EXACTIN – A SPECIFIC INHIBITOR OF FACTOR X ACTIVATION BY EXTRINSIC TENASE COMPLEX ISOLATED FROM HEMACHATUS HAEMACHATUS VENOM

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M. TECH.

BIOTECHNOLOGY

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

FACULTY OF SCIENCE

NATIONAL UNIVERSITY OF SINGAPORE

MAY, 2012

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Professor R Manjunatha Kini for his constant encouragement and scientific input throughout my four years of PhD. He has provided me an opportunity to work in his lab as well as guided me through various critical experiments. His golden words "sit, think, plan and write" has always helped me while starting new experiments. From 2007 to this day, I see myself in a position where I can take new challenges as well as independently do my work. Thanks a lot Prof. Kini for making be eligible in this scientific world.

I extend my gratitude to Bee Ling and Say Tin. Both of them have helped a lot during my studies. Bee Ling, an efficient lab officer has always fascinated me by her management skills. Say Tin has taught me to use the protein sequencer as well as given me the opportunity to handle it. Thanks a lot you both!

I would like to thank National University of Singapore for their financial support for the past four years. I also extend my gratitude to the Biomedical Research Council (BMRC), Singapore for providing a generous grant to Prof. Kini which funded my work described in this thesis.

I would like to thank Prof. Sivaraman and his group members, Thangavelu and Dr. Jobichen for helping me with crystallization studies of exactin. I extend my gratitude to Prof. Daniel Bertrand, for helping me out with all electrophysiology experiments related to exactin.

I would like to thank Dr. Koh Cho Yeow (Koh Cho) for teaching me initially purification and enzyme kinetics. He has always been there with me as a good friend and a mentor. I am extremely thankful to Sheena (Kappu) for helping me with CBCM studies. I would like to thank Bhaskar as well as Bidhan for helping me with *in vivo* toxicity studies and Garvita for teaching me CD spectroscopy. I would like to thank my dear and near friends Amrita and Vivek (and of course Bhaskar, Garvita, Debu, Sheena, Angelina). They have been a great support in all my hard times. I would like to thank all my present and past lab mates for all the help they have given to me while I was a student: Sindhuja, Bidhan, Ryan, Akthar, Summer, Hoi, Varuna, Ritu, Reza, Robin, Shiyang, Shifali, Nazir, Alex, Tzer Fong, Guna, Pushpa, Kishore, Raghu, Ming Zi, Bee Haar, Ee Xuan, Maulana. I would also like to thank all the members of structural biology lab 4 and lab 5 as well as plant morphogenesis lab for their help during various stages of my work. Thanks to Pradeep for the healthy scientific discussions we used to have at home.

I am grateful to my family for their support. Thanks to my Appa (Balakrishnan) and Amma (Girija) for being with me all the time. Thanks for the good deeds I have received from them. Thanks to my sister, my brother-in-law and my little devils Ammu and Appu for giving me good times whenever I visit them.

Finally, my gratitude to goddess “Mookambika”. She has supported me in all my bad and good moments of life. An unseen friend and mother, she has been with me throughout my life. Thanks a lot Amma.

V.M Girish

January, 2012

TABLE OF CONTENTS

SUMMARY

The formation of unwanted clots leads to heart attack and stroke that result in a large number of mortalities and morbidities in developed countries, including Singapore. Currently available anticoagulant drugs have some drawbacks including their non-specific actions. Therefore novel anticoagulants that target specific steps in the coagulation pathway are being sought. We have been searching for new anticoagulants from snake venoms. Here, we report the purification and characterization of a novel anticoagulant protein, exactin from the venom of *Hemachatus haemachatus* (African ringhals cobra). The protein was purified from the crude venom using gel filtration and reverse-phase high performance liquid chromatography. The molecular mass of the purified protein was determined to be 6621.12 ± 0.22 Da. Based on its sequence similarity and cysteine positioning it can be concluded that exactin belongs to three-finger toxin family. A dissection approach on the coagulation cascade revealed the target of exactin as extrinsic activation complex (FVIIa/TF/FX). To better understand the molecular mechanism, we examined the effect of exactin on various assays in which each part of the complex was removed sequentially. Removal of TF did not affect the inhibitory potency of exactin (IC_{50} value 102.70 ± 11.71 nM compared to 116.49 ± 3.28 nM for the complete complex). However, in the absence of phospholipids the inhibition dropped by >1000 folds. Exactin also poorly inhibited the amidolytic activities of FVIIa and FXa, suggesting it exerts its inhibitory potency towards the entire complex. Kinetic studies showed that exactin exhibits a mixed-type inhibition towards FX activation by FVIIa/TF_{PL}, FVIIa in presence of PL and FVIIa/sTF. The affinity of the inhibitor towards the enzyme-substrate complex (FVIIa/TF_{PL}/FX, Ki' 30.62 ± 7.73 nM) was 5-fold higher compared to the enzyme complex (FVIIa/TF_{PL}, Ki 153.75 ± 17.96 nM) suggesting its preference to [ES] complex. In the absence of TF, Ki' dropped 3-fold to 103 ± 13.49 nM with a

slight decrease in Ki of 184.25 ± 6.13 nM. Thus, exactin appears to bind to the complete complex better than FVIIa/FX complex. The affinity of exactin was drastically reduced by >1000 fold for FX activation in the absence of phospholipids (Ki', 295 ± 7.07 µM; Ki, 1250 ± 56.6 µM), suggesting its preference towards membrane-bound complex. Its poor ability to inhibit the hydrolysis of chromogenic substrate by these complexes suggests that exactin preferably inhibits the macromolecular substrate complex. To understand macromolecular specificities, we examined its effects on FIX activation by FVIIa/TF_{PL} complex. Exactin preferably inhibits FX activation than FIX activation (IC_{50} 29.66 ± 5.27 µM; Ki', 38.66 ± 10.27 µM**;** Ki, 128.6 ± 12.54 µM). Interestingly, exactin also inhibits, but not potently, FX activation by the intrinsic tenase complex $(FIXa/FVIIIa)_{PL}$ as well as a metalloproteinase, RVV-X non-competitively (IC_{50} 4.05 ± 0.32 µM; Ki, 1.67 ± 0.35 µM and IC_{50} 6.1 ± 2.9 µM; Ki, 2.79 ± 0.29 µM, respectively). These results indicate that exactin may bind to FX, (as distinct exosites are recognized by these enzyme/enzyme-cofactor complexes) thereby preventing its proteolytic cleavage. To understand its usefulness as the anticoagulant lead, we also studied its toxicity. Exactin showed weak neurotoxicity in mice. The *ex vivo* studies with chick biventer cervicis muscle preparations, suggested that it is a reversible, postsynaptic neurotoxin. Thus we conclude that exactin is a novel three-finger toxin with dual function; a potent and specific anticoagulant effect on the FX activation by the complete extrinsic tenase complex and a weak, reversible, postsynaptic neurotoxicity.

RESEARCH COLLABORATIONS

We have made collaborations with the following laboratories. Their contribution is greatly acknowledged.

1) Crystallographic studies with exactin

Associate Professor Jayaram Sivaraman, Mr. Thangavelu and Dr. Jobichen

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2) Electrophysiology studies on exactin

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LIST OF FIGURES

Chapter 1: Introduction

Chapter 2: Anticoagulant properties of exactin

Chapter 3: Neurotoxic properties of exactin

Chapter 4: Crystallization of exactin

LIST OF TABLES

Abbreviations

Ach	Acetylcholine
ACS	Acute coronary syndrome
ADP	Adenosine di phosphate
APC	Activated protein C
APTT	Activated partial thromboplastin time
AT-III	Antithrombin-III
BSA	Bovine serum albumin
Cch	Carbamyl choline (carbachol)
CD	Circular dichroism
DVE	Deep-vein thrombosis
ESI-MS	Electrospray ionization mass spectrometry
FA	Formic acid
FIX, FIXa	Factor IX, activated factor IX
FV, FVa	Factor V, activated factor V
FVII, FVIIa	Factor VII, activated factor VII
FVIII, FVIIIa	Factor VIII, activated factor VIII
FX, FXa	Factor X, activated factor X
FXI, FXIa	Factor XI, activated factor XI
FXII, FXIIa	Factor XII, activated factor XII
FXIIIa	Activated factor XIII
Gla	Gamma-carboxyglutamic acid
HCII	Heparin cofactor II
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HIT	Heparin-induced thrombocytopenia
HMWK	High-molecular weight kallikrein
i.p	Intraperitoneal
KCl	Potassium chloride
LMWH	Low-molecular-weight heparin
MI	Myocardial infarction
nAChRs	Nicotinic acetylcholine receptors
NO	Nitric oxide
Par4	Protease-activated receptor 4

PCI	Percutaneous coronary intervention
PDB	Protein Data Bank
PE	Pulmonary embolism
PEG	Polyethylene glycol
PK	Prekallikrein
PL	Phospholipids
*p*NA	*p*-nitroaniline
PTT	Prothrombin time
PT	Prothrombin
RP-HPLC	Reverse-phase high performance liquid chromatography
RVV-X	Russell's viper venom factor X activator
S.D.	Standard deviations
S2222	benzoyl-IIe-Glu (Glu-γ -methoxy)-Gly-Arg-*p*-nitroanilide (*p*NA) hydrochloride (HCl)
S2238	*H*-D-Phe-pipecolyl (Pip)-Arg-*p*NA•2HCl
S2288	*H*-D-IIe-Pro-Arg-*p*NA•2HCl
Serpin	Serine proteinase inhibitor
Spectrozyme® FIXa	*H*-D-Leu-phenylalanyl-Gly-Arg-*p*NA•2-AcOH
TxA_2	Thromboxane A_2
TAFI	Thrombin activatable fibrinolysis inhibitor
TF	Tissue factor
TFA	Trifluoroacetic acid
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TT	Thrombin time
UFH	Unfractionated heparin
u-PA	Urokinase –type plasminogen activator
VWF	Von Willebrand factor

Chapter 1

Introduction

1. Hemostasis

The maintenance of blood fluidity in circulation is pivotal for the survival of an organism. Hemostasis, a process designed to arrest bleeding from injured blood vessels is a tightly regulated process and involves interactions between plasma proteins (coagulations factors, physiological inhibitors and fibrinolytic proteins) and blood cells (platelets, endothelial cells and monocytes) (Mackie and Bull, 1989; Hopper and Bateman, 2005; Sere and Hackeng, 2003). The various steps that are involved in the final accomplishment of clot formation are described below in detail:

1.1. Mechanism of Hemostasis

1.1.1. Vasoconstriction

The vascular lumen is lined by endothelium that provides a non-thrombogenic surface maintaining the proper blood flow. This is achieved through the production of various anti-thrombotic agents like prostacyclin (PGI_2), NO, ADPase, thrombomodulin, TFPI, heparan sulphate and TPA. Prostacyclin, NO and ADPase act by inhibiting the platelet adhesion (Becker *et al*., 2000) whereas thrombomodulin (Dahlbäck and Villoutrix, 2005), TFPI (Girard *et al*., 1989), heparan sulphate (Fredenburgh *et al*., 2002) and tPA (Dobrovolsky and Titaeva, 2002) mediate their action through interaction with the coagulation proteins.

