.

References

1. Riddel, J.P., Jr., et al., *Theories of blood coagulation.* J Pediatr Oncol Nurs, 2007. **24**(3): p. 123-31.
2. Dargaud, Y., et al., *Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B.* Thromb Haemost, 2005. **93**(3): p. 475-80.
3. Mackman, N., R.E. Tilley, and N.S. Key, *Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(8): p. 1687-93.
4. Wikimedia, *The coagulation cascade.* wikimedia.org/wiki/File:Coagulation_full.svg
5. Wu, Y., *Contact pathway of coagulation and inflammation.* Thromb J, 2015. **13**: p. 17.
6. Gabriel, D.A., et al., *Recombinant human factor VIIa (rFVIIa) can activate factor FIX on activated platelets.* J Thromb Haemost, 2004. **2**(10): p. 1816-22.
7. Baker, C.J., S.A. Smith, and J.H. Morrissey, *Polyphosphate in thrombosis, hemostasis, and inflammation.* Res Pract Thromb Haemost, 2019. **3**(1): p. 18-25.
8. Kravtsov, D.V., et al., *Factor XI contributes to thrombin generation in the absence of factor XII.* Blood, 2009. **114**(2): p. 452-8.
9. Cheng, Q., et al., *A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo.* Blood, 2010. **116**(19): p. 3981-9.
10. He, R., D. Chen, and S. He, *Factor XI: hemostasis, thrombosis, and antithrombosis.* Thromb Res, 2012. **129**(5): p. 541-50.
11. Seligsohn, U., *Factor XI deficiency in humans.* J Thromb Haemost, 2009. **7 Suppl 1**: p. 84-7.
12. Zhou, Z.Y., et al., *Burden of illness: direct and indirect costs among persons with hemophilia A in the United States.* J Med Econ, 2015. **18**(6): p. 457-65.
13. Kane, W.H. and E.W. Davie, *Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders.* Blood, 1988. **71**(3): p. 539-55.
14. Bos, M.H. and R.M. Camire, *A bipartite autoinhibitory region within the B-domain suppresses function in factor V.* J Biol Chem, 2012. **287**(31): p. 26342-51.
15. Schuijt, T.J., et al., *Factor Xa activation of factor V is of paramount importance in initiating the coagulation system: lessons from a tick salivary protein.* Circulation, 2013. **128**(3): p. 254-66.
16. Camire, R.M. and M.H. Bos, *The molecular basis of factor V and VIII procofactor activation.* J Thromb Haemost, 2009. **7**(12): p. 1951-61.
17. Suzuki, K., B. Dahlback, and J. Stenflo, *Thrombin-catalyzed activation of human coagulation factor V.* J Biol Chem, 1982. **257**(11): p. 6556-64.
18. Schreuder, M., P.H. Reitsma, and M.H.A. Bos, *Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding.* J Thromb Haemost, 2019. **17**(8): p. 1229-1239.
19. Mann, K.G., et al., *Surface-dependent reactions of the vitamin K-dependent enzyme complexes.* Blood, 1990. **76**(1): p. 1-16.