Vasoconstriction functions as an effective way to reduce blood loss immediately after a vessel injury via neurogenic vasospasm, precapillary sphincter constriction, humoral vasospastic phenomena (Mason and Saba, 1978; Mason *et al*., 1977) as well as by the release of vasoconstrictor endothelins (Agapitov and Haynes, 2002). This is usually followed by action of pro-thrombotic factors leading to the formation of a stable hemostatic plug that prevent the oozing out of blood from the injured surface. The various pro-thrombotic factors involved in this process include expression of TF on the endothelial cell luminal surface, release of vWF from endothelial Wiebel-Palade bodies resulting in the platelet adhesion to sub endothelial collagen followed by the action of thromboxane A_2 and platelet activation factor which further leads to platelet activation, aggregation and plug formation, involvement of plasminogen activator inhibitor-1 in preventing fibrinolysis and expression of P-selectin, an adhesion molecule that helps binding of platelets to endothelial surface (Maria *et al*., 1986).

1.1.2. Platelet plug formation

Primary hemostasis starts with recruitment and activation of platelets to the site of vascular injury forming a platelet plug via stimulation by collagen, ADP, TxA_2, epinephrine, serotonin and thrombin (Jackson *et al*., 2009; Wei *et al*., 2009) (**Fig. 1.1**). This multi-activation process culminates into a stable platelet rich thrombus. The initial capture of platelets to collagen is facilitated by the interaction between collagen bound vWF and GPIb/V/IX receptor complex under conditions of high shear as found in arterioles and arteries (Jennings, 2009; Lenting *et al*., 2010; Nuyttens, 2011). The platelet-collagen binding is stabilized by the interaction of two major receptors, $\alpha_2\beta_1$ and GPVI to collagen surfaces. Integrin, $\alpha_2\beta_1$ is known for its role in platelet adhesion and anchoring, whereas GPVI is known for platelet activation (Clemetson and Clemetson, 2001; Nieswandt and Watson, 2003). The cumulative effect of the above mentioned platelet activators also leads to the activation of the receptor GPIIb/IIIa that mediates platelet adhesion, aggregation and finally stable thrombus formation via the interaction of the receptor to fibrinogen, bridging the platelet cells (Jennings, 2009). During the platelet activation, ADP is released from granules and exhibit profound effects like platelet recruitment to site of injury, change in platelet shape, platelet aggregation and release of TxA_2. This release of TxA_2 further enhances platelet recruitment, activation and finally aggregation of primary hemostatic plug (Brass, 2010).

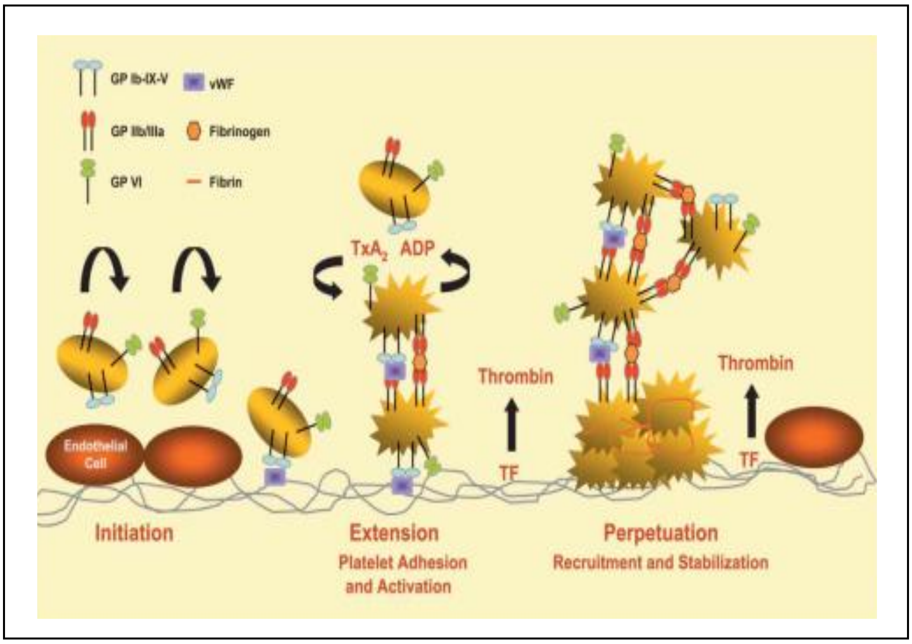


Figure 1.1: Platelet plug formation. Upon vascular rupture, circulating platelets are captured on the exposed collagen and vWF in the vessel wall leading to a monolayer of activated platelets. The activated platelets formed induce the secretion of ADP and TxA_2 from the adherent platelets resulting in changes in platelet shape and enhancement of platelet activation. Like collagen, thrombin also activates platelets by cleaving PAR-1 and PAR-4 receptors. In the continuance, platelet contacts promote growth and stabilization of the platelet plug [*Adapted from Jennings, Thromb Haemost (2009) 102: 248–257*].

Molecules like serotonin and epinephrine also play minor roles in platelet activation by recruiting the platelet cells to the injury site and promoting procoagulant activity via retention of fibrinogen and thrombospondin to cell surface (Stegner and Nieswandt, 2011). Like collagen, thrombin also play major role in platelet activation by the cleavage of protease-activated receptors, PAR-1 and PAR-4 at the N-termini. However, it is independent of endothelial rupture and vWF mediated activation (Furie and Furie, 2008). Thrombin assisted activation results in release of ADP, serotonin and TxA2 that also activates other platelets and by so enhances thrombus formation (Schmidlin and Bunnett, 2001; Brass, 2003).

Activation of both platelets and coagulation factors are interconnected in that latter can bind to platelets either through glycoprotein receptors or through exposed phospholipids during platelet activation as seen with thrombin formation on exposed PS surface by collagen-GPVI interaction (Siljander *et al*., 2001; Bouchard *et al*., 2001; Monroe and Hoffman, 2002; Heemskerk *et al*., 2002). This interaction can also mediate the release of procoagulant membrane blebs into coagulation system (Heemskerk *et al*., 2002) which is also facilitated by enhanced intracellular Ca^{2+} via platelet activation by ADP, TxA_2 and thrombin (Brass, 2003). Platelet secretions are known to promote procoagulant activity of activated platelets by contributing FV, FVIII and fibrinogen. Activated platelets also support the contact activation pathway (Offermanns, 2006; Caen and Wu, 2010).

1.1.3. Role of coagulation factors

The first account of blood clotting was given by Hippocrates, Aristotle, Celsius and Galen who associated blood coagulability to cooling. However, they did not explain the concept of hemostasis in relation to coagulation. In 1720, French surgeon Jean Louis-Petit associated hemostasis to blood coagulation which was further supported with observations about hemophilia by Friedrich Hopff in 1828 (Owen, 2001). The discovery of various components of coagulation process then has led to the postulation of the classic theory of blood coagulation by Paul Merowitz in 1945. According to his theory, prothrombin was converted to thrombin by thromboplastin in presence of Ca^{2+}. The thrombin formed finally converts fibrinogen to fibrin. The theory also explained the presence of components of coagulation in the circulating blood and the absence of clots in normal blood flow was due to a lack of wettable surface on blood vessels (Beck, 1977). Though a number of coagulation factors (vWF, FV, FVII, FVIII, FIX and FXI) were discovered since then, how these factors sequentially played to convert fibrinogen to fibrin was not clear.

In 1964, two separate models: the Cascade model (Macfarlane, 1964) and Waterfall model (Davie and Ratnoff, 1964) were proposed to explain the step-by-step sequential activation of coagulation factors. Both these models, starting from surface contact to fibrin formation, segregated the coagulation factors into an intrinsic and extrinsic pathway which converges in FXa formation, ultimately leading to fibrin clot via thrombin cleavage of fibrinogen (**Fig. 1.2**). The cascade model also emphasized the importance of phospholipids like phoshatidylserine in coagulation (Zwaal, 1978). However, these models failed to highlight hemostasis *in vivo* accurately as it could not explain why haemophiliacs bleed due to deficiency of FVIII and FIX, though FVIIa/TF would be expected to bypass the role of FVIII and FIX. Similarly, people with FVII deficiency have high tendency of bleeding even when intrinsic pathway functions normally (Hoffman, 2003). Also the deficiency of FXII, prekallikrein and HMWK prolonged clotting time *in vitro* however, failed to affect coagulation *in vivo* (Hoffman, 2003). It was with the finding of FIX activation by FVIIa/TF (Osterud and Rapaport, 1977), FXI activation by thrombin on activated platelets (Gailani and Broze,

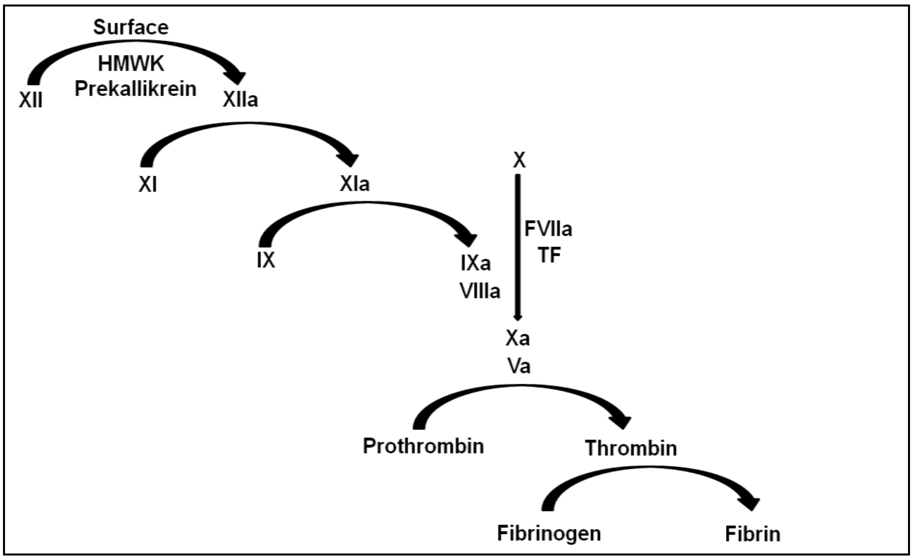


Figure 1.2: The cascade and waterfall hypothesis of blood coagulation. The intrinsic pathway of coagulation is initiated by exposure of the contact factors (FXII, prekallikrein, HMWK) to an appropriate surface with subsequent activation of factor XI by factor XIIa. The extrinsic pathway of coagulation is initiated by exposure of factor VIIa to TF. Both pathways merge at the formation of FXa, finally culminating into fibrin clot formation. Phospholipids and calcium ions in certain reactions are not indicated.

1993; Oliver *et al*., 1999; Baglia and Walsh, 1998), importance of FVIIa/TF in initiating clotting *in vivo* (Nemerson and Esnouf, 1973; Nemerson, 1992) and discovery of TFPI (Broze, 1992) that has led to a revised view of coagulation cascade. These observations have derived the conclusion that intrinsic and extrinsic pathways are interrelated *in vivo* rather than functioning independently.

1.1.3.1. Modern view of coagulation cascade

A cell-based model of blood coagulation has been proposed, where the cellular components provide a phospholipid substratum on which coagulation proteins can exhibit their activity (Monroe *et al*., 1996; Kjalke *et al*., 1998; Allen *et al*., 2000; Hoffman and Monroe, 2001) (**Fig. 1.3**). The coagulation cascade can be divided into three phases: initiation, amplification and propagation.

1.1.3.1.1. Initiation

Coagulation is initiated when flowing blood comes in contact with cell-exposed TF to produce minute amounts of thrombin. There are two pathways by which TF can initiate thrombus formation, via vascular TF and microparticle derived TF. However, their relative roles *in vivo* are unknown (Chou *et al*., 2004). TF, a membrane protein is constitutively expressed on fibroblasts and smooth muscle cells of vessel walls (Drake *et al*., 1989). However, its expression on monocytes and endothelial cells can be induced by chemical stimuli (Semeraro *et al*., 1983; Bevilacqua *et al*., 1984). In the absence of vascular injury TF is masked from FVIIa by endothelial surface to prevent unwanted clot formation (Morrissey et al., 1987), but coagulation is initiated as soon as the endothelium ruptures to expose TF to FVIIa. TF associated with microparticles in the circulating blood are derived from leukocytes, monocytes, endothelial cells, platelets and smooth muscle cells (Giesen *et al*., 1999; Chou *et al*., 2004; Morel *et al*., 2006, Morel *et al*., 2008).

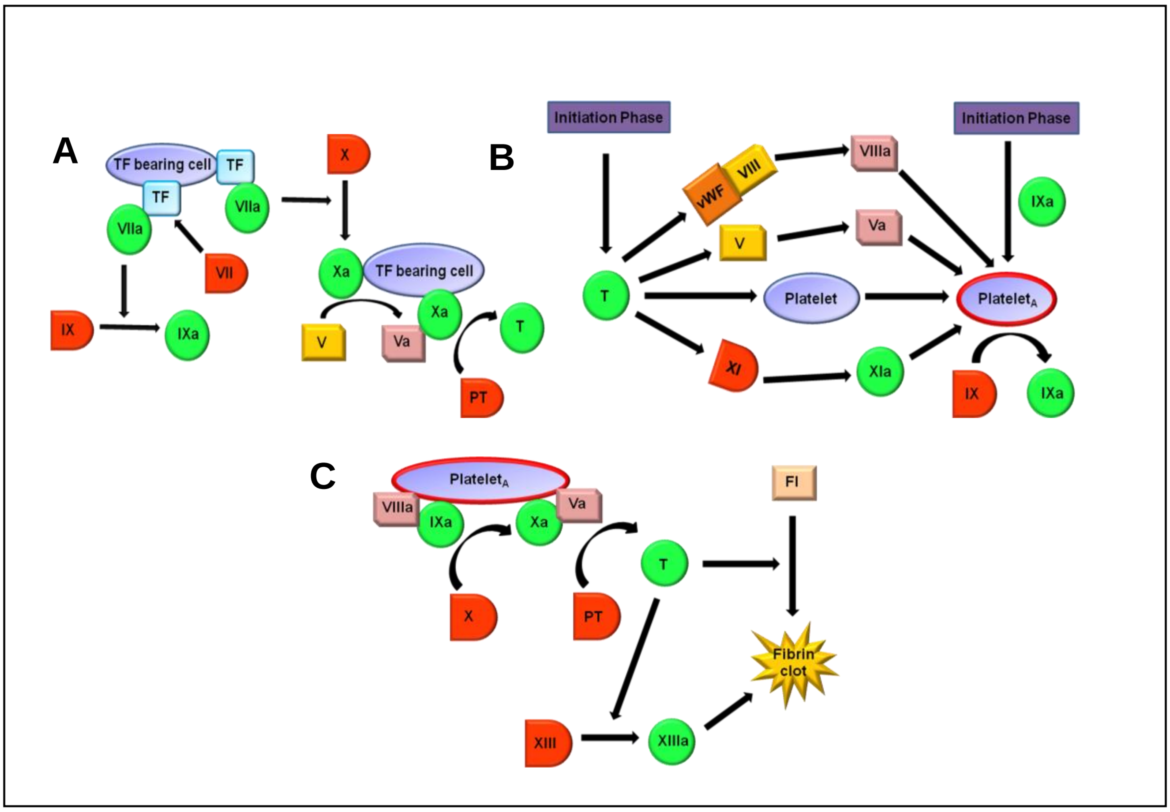


Figure 1.3: Cell based model of coagulation. The recent cell based model of blood coagulation explains the role of both coagulation factors and cells for preventing the blood loss during vascular injury. The different phases involved are A**) Initiation phase** where the encrypted TF on the TF bearing cell gets activated upon vascular rupture and captures circulating FVII and activates it. The activated FVIIa/TF complex (extrinsic tenase complex) generates small amounts of FXa and FIXa. The FXa produced is used to generate thrombin in small amounts that plays a major role in the next phase. **B) Amplification phase** where the small amounts of thrombin generated during initiation performs multiple roles like activating platelets, FVIII, FV and FXI. The activated FXI inturn activates FIX. Activated FIX is also contributed from initiation phase. **C) Propagation phase** is characterized by the thrombin burst that occurs when FX is activated by intrinsic tenase complex (FIXa/FVIIIa). The activated FX complex with activated FV to form the prothrombinase complex (FXa/FVa) converting prothrombin to thrombin. The thrombin produced converts soluble fibrinogen to insoluble fibrin. It also helps to stabilize fibrin clot by activating FXIII. Fibrin clot is also stabilized by FXIIa according to the recent findings.

They exist in an inactive form (lacks cofactor activity) in the blood stream due to yet to be confirmed reasons (Furie and Furie, 2008). It has been proposed that activated platelets and endothelial cells at the site of vascular injury expresses protein disulfide isomerise (PDI) that activates the inactive TF, thus helping in thrombus formation (Reinhardt *et al*., 2008; Cho *et al*., 2008). During platelet activation at the site of vascular injury, P-selectin, a cell adhesion molecule gets expressed on the cell surface. This captures the circulating TF expressed microparticles via the receptor P-selectin glycoprotein ligand 1 (PSGL-1) and thus increases the local concentration of TF (Falati *et al*., 2003; Furie and Furie, 2004) leading to fibrin formation. However, this pathway may operate only in the presence of low concentration of vascular TF (Chou *et al*., 2004).

During vascular injury, plasma FVII binds to TF exposed on cell surface and readily gets activated (Rao and Rapaport, 1988; Rao *et al*., 1996). Apart from the cofactor induced activation, FXa, FIXa and less predominantly, FVIIa present in the plasma can also activate FVII (Masys *et al*., 1982; Wildgoose and Kisiel, 1989; Neuenschwander *et al*., 1993). Recently, a novel protease has been characterized in the human plasma called as factor VII activating protease (FSAP) that can also activate FVII. However, it has been shown to activate pro-urokinase as well (Römisch, 2002; Kanse *et al*., 2008). FSAP circulates in plasma as an inactive zymogen and gets activated by auto-catalytic mechanism that is enhanced by heparin (Muhl *et al*., 2009). The FVIIa/TF complex also known as extrinsic tenase complex can activate both FX and FIX (Fujikawa et al., 1975; Osterud and Rapaport, 1977).The FIXa recruited to the platelet surface mediates the amplification phase (discussed below), while FXa generated on TF bearing cell can activate FV (Monokovic and Tracy, 1990) and complex with it to form the prothrombinase complex (discussed under propagation phase). The prothrombinase complex can generate small amounts of thrombin which can subsequently activate FXI (Walsh, 2001; Matafonov *et al*., 2011) and FVIII during amplification phase (Monroe *et al*., 1996; Camire and Bos, 2009).

1.1.3.1.2. Amplification

The second phase in the coagulation cascade occurs on platelet surface (Hoffman and Monroe, 2001; Furie and Furie, 2008). Thrombin produced in the initiation phase activates both platelets (Hung et al., 1992; Schmidlin and Bunnett, 2001) as well as the coagulation factors FV, FVIII and FXI leading to a thrombin surge as discussed in propagation phase. Apart from plasma, FV is also found in alpha granules of platelets and is secreted on to the surface during platelet activation by thrombin. This FV is activated either by thrombin or FXa (Monokovic and Tracy, 1990; Camire et al., 1998). Recent reports have shown that poly phosphates, a linearized polymer of inorganic phosphate released from platelets can also enhance FV activation (Smith *et al*., 2006). FVIII on the other hand is observed in plasma as an inactive complex with vWF. During initiation, through a proteolytic cleavage by thrombin, FVIII gets activated and detached from vWF. The activated FVIII is then recruited on to the platelet surface (Myles *et al*., 2002; Fay, 2006). FVIII is also shown to be activated by FIXa (Rick, 1982), FXIa (Whelihan *et al*., 2010), FXa (Saenko et al., 1999) and FVIIa/TF (Soeda *et al*., 2010), however their physiological significance is unknown. The current cell-based model of coagulation does not support FXI activation by FXIIa as seen with cascade/waterfall hypothesis (Macfarlane, 1964; Davie and Ratnoff, 1964; Kravtsov *et al*., 2009). This is based on the observation that unlike FXIIa, FXIa deficiency causes bleeding in patients (Seligsohn, 2007; Gailani and Neff, 2009). Also FXII is not involved in hemostasis; however recent findings show its role in thrombosis (Schousboe, 2008; Schmaier, 2008). Thus it is postulated that FXI is activated during haemostasis by proteases rather than by FXIIa. FXI is activated by α-thrombin (Naito and Fujikawa, 1991; Gailani and Broze, 1991), meizothrombin (Matafonov *et al*., 2011) and through auto-activation (Naito and Fujikawa, 1991; Gailani and Broze, 1991) on platelet surface. The platelet surface is important for FXI activation as thrombin fails to activate circulating FXIa (Pedicord *et al*., 2007). The FXIa formed sustains thrombin generation through activation of FIX. Amplification phase there by prepares activated cofactors FV and FVIII for the next step where procoagulant complexes are assembled to produce a thrombin burst.

1.1.3.1.3. Propagation

A large number of platelets are recruited at the site of injury. The intrinsic tenase (FIXa/FVIIIa) and prothrombinase complex (FXa/FVa) are assembled on platelet surface (Hoffman and Monroe, 2001). The local concentration of FIXa is enhanced on the platelet surface by FXIa as well as from TF bearing cell, where it was produced during initiation (Mannhalter *et al*., 1984; Osterud and Rapaport, 1977). The intrinsic tenase activates FX which complexes with FVa to produce thrombin via cleavage of prothrombin (Mann *et al*., 2003). The formation of a stable hemostatic fibrin clot by thrombin marks the final step in blood coagulation. The fibrin clot formed from fibrinogen fills the injured site and prevents blood loss. Fibrinogen is a dimer of a trimer having two Aα, Bβ and γ chains linked by disulfide bonds. The $-NH_2$ termini of all the chains are closer together at the centre constituting the E domain whereas –COOH termini are present in the globular regions at the ends constituting D domain. Thrombin cleaves the Aα and Bβ chains without touching γ chains forming fibrin monomers comprising of two α, β and γ chains. These monomers polymerizes immediately forming fibrin protofibrils in a half-staggered overlap fashion which later associate laterally and longitudinally to form a thick fibrin mesh. During clot formation, thrombin catalyzes the activation of FXIII. FXIIIa, a transglutaminase catalyzes the formation of intermolecular γ-glutamyl-ε -lysine crosslinks in the fibrin network there by stabilizing blood clot. Thrombin also helps in stabilizing the fibrin clot by activating TAFI (Bajzar *et al*., 1995). Apart from its role in isopeptide bond formation, FXIIIa can also cross-link a variety of other cell surface proteins and lipoproteins into the formed fibrin clot helping in wound healing and tissue repair (Nesheim, 2003; La Corte et al., 2011; Komaromi et al., 2011). Recent findings show that FXIIa mediates the regulation of fibrin structure, elasticity and susceptibility to lysis via binding to fibrin(ogen). Upon activation, FXII can yield α-FXIIa (with a heavy and light chain linked by disulfide bond) and β-FXIIa after further proteolysis (with light chain alone). The α-FXIIa can mediate the activation of FIX, finally resulting in fibrin formation via thrombin. α-FXIIa can directly bind to fibrinogen (site unknown) and results in a denser and stable fibrin clot (Konings et al., 2011).

1.1.3.1.4. Fibrinolytic system

Once a fibrin clot is formed over the site of injury, the clotting process must be restricted spatially to avoid thrombotic occlusion in adjacent normal areas of the vasculature. This is aided by a fibrinolytic system that can mediate clot dissolution once the wound gets healed. The system is composed of plasminogen, several activators and inhibitors. Plasmin, the key fibrinolytic enzyme is formed by the proteolytic activation of plasminogen. Fibrin has binding sites for both plasminogen and t-PA where proteolytic reaction occurs (Robbins et al., 1967; Castellino, 1984). t-PA release at the site of injury from endothelial cells is stimulated by fibrin, thrombin and by blood vessel occlusion (Amery *et al*., 1962; Levin *et al*., 1984; Kaplan *et al*., 1989). t-PA by itself is a poor activator of plasminogen until and unless it gets bound to fibrin (Camiolo et al., 1971). u-PA also a plasminogen activator plays significant role in tissue degradation that facilitates cell migration during wound healing and tissue repair (Dano *et al*., 1985). FXIIa, FXIa and kallikrein can also activate plasminogen, however their physiological relevance is still under conformation (Colman *et al*., 1975; Mandle and Kaplan, 1979; Colman, 1969). Plasmin cleaves fibrin sequentially to release the degradation products. Lys/Arg residues on the α and β chains are prone for proteolysis (Dobrovolsky and Titaeva, 2002). The plasmin activity is regulated through the effect of α2-antiplasmin and α2-macroglobulin with α2-antiplasmin having a major role (Wiman and Collen, 1978; Aoki and Harpel, 1984). AT, α1-antitrypsin and C1 inhibitor have also shown to inhibit plasmin in *in vitro* studies (Aoki and Harpel, 1984). Inhibitors regulating the activity of plasminogen activators involve PAI-1, PAI-2, PAI-3 and the protease nexin of which PA-1 has significant role in inhibiting t-PA (Baker *et al*., 1980; Chandler *et al*., 1990) and FVIIa/TF (Sen *et al*., 2011). Recently thrombin-activatable fibrinolysis inhibitor (TAFI) or plasma carboxypeptidase B has been characterized. TAFI is activated by T-TM complex (Bajzar *et al*., 1995). TAFI removes the carboxy terminal end of fibrin there by making fibrin inaccessible for plasminogen activation via t-PA (Wang *et al*., 1998). Activation of TAFI is controlled by APC (Bajzar *et al*., 1996). Recently, it was shown that poly phosphates can enhance the activity of TAFI there by delaying the clot lysis (Smith *et al*., 2006).

1.2. Regulation of hemostasis

Uncontrolled coagulation can lead to thromboembolic disorders after receiving a modest procoagulant stimulus (Hoffman, 2003). This explains the significance of an *in vivo* anticoagulation system for tightly regulating the hemostatic pathway (**Fig. 1.4**).

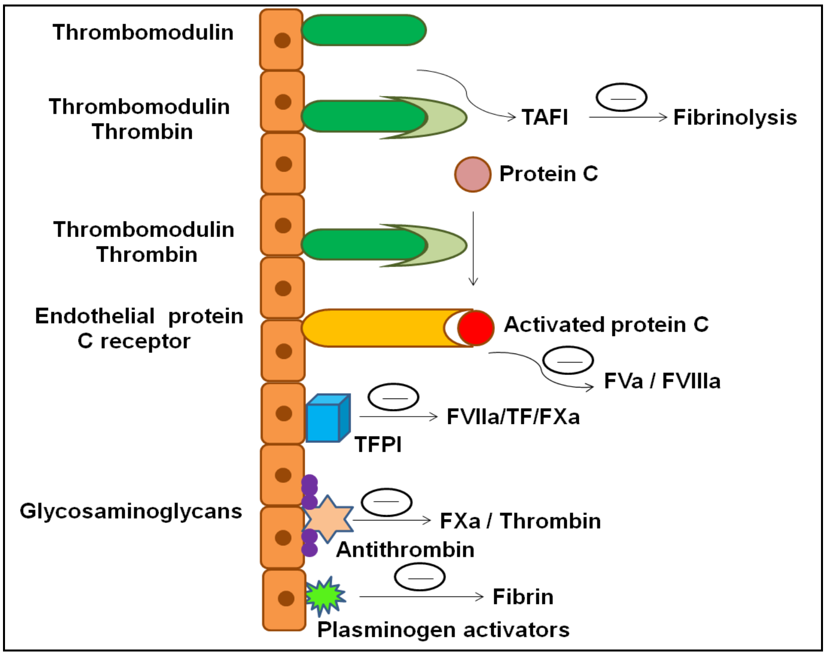


Figure 1.4: Physiological regulation of hemostasis. Thrombin/ thrombomodulin complex plays a major role in activating TAFI and protein C. Activated protein C and TAFI regulates coagulation/fibrinolysis. TFPI is the endothelial cell–associated inhibitor of tissue factor pathway where it inhibits both FXa and FVIIa to form a quaternary complex. Antithrombin binds to endothelial glycosaminoglycans, which causes a more efficient inhibition of activated coagulation proteases like FXa and thrombin. Release of plasminogen activators from endothelial cells will affect local fibrinolysis. Minus sign indicate inhibitory effects. [*Adapted and modified from Levi et al., Circulation 2004, 109:2698-2704*]

1.2.1. Tissue factor pathway inhibitor

TFPI is an endogenous serine protease inhibitor for the tissue factor pathway. A major proportion of TFPI (75-90 %) is localized in endothelial cells where as the remaining circulates bound to plasma lipoproteins (Gentry *et al*., 2008). Because of its association with lipoproteins, TFPI was formerly known as lipoprotein-associated coagulation inhibitor or LACI (Girard *et al*., 1989). TFPI is also found to be present in platelets at lower concentrations and is secreted out upon activation by thrombin during vascular injury (Novotny *et al*., 1988; Mast *et al*., 2000). TFPI, a single-chain 32-kDa glycoprotein consisting of three multivalent Kunitz type domains (K1, K2 and K3) is characterized by an acidic N-terminus and a basic C-terminal tail (Broze, 1992). It is a bi-directional inhibitor in that it inhibits both FXa and FVIIa/TF (Broze *et al*., 1990). The mechanism of inhibition is explained as follows (**Fig. 1.5**). TFPI with its K2 domain inhibits the active site of FXa to form a 1:1 complex. This is followed by the recruitment of K1 domain to the active site of FVIIa/TF forming a stable quaternary complex (Salemink et al., 1999). The function of the K3 domain however has remained an enigma, but is suggested to localize TFPI to cell surface. K3 domain as well as basic C-terminal tail are also believed to interact with lipoproteins, glycosaminoglycans and heparins (Piro and Broze, 2004; Lwaleed and Bass, 2006) and are known to have a role in anticoagulation by TFPI (Nordfang *et al*., 1991; Wun, 1992; Valentin *et al*., 1993; Enjyoji *et al*., 1995). During the TF mediated coagulation, TFPI functions as a feedback regulator of FVIIa/TF. The production of activated FIX and FX by FVIIa/TF makes TFPI to get manifested and prevents further generation of FIXa and FXa. The FXa formed can produce small amounts of thrombin that can mediate its role in amplification phase as well as platelet activation.

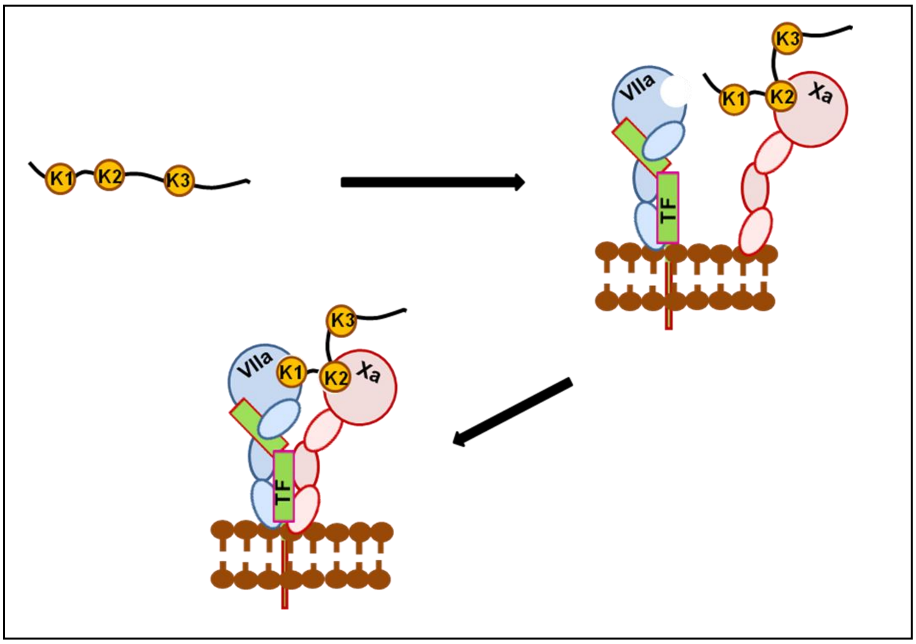


Figure 1.5: Tissue factor pathway inhibitor. The tissue factor pathway inhibitor (TFPI) is a three Kunitz domain (represented as K) endogenous inhibitor. It inhibits both FXa and FVIIa to form a quaternary complex. With its K2 domain, TFPI first binds to the active site of FXa and later recruits K1domain into the active site of FVIIa. However, the K3 domain and the C-terminal tail are suggested to interact with proteoglycans.

Further production of FXa is achieved through intrinsic tenase complex, when FIXa formed is recruited to the platelet surface (Broze, 1995; Kalafatis *et al*., 1997).

1.2.2. Serine protease inhibitors (serpins)

A number of serpins has been characterized that can regulate the hemostatic system (Silverman *et al*., 2001). This includes antithrombin (AT also known as antithrombin III/ATIII) along with its cofactor heparin inhibiting the activity of several coagulation proteases (FXa, FIXa, FXIa, FXIIa, thrombin, FVIIa, plasmin and kallikrein) (De Agostini *et al*., 1990; Gettins and Olson, 2009); Heparin cofactor II (HCII) specifically inhibiting thrombin via binding to thrombin exosite 1 with its acidic N-terminal tail (Colwell *et al*., 1999; Tollefsen, 2004; O'Keeffe *et al*., 2004; Tollefsen, 2007); α_1-antitrypsin and α_1-protease inhibitor inhibiting thrombin, FXIa, FXa, APC and plasmin (Scott *et al*., 1982; Long *et al*., 1984); α_2-Macroglobulin inhibiting FXa (Fuchs and Pizzo, 1983); C1 inhibitor inhibiting FXIa, FXIIa and kallikrein (Harpel *et al*., 1985; Pixley *et al*., 1985); protein C inhibitor (PCI) also known as plasminogen activator inhibitor-3 inhibiting thrombin, FXa, FXIa, APC, tissue-type plasminogen activator and urokinase-type plasminogen activator (Meijers *et al*., 2002; Pike *et al*., 2005). Though PCI inhibits APC in presence of heparin, it's physiologically relevant role is in inhibiting the thrombin-thrombomodulin complex (Yang *et al*., 2003); protein Z-dependent protease inhibitor (ZPI), a cofactor dependent serpin inhibiting FXa and FIXa (Huang et al., 2011; Vasse, 2011); protease nexin-1 (PN-1) inhibiting thrombin in the presence or absence of heparin (Evans *et al*., 1991). It can also bind to TM and mediate the inhibition of thrombin catalytic activity as well as thrombin induced fibrin clot formation (Bouton *et al*., 2007).

1.2.3. Protein C system

The thrombin generation during the coagulation cascade is regulated via keeping a check on FVIIIa and FVa by protein C anticoagulant system as they are cofactors for intrinsic tenase and prothrombinase complex respectively. The system involves thrombin-thrombomodulin (T-TM) complex, protein C, protein S and endothelial cell protein C receptor (EPCR) (Esmon, 2001; Esmon, 2003). Protein C, a vitamin K-dependent serine protease circulates as a zymogen in the plasma. It is composed of two chains: a heavy chain and light chain linked by disulfide bridges. The γ-carboxyglutamic acid (Gla) residues in the light chain help protein C to interact with endothelial receptor EPCR, which is essential for its activation. The Gla residues also help the activated protein C (APC) to interact with negatively charged phospholipids during its inhibition of FVIIIa and FVa (Dahlbäck and Villoutreix, 2005; Wildhagen et al., 2011). The T-TM-EPCR complex removes the activation peptide from protein C's heavy chain there by converting the serine protease domain to an active conformation. APC is highly specific in inactivating FVa and FVIIIa. Both the cofactors share similar domain arrangement of A1–A2–A3–C1–C2, and gets activated upon removal of the carbohydrate rich B domain during cleavage by thrombin or FXa. Three APC cleavage sites have been identified with Arg306, Arg506, Arg679 on FVa and Arg336, Arg562, Arg740 on FVIIIa (Fay *et al*., 1991; Hockin *et al*., 1999). Cleavage at Arg506 results in partial loss of FVa activity compared to that of Arg306, upon cleavage of which results in severe loss of FVa activity. The total loss of activity is however accounted to the removal of A2 domain after cleavage at Arg306 (Mann *et al*., 1997). In the case of FVIIIa the inactivation by APC requires protein S and FV as cofactors (Shen and Dahlbäck, 1994; Shen *et al*., 1997; Nicolaes and Dahlback, 2002). However, the molecular mechanism by which APC along with the two cofactors inactivate FVIIIa in the tenase complex is not understood completely.

1.3. Thrombotic disorders

Blood coagulation, a hemostatic response to vascular injuries is a highly synchronized and tightly regulated cascade that involves sequential activation of various blood coagulation factors ultimately leading to the formation of fibrin clot. Any imbalance in its regulation can lead to either excessive bleeding through a cut (hemorrhage) or unwanted clot inside a blood vessel (thrombosis) (Mann *et al*., 2003; Furie and Furie, 2008). Thrombosis via vascular occlusion is the major cause of morbidity and mortality in western populations (Gross and Weitz, 2008; Streiff *et al*., 2011). Thrombosis can be classified into venous and arterial with venous thrombosis triggered by changes in coagulation factors and their inhibitors resulting in restricted blood flow. While arterial thrombosis is due to rupture of vascular endothelium and increased platelet activity. However, these are not mutually exclusive in that increased fibrinogen in the blood and platelet activity can be associated with arterial and venous thrombosis respectively. Sometimes the thrombus formed can break off from the site of formation into the circulatory system obstructing the blood flow and are often called as an embolus/moving clot (Phillips *et al*., 2001). This results in thromboembolic disorders characterized as deep venous thrombosis (DVT), pulmonary embolism (PE), myocardial infarction (MI) and stroke (Kuntz *et al*., 2006; Furie and Furie, 2008). It has been estimated that out of the 16.7 million deaths in 2002, 7.2 and 5.5 million deaths were due to MI and stroke respectively, making them globally the lead killers (Anderson and Chu, 2007). On the other hand, PE and DVT together known as venous thromboembolism (VTE) has an incidence of 1-3 out of every 1000 of the general population per year and constitutes the third most common cardiovascular disorder (Kearon, 2001; Naess et al., 2007).

1.4. Antithrombotic agents

Arterial (MI and stroke) and venous thrombotic (DVT and PE) disorders are the leading causes of debilitation and death all over the world. Thus there is the need for effective antithrombotic therapy for the prevention and treatment of these disorders. Over the years, many antiplatelets, anticoagulants and thrombolytics have been developed and commercialized. By analyzing the nature of thrombus formed, the antithrombotic therapy can be instituted. Incidence of arterial and venous thrombosis, mainly thrombus extension, recurrence and embolic complications can be treated using antiplatelets and anticoagulants respectively. However, these agents cannot dissolve the existing clot in the system for which thrombolytics are used (Sikka and Bindra, 2010). A list of antithrombotics, currently in clinical trials and therapy has been discussed:

1.4.1. Antiplatelets

The conversion of coronary-artery plaque from stable to unstable with the subsequent plaque rupture leads to acute coronary syndrome (ACS), ranging from unstable angina to acute myocardial infarction (AMI). This plaque rupture results in the exposure of the thrombogenic surface, leading to platelet plug formation and finally arterial thromboembolic disorders (Fitzgerald and Shipp, 1992). Currently used antiplatelet agents include aspirin, P2Y12 inhibitors (ticlopidine and clopidogrel) and glycoprotein IIb/IIIa inhibitors. Aspirin irreversibly acetylate cyclooxygenase-1 thereby inhibiting arachidonate induced platelet activation and aggregation and greatly reduce TxA_2 synthesis (Patrono *et al*., 2005). Aspirin considered as a gold-standard in antiplatelet therapy due to its cost effectiveness and low risk factors, however has exhibited many pitfalls. Aspirin has shown to be ineffective to many patients during treatment, especially in inhibiting TxA_2 production (Moser and Bode, 2010; Lopes, 2011). Strategies were devised for either inhibiting thromboxane synthetase or blocking thromboxane receptors (Gaussem *et al*., 2005). Targeting thromboxane synthetase (final step in synthesis of TxA_2) has got limited efficacy, as its inhibition will result in endoperoxide precursors that can act as platelet agonists (Fitzgerald *et al*., 1988). These inhibitors can also be used in association with receptor antagonists to achieve maximum efficacy. However, these two strategies have not performed well in humans as compared to aspirin (Ghuysen *et al*., 2005). A thromboxane antagonist, S18886 was found to be orally active in preventing platelet aggregation and has been pushed for large phase II trials (Gaussem *et al*., 2005; Weitz *et al*., 2008).

The $P2Y_{12}$ receptor that mediates ADP-induced platelet activation and aggregation has been targeted for antiplatelet therapy (Kunapuli *et al*., 2003; Kim and Kunapuli, 2011). Thioenopyridines like ticlopidine and clopidogrel were used as receptor antagonists (Becker *et al*., 2008; Cairns and Eikelboom, 2008; Mega *et al*., 2009).

Human platelets express both PAR-1 and PAR-4 receptors which when activated by thrombin results in platelet activation and aggregation. However, affinity of thrombin for PAR-1 is higher than that for PAR-4. Even PAR-1 is activated with lower amounts of thrombin as compared to that of PAR-4 activation (Bahou, 2003). This has made PAR-1 a better target in antiplatelet therapy. The two PAR-1 antagonists undergoing phase II evaluation are SCH-530348 and E5555. Both these antagonists exhibited mild bleeding and better protection from adverse cardiovascular events when administered orally to healthy volunteers (Seiler and Bernatowicz, 2003; Weitz *et al*., 2008; Moser and Bode, 2010).

Platelet aggregation, independent of agonists like ADP and TxA2 are mediated through the interaction of GP IIb/IIIa receptor on adjacent platelets to a molecule of fibrinogen representing the final step in the formation of platelet-rich thrombi (Jennings, 2009). By preventing this interaction, platelet aggregation can be checked. The inhibitors falling in this category include eptifibatide, tirofiban, and abciximab with eptifibatide and tirofiban as competitive inhibitors and abciximab permanently blocking the receptor (Hirsh and Weitz, 1999; Van de Werf, 2009). cAMP promotes suppression of platelet activation and subsequent aggregation. Drugs like dipyridamole and cilostazol inhibits phosphodiesterase, thereby increasing the level of cAMP. Since the risk of bleeding is low in this category of antiplatelets, they have been extensively evaluated in human trials for their use in stroke (Gross and Weitz, 2009).

1.4.2. Anticoagulants

Anticoagulants have been used for more than 70 years for the treatment of deadly blood clots (**Fig. 1.6**). It was estimated that approximately 0.7% of the western population receives the oral anticoagulation therapy with heparin and vitamin K antagonists as the mainstays. (Gustaffson *et al*., 2004). Both these anticoagulants are highly effective when administered at proper dosages and relatively safe when monitored efficiently. Thus they have become the cornerstones in anticoagulation therapy. However, their clinical use is limited due to number of factors.

1.4.2.1. Heparin

Unfractionated heparin (UFH) is a heterogeneous mixture of polysaccharide chains of varying molecular weight. Chemical or enzymatic cleavage of UFH yields heparin chains of low molecular weight and hence the name low molecular weight heparin /LMWH. The anticoagulant activity of UFH and LMWH is mediated through its interaction with the physiological anticoagulant AT via a distinct pentasaccharide sequence (Cosmi and Palareti, 2011). Thus heparin via AT can inhibit multiple targets like FXa, FIXa, FXIa and thrombin (explained in the section 1.2.2). The use of UFH has been greatly withdrawn due to the complications it caused in patients (Laux *et al*., 2009). UFH exhibit tighter binding to plasma proteins and cells as compared to LMWH, resulting in variable pharmacokinetics. In addition, UFH also induces a high risk of heparin-induced thrombocytopenia (HIT) when compared to LMWH. HIT is an immune response generated when heparin interacts with the platelet factor-4. This interaction can also neutralize the heparins anticoagulant activity (Warkentin *et al*., 2008). Heparin therapy also requires intense coagulation monitoring and cases of osteoporosis has also been reported (Hawkins, 2004).

1.4.2.2. Vitamin K antagonists

Coumarin derivatives like warfarin are used as vitamin K antagonists (VKAs) that exhibit their anticoagulant activity by targeting the hepatic synthesis of vitamin K dependent coagulation factors (Haas and Schellong, 2007). They does so by interfering with cyclic inter-conversion of vitamin K and its 2, 3-epoxide. Vitamin K functions as a cofactor for the γ-carboxylation reaction of clotting factors in their N-terminal glutamic acid residues. The γ-carboxyglutamic acid (Gla) residues help these proteins to bind to the phospholipid membranes and enhance coagulation (O'Reilly, 1976; Vermeer, 1984; Furie *et al*., 1999; Hirsh *et al*., 2001). However, the anticoagulant response of warfarin is challenged by many factors. In addition to their anticoagulant effect, the vitamin K antagonists exert procoagulant effect as well since they inhibit carboxylation of the regulatory anticoagulant proteins C and S.

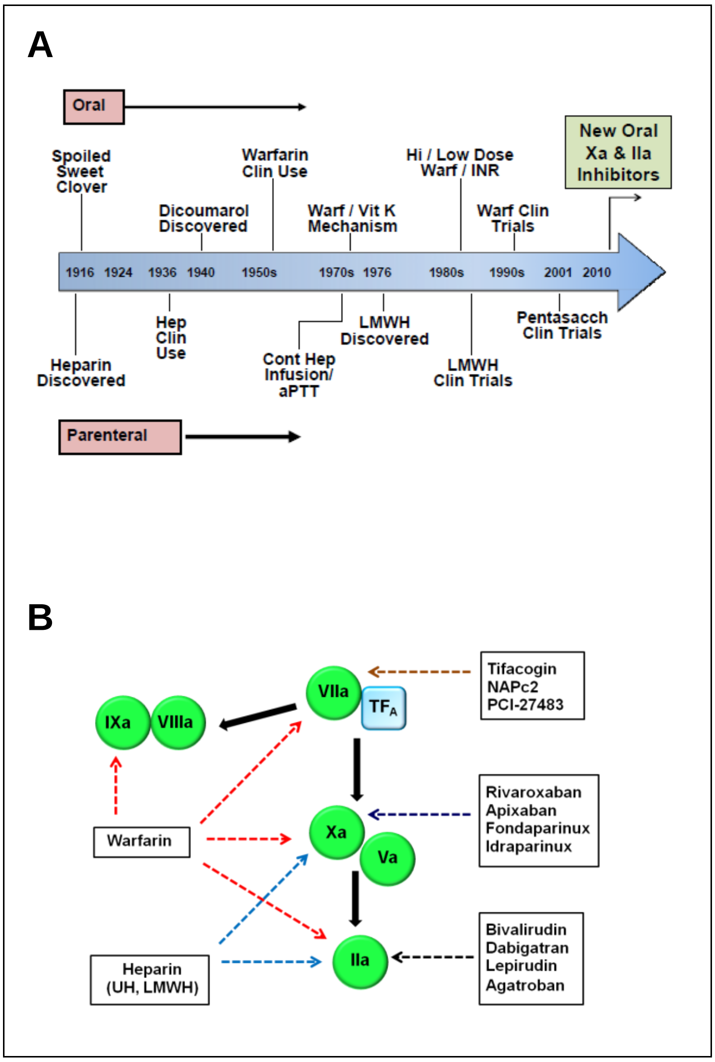


Figure 1.6: Cornerstones of anticoagulation therapy. **A)** The history of anticoagulants used for the treatment of arterial and venous thromboembolic disorders. Heparin and warfarin were the mainstays of coagulation therapy for more than 70 years. However, they have been replaced recently by direct thrombin inhibitors and FXa inhibitors to overcome the disadvantages posed by heparin and warfarin. **B**) The various anticoagulants in clinical use (or trials) targeting the different stages in the coagulation cascade. Note that heparin and warfarin can target different coagulation proteases. Due to their limitations, inhibitors targeting directly the extrinsic tenase complex, FXa or thrombin have been sought [*Fig. 1.6a is adapted from Ansell J. 'New oral anticoagulants: The future is here'*]

Warfarin's use is limited due to its narrow therapeutic index. The anticoagulant response of warfarin is analysed by its effect on international normalized range (INR) that is derived from patient's plasma prothrombin time. Maintaining the INR value of within the recommended therapeutic range requires frequent laboratory monitoring and appropriate dosage adjustments. Warfarin is known to be involved in drug and food interactions that affect its anticoagulant activity. Its drug interaction can cause an either a decrease or increase of anticoagulant activity. Certain herbal medicines are known to enhance warfarin effect thereby causing excessive bleeding. Warfarin's effect is also limited by dietary consumption of vegetables rich in vitamin supplements. Apart from these, several disease states are also known to alter warfarin's effects (Hirsh *et al*., 2001; Hawkins, 2004; Nutescu *et al*., 2006, Tsiara *et al*., 2011).

1.4.2.3. FXa inhibitors

FXa represents the common point in the coagulation cascade and inhibiting FXa can put a check on the thrombin burst. FXa inhibitors can be either direct (examples: rivaroxaban, apixaban etc.) or indirect (examples: fondaparinux and idraparinux). Rivaroxaban (Xarelto®; Bayer Schering Pharma) is a orally administered small-molecule direct Factor Xa inhibitor that selectively and reversibly inhibits both free and clot-associated Factor Xa activity, as well as prothrombinase activity (Laux *et al*., 2009). With its fast on-set of action, high oral bioavailability, fast clearance from the physiological system and low incidence of food and drug interactions, it has been approved for the treatment of VTE in several countries (Galanis *et al*., 2011). Otamixaban, a non-competitve inhibitor of FXa was subjected for phase IIa trials in patients with coronary artery disease and was found to exhibit sustained anti-FXa activity with no incidence of bleeding. It was also compared with heparin in phase II trials where patients having PCI demonstrated reduction in Xa activity. Razaxaban, an oral FXa inhibitor underwent phase II trials for thromboprophylaxis after knee arthroplasty. However, it exhibited increased incidence of bleeding in patients given with higher doses and further development was stopped (Weitz *et al*., 2008). Apixaban, a variant of razaxaban exhibited greater bioavailability and pharmacokinetic properties. Like rivaroxaban, it is also a small molecule reversible inhibitor of FXa and prothrombinase activity. It is currently under phase III trials against VTE after orthopaedic surgery (Laux *et al*., 2009; Weitz, 2010). Other direct inhibitors of FXa include betrixaban and edoxaban, both of which are undergoing phase II trials (Harenberg and Wehling, 2011; Tsiara *et al*., 2011). Fondaparinux and idraparinux are indirect FXa inhibitors administered sub-cutaneously that mediate FXa inhibition through AT. Fondaparinux is a synthetic analogue of AT binding pentasaccharide found in heparin or LMWH where as idraparinux is a polymethylated derivative of the former (Harenberg, 2008). Fondaparinux is the most successful FXa inhibitor and has been approved recently for the treatment of thromboprophylaxis and VTE. The recent trials have confirmed its efficacy and safety in acute coronary syndrome. It does not induce HIT during treatment. Its high bioavailability, pharmacokinetic properties and non-requirement of timely coagulation monitoring make it advantageous (Haas and Schellong, 2007). Idraparinux on the other hand exhibits higher AT activity and a prolonged plasma half-life compared to fondaparinux (Alban, 2005; Kanagasabapathy et al., 2011). It was highly efficacious in patients with DVT compared to that with patients having PE. Idraparinux was also associated with higher risk of bleeding though it was effective in preventing recurrent VTE. Idrabiotaparinux, a biotinylated version of idraparinux has subsequently been developed that has a specific neutralizing agent. However, there were no further trials planned with either idraparinux or idrabiotaparinux (Levy et al., 2010).

1.4.2.4. Direct thrombin inhibitors

Thrombin plays a critical role in blood clotting in that it converts soluble fibrinogen to insoluble fibrin. It also exerts other thrombogenic properties like activating platelets, FXIII, FV, FXI and FVIII. Activated FXIII is required for fibrin clot stabilization. This explains the importance of thrombin as a target for anticoagulation therapy. Direct thrombin inhibitors (DTIs) inhibit thrombus formation and produce a more predictable anticoagulant effect. When compared to heparin, they neither bind to plasma proteins nor interact with platelet factor-4 (Harenberg, 2008). Some of these that are currently in the market are hirudin, bivalirudin, argatroban and dabigatran etexilate. Hirudin (Refludan®; Bayer HealthCare Pharmaceuticals) is a 65-residue protein isolated from the medicinal leech, *Hirudo medicinalis*. The recombinant hirudin is approved for the treatment in patients with HIT and for thromboprophylaxis after an orthopaedic surgery. However, its usage was limited due to risk of bleeding, lack of antidote, immunogenicity and pharmacokinetics that depend on renal function (Greinacher and Warkentin, 2008; Laux *et al*., 2009). Bivalirudin (Angiox®; The Medicines Company), a 20-residue peptide was designed based on hirudin structure. It was basically assigned for percutaneous coronary intervention (PCI). Compared to hirudin, bivalirudin was easily cleared off from the physiological system and exhibited negligible immunogenicity. It has been clinically accepted for both arterial and venous thrombotic disorders (Warkentin et al., 2008). Pegmusirudin, a chemically modified hirudin derivative prolongs the half-life of hirudin even in patients with renal disease. Like hirudin, pegmusirudin is cleared off via urinary system. Thus in phase II studies, it has been evaluated for its anticoagulant activity in patients with end stage renal deficiency and as a strategy to reduce the risk of vascular graft occlusion (Avgerinos *et al*., 2001; Weitz *et al*., 2008). Argatroban (Argatra®; Mitsubishi Pharma), a small molecule inhibitor of thrombin has been approved for the treatment of patients with HIT (Yeh and Jang, 2006; Hirsh *et al*., 2008). Flovagatran, a synthetic small molecule reversibly inhibiting thrombin has been found to show predictable pharmacokinetics. Since its clearance from the system is via an extra-renal mechanism, it has been investigated in phase II trials as an alternative to heparin in patients undergoing hemodialysis with renal disease who have antibodies to heparin/platelet factor-4 complex (Weitz et al., 2008, Gomez-Outes *et al*., 2011). However, these DTIs were administered intravenous which have limited their long term use. This has led to the development of oral DTIs such as Ximelagatran (Exanta®; AstraZeneca) and dabigatran etexilate (Pradaxa®; Boehringer Ingelheim). Ximelagatran, a prodrug of thrombin inhibitor melagatran was evaluated for the treatment of VTE after orthopaedic surgery. It had undergone a phase III trial for stroke prevention in patients with atrial fibrillation. However, due to reports of hepatotoxicity and other adverse cardiovascular complications, Ximelagatran was withdrawn from further trials and development (Weitz *et al*., 2008). Dabigatran etexilate, the recently launched DTI in the market has been accepted by the European Commission for the treatment of VTE in patients who have undergone total hip or knee replacement surgery. Despite of its low bioavailability (5%), dabigatran etexilate does not require regular coagulation monitoring. The anticoagulant effect of dabigatran etexilate is associated with low bleeding risk without major problems of interactions with other drugs (Harenberg, 2008; Weitz *et al*., 2008; Gomez-Moreno *et al*., 2010).

1.4.2.5. Extrinsic complex inhibitors

Drugs that target extrinsic tenase complex (FVIIa/TF) prevent the initiation of coagulation and have reached the phase II and III trials. These include tifacogin, recombinant NAPc2 and active site inhibited FVIIa (factor VIIai). Tifacogin, a recombinant form of TFPI expressed in *Saccharomyces cerevisae* has been used in patients with sepsis and it is undergoing phase III trials. Initial clinical trials with NAPc2 on the other hand focused on venous thromboprophylaxis. In the phase II trials NAPc2 exhibited high efficacy and minimal bleeding when compared to historical anticoagulants. Currently, in phase II trials NAPc2's role on arterial thrombosis is being evaluated. FVIIai function by competing with normal FVIIa in binding to TF and initiating coagulation. However, in human trials FVIIai exhibited poor performance towards arterial thrombosis and its further development was suspended (Weitz et al., 2008). PCI-27483 is a small molecule inhibitor of FVIIa/TF complex reported to exhibit antitumor and antithrombotic activities in preclinical trials. Currently, its phase Ib/II trials are carried out in patients with pancreatic cancer.

1.4.2.6. Other new anticoagulants

A human monoclonal antibody (TB-402) partially inhibiting FVIIIa was analyzed in phase II trials upon IV injection in patients with VTE undergoing total knee replacement surgery. TB-402 exhibited a high efficacy and reduced risk of bleeding at lower doses. Phase III trials are yet to be conducted with TB-402 (Gomez-Outes *et al*., 2011).

Inhibitor targeting FIXa (RB006) has been developed as RNA aptamer, that when administered parenterally binds FIXa with high affinity. Phase I trials has shown a prolongation of plasma APTT where it exhibited a fast neutralization in presence of complementary oligonucleotides. Currently, its role in cardiopulmonary bypass surgery has been studied. TTP889 is an orally administered FIXa inhibitor that failed to pass the phase II trials (Rusconi *et al*., 2002; Eriksson and Quinlan, 2006, Weitz *et al*., 2008).

Drotrecogin alfa (activated), a recombinant APC has been initially licensed in 2001 to be used as a FVa inhibitor in patients with severe sepsis and high risk of death. It indeed reduced the mortality rate though incidence of high bleeding was reported. However, later clinical trials in patients with severe sepsis and low risk of death have proven it to have low efficacy and higher risk of bleeding, thus limiting its use (Bernard *et al*., 2001; Abraham *et al*., 2005; Boyle *et al*., 2011). ART-123, a recombinant analogue of TM can bind to thrombin there by activating protein C. During phase II trials in patients with hip arthroplasty, it showed high efficacy against VTE but associated with major bleeding. It also exhibited high bioavailability when administered subcutaneously (Kearson *et al*., 2005; Gomez-Outes *et al*., 2011).

1.4.3. Fibrinolytics

Although antiplatelets and anticoagulants are meant for preventing the recurrent incidence of thromboembolism, they have a negligible role in removing already formed clots in the arteries or veins. Clot removal is thus mediated through either indirect or direct fibrinolytic/thrombolytic agents.

Indirect fibrinolytic agents are fibrinolysis enhancers. PAI-1 is the physiological inhibitor for t-PA that converts plasminogen to plasmin and thereby increasing the fibrinolysis. Lipid lowering drugs has been reported to reduce the expression of PAI-1 along with other proteins which makes them non-ideal (Brown *et al*., 1995). Peptides and small molecules that can block PAI-1 activity have been devised; however they require in vivo studies (Eitzman *et al*., 1995; Friederich *et al*., 1997). Inhibitors characterized from potato as well as small molecules were found to inhibit TAFI but were limited with the fact that low doses of the inhibitor enhanced TAFI activity (Nagashima et al., 2000; Schneider and Nesheim, 2003; Guimaraes et al., 2006). Inhibition of FXIIIa can destabilize the fibrin clot that can be prone to lysis later. FXIIIa inhibitors involve tridegin and destabilase enzyme from leech (Baskova and Nikonov, 1991; Seale *et al*., 1997; Finney *et al*., 1997). However, these inhibitors haven't reached human trials. Recently, variants of t-PA have been licensed to be used as fibrinolytic agents. These include reteplase and tenecteplase (Noble *et al*., 1996; Dunn and Goa, 2001; Llevadot *et al*., 2001). Desmoteplase is a plasminogen activator analogue from the vampire bat *Desmodus rotundus*. Like t-PA, desmoteplase binds to fibrin and activate plasminogen to plasmin leading to fibrin degradation (Stewart *et al*., 1998). Currently, it is under phase III trials with patients suffering from acute ischemic stroke though it has been reported to have exhibited low efficacy (Weitz *et al*., 2008).

Direct fibrinolytic agents directly lyse the clot and include Alfimeprase. It is derived from fibrolase, a zinc-metalloprotease isolated from the venom of *Agkistrodon contortrix contortrix*. It directly degrades fibrin and fibrinogen. Since alfimeprase is inhibited physiologically by circulating α_2-macroglobulin, it has to be administered directly to the thrombus site in order to prevent its systemic limitations. Clinical trials with alfimeprase have been carried out using catheter-directed lysis of peripheral arterial occlusions. Phase III trials have been suspended temporarily because of its inadequacy in meeting the standards (Moll *et al*., 2006; Weitz *et al*., 2008).

1.5. Choice of the antithrombotic agent

As the famous saying 'Prevention is better than cure', use of antiplatelets and anticoagulants are highly advantageous in treating thromboembolic disorders when compared to fibrinolytics as the latter is used to dissolve the existing thombi. Antiplatelets are effective in initial and long term management of ACS as well as coronary, cerebral and peripheral artery disease (PAD) respectively. They do prevent thromboembolic events in atrial fibrillation (AF) and VTE but when compared to anticoagulants are less effective. On the contrary, anticoagulants are effective for initial and long term management of both arterial (ACS and stroke) and venous thrombotic disorders (DVT and PE). Though they exhibit high risks of bleeding; by optimizing dosage regimens and developing new anticoagulants, the bleeding problems can be counteracted (Eikelboom and Hirsch, 2007; Vedovati *et al*., 2010). Thus anticoagulants are pivotal for the prevention and treatment of thrombotic disorders.

1.6. Ideal target for anticoagulation

Traditional anticoagulants like warfarin and heparins have many pitfalls in that it targets many coagulation factors at a time, there by affecting the normal hemostasis. Strategy has been adopted to design new anticoagulants that can target a specific coagulation factor or step in the cascade without hindering hemostasis. Currently, anticoagulants are designed targeting FXa and thrombin. Thrombin represents the final stage in coagulation where it converts soluble fibrinogen to insoluble fibrin. It also helps in clot stabilization via activating FXIII and TAFI. It also has role in activating platelets along with FV, FXI and FVIII. Thus inhibiting the central role of thrombin can ensure proper anticoagulation, however can exhibit pleiotropic effects (Gurm and Bhatt, 2005; Bauer, 2006). FXa occupies a critical juncture in the coagulation cascade and is responsible for the thrombin burst. Thus a check on FXa can inhibit thrombin generation while allowing existing thrombin to continue its vital functions in normal hemostasis. FXa inhibitors also exhibit less bleeding compared to

DTIs (Bauer, 2006; Borensztajn *et al*., 2008; Borensztajn and Spek, 2011). It was hypothesized that selective inhibition of coagulation factors located further upstream (FVIIa/TF) in the cascade might be safer with respect to bleeding risk. Also, by not inhibiting thrombin activity directly can ensure a proper hemostasis. Furthermore, it was hypothesized that, as the amount of coagulation factors is amplified at each step of coagulation, targeting the upstream especially extrinsic tenase complex, might be more effective than those directly inhibiting FXa and thrombin. The critical nature of extrinsic tenase complex in initiating the coagulation process makes it an attractive target for anticoagulation therapy (Bates and Weitz, 2005; Mackman *et al*., 2007; Shirk and Vlasuk, 2007). The significance of FVIIa/TF in coagulation was further supported by the fact that recombinant FVIIa therapy provided normal hemostasis in patients with severe haemophilia in the absence of FIX and FVIII (Hedner and Ezban, 2008; Hedner, 2008). Also exposure of TF during orthopaedic surgery and in certain patients with cancer may contribute to the high rates of VTE in these groups, making it an important target for anticoagulation therapy (Spyropoulos. 2007). FVIIa/TF is also known to be involved in cancer metastasis (Mueller *et al*., 1992), tumor angiogenesis (Zhang *et al*., 1994) and sepsis (Taylor *et al*., 1991). FIXa is also an attractive target for anticoagulants as most of FXa is produced by the intrinsic tenase complex. However, targeting the extrinsic tenase complex can put a check on both FXa and FIXa generation as both of them are the physiological macromolecular substrates for the tenase complex.

1.7. Extrinsic tenase complex

The extrinsic tenase complex or the initiation complex is comprised of FVIIa with its physiological cofactor; membrane bound TF that converts the macromolecular substrates FX and FIX to their active forms. A detailed account of the structure and function of the tenase complex with respect to FX has been discussed.

1.7.1. Tissue factor

TF also known as factor III or thromboplastin or CD142 (Type II cytokine receptor) is a 263-residue transmembrane glycoprotein, consisting of an extracellular domain (1-219), a transmembrane domain (220-242) and a cytoplasmic tail (243-263) (Morrissey *et al*., 1987; Spicer *et al*., 1987). TF is abundantly found on fibroblasts, advential pericytes and medial smooth muscles of vessel wall. It is also profusely found on many non-vascular cells, monocytes and endothelial cells. However, their presence on granulocytes and platelets requires further clarification. They are also present in circulating blood as microparticles derived from leukocytes, monocytes, platelets, endothelial cells and smooth muscle cells (Daubie et al., 2007; Furie and Furie, 2008). In addition to initiating coagulation, TF is also involved in intracellular signalling associated with angiogenesis, metastasis and maintenance of yolk-sac vasculature (Carmeliet et al., 1996; Belting et al., 2004; Dorfleutner et al., 2004). The extracellular part of TF consists of two compact fibronectin type III (FNIII) domains each folded into a β-sandwich containing a four-stranded and a three-stranded antiparallel sheet. These two domains, called TF1 (residues 1-107) and TF2 (residues 108-219), are connected by a polypeptide linker and the angle between the two is approximately 125º. The interface between the domains is extensive, with a large hydrophobic core, which is likely to be rigid, thus making TF a firm template for FVIIa binding (Harlos et al. 1994, Muller et al. 1994, Muller et al. 1996). TF has four N-linked glycosylation sites: three at the extracellular domain (Asn^{11}, Asn^{124} and Asn^{137}) and one at the cytoplasmic tail (Asn^{261}), however their functional role in cofactor activity is not known as recombinantly expressed TF also expresses full activity (Paborsky et al., 1989; Paborsky and Harris, 1990). Though there were many reports regarding the influence of glycosylation on TF procoagulant activity (Egorina *et al*., 2008), it was shown recently that glycosylation does not have any effect on TF in terms of its transportation as well as procoagulant activity (Kothari *et al*., 2011), thus validating the findings of Paborsky *et al*. The cytoplasmic tail is modified by phosphorylation of several serine residues as well as palmitoylation of cys^{245}. The phosphorylation plays an important role in upregulation of procoagulant activity and PAR-2 mediated cell signalling whereas palmitoylation regulates phophorylation of TF cytoplasmic tail as well as targeting TF towards lipid rafts, there by modulating TF procoagulant activity (Egorina *et al*., 2008). It has been postulated that TF is expressed as an encrypted form on the membrane surface that lacks coagulant activity. Two types of disulfide bonds are present in TF. Cys^{49}-Cys^{57} present between the two FNIII domains provides structural stability where as the C-terminal allosteric disulfide bond provides functional stability to TF. Cys^{186}-Cys^{209} also links the C-terminal FNIII to transmembrane domain there by stabilizing TF on the cellular membrane. There are contradictory views related to TF procoagulant activity regulated by PDI. According to one view that supports the role of PDI, oxidation of free thiols in the encrypted/inactive form of TF to form the disulfide linkage can conformationally activate TF such that it can bind to FVIIa and initiate coagulation (Chen *et al*., 2006; Chen and Hogg, 2006). Activated platelets and endothelial cells at the site of vascular injury release PDI that can mediate in decryption of TF (Cho *et al*., 2008; Reinhardt *et al*., 2008). The other view proposed by Pendurthi *et al*., challenged the existing hypothesis of PDI mediated TF decryption (Pendurthi *et al*., 2007). According to them, surface exposed phosphatidylserine rather than PDI