20. Rau, J.C., et al., *Serpins in thrombosis, hemostasis and fibrinolysis.* J Thromb Haemost, 2007. **5 Suppl 1**: p. 102-15.
21. Bjork, I. and S.T. Olson, *Antithrombin. A bloody important serpin.* Adv Exp Med Biol, 1997. **425**: p. 17-33.
22. Broze, G.J., Jr., T.J. Girard, and W.F. Novotny, *Regulation of coagulation by a multivalent Kunitz-type inhibitor.* Biochemistry, 1990. **29**(33): p. 7539-46.
23. Wood, J.P., et al., *Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation.* Proc Natl Acad Sci U S A, 2013. **110**(44): p. 17838-43.
24. Kisiel, W., et al., *Anticoagulant properties of bovine plasma protein C following activation by thrombin.* Biochemistry, 1977. **16**(26): p. 5824-31.
25. Furie, B. and B.C. Furie, *Mechanisms of thrombus formation.* N Engl J Med, 2008. **359**(9): p. 938-49.
26. Reiner, A.P., D.S. Siscovick, and F.R. Rosendaal, *Hemostatic risk factors and arterial thrombotic disease.* Thrombosis and Haemostasis, 2001. **85**(4): p. 584-595.
27. Previtali, E., et al., *Risk factors for venous and arterial thrombosis.* Blood Transfusion, 2011. **9**(2): p. 120-138.
28. Franco, R.F. and P.H. Reitsma, *Genetic risk factors of venous thrombosis.* Hum Genet, 2001. **109**(4): p. 369-84.
29. Beckman, M.G., et al., *Venous thromboembolism: a public health concern.* Am J Prev Med, 2010. **38**(4 Suppl): p. S495-501.
30. Budnitz, D.S., et al., *Emergency hospitalizations for adverse drug events in older Americans.* N Engl J Med, 2011. **365**(21): p. 2002-12.
31. Wysowski, D.K., P. Nourjah, and L. Swartz, *Bleeding complications with warfarin use: a prevalent adverse effect resulting in regulatory action.* Arch Intern Med, 2007. **167**(13): p. 1414-9.
32. Sardar, P., et al., *Risk of major bleeding in different indications for new oral anticoagulants: insights from a meta-analysis of approved dosages from 50 randomized trials.* Int J Cardiol, 2015. **179**: p. 279-87.
33. Pollack, C.V., Jr., et al., *Idarucizumab for Dabigatran Reversal - Full Cohort Analysis.* N Engl J Med, 2017. **377**(5): p. 431-441.
34. Arachchillage, D.R.J., et al., *Efficacy and safety of prothrombin complex concentrate in patients treated with rivaroxaban or apixaban compared to warfarin presenting with major bleeding.* Br J Haematol, 2019. **184**(5): p. 808-816.
35. Schulman, S., et al., *Prothrombin Complex Concentrate for Major Bleeding on Factor Xa Inhibitors: A Prospective Cohort Study.* Thromb Haemost, 2018. **118**(5): p. 842-851.
36. Gerner, S.T., et al., *Association of prothrombin complex concentrate administration and hematoma enlargement in non-vitamin K antagonist oral anticoagulant-related intracerebral hemorrhage.* Ann Neurol, 2018. **83**(1): p. 186-196.
37. Lu, G., et al., *A specific antidote for reversal of anticoagulation by direct and indirect inhibitors of coagulation factor Xa.* Nat Med, 2013. **19**(4): p. 446-51.
38. Favresse, J., et al., *Andexanet alfa for the reversal of factor Xa inhibitors.* Expert Opin Biol Ther, 2019. **19**(5): p. 387-397.

39. Vetter, I., et al., *Venomics: a new paradigm for natural products-based drug discovery.* Amino Acids, 2011. **40**(1): p. 15-28.
40. Vonk, F.J., et al., *Snake venom: From fieldwork to the clinic: Recent insights into snake biology, together with new technology allowing high-throughput screening of venom, bring new hope for drug discovery.* Bioessays, 2011. **33**(4): p. 269-79.
41. Koh, C.Y., et al., *Toxins Are an Excellent Source of Therapeutic Agents against Cardiovascular Diseases.* Semin Thromb Hemost, 2018. **44**(7): p. 691-706.
42. Markland, F.S., *Snake venoms and the hemostatic system.* Toxicon, 1998. **36**(12): p. 1749-800.
43. St Pierre, L., et al., *Comparative analysis of prothrombin activators from the venom of Australian elapids.* Mol Biol Evol, 2005. **22**(9): p. 1853-64.
44. Rao, V.S., S. Swarup, and R.M. Kini, *The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (Pseudonaja textilis) venom, shows structural similarity to mammalian coagulation factor V.* Blood, 2003. **102**(4): p. 1347-54.
45. Rao, V.S., S. Swarup, and R. Manjunatha Kini, *The catalytic subunit of pseutarin C, a group C prothrombin activator from the venom of Pseudonaja textilis, is structurally similar to mammalian blood coagulation factor Xa.* Thromb Haemost, 2004. **92**(3): p. 509-21.
46. Masci, P.P., A.N. Whitaker, and J. de Jersey, *Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, Pseudonaja textilis textilis.* Biochem Int, 1988. **17**(5): p. 825-35.
47. Bos, M.H., et al., *Venom factor V from the common brown snake escapes hemostatic regulation through procoagulant adaptations.* Blood, 2009. **114**(3): p. 686-92.
48. Bos, M.H. and R.M. Camire, *Procoagulant adaptation of a blood coagulation prothrombinase-like enzyme complex in australian elapid venom.* Toxins (Basel), 2010. **2**(6): p. 1554-67.
49. Johnson DJ, H.J., *Pseudonaja textilis venom FXa is poorly inhibited by human antithrombin.* Abstracts of the XXIV congress of the International Society on Thrombosis and Haemostasis, 2013: p. PB 4.58-2.
50. Lechtenberg, B.C., et al., *Crystal structure of the prothrombinase complex from the venom of Pseudonaja textilis.* Blood, 2013. **122**(16): p. 2777-83.
51. Hedstrom, L., *Serine protease mechanism and specificity.* Chem Rev, 2002. **102**(12): p. 4501-24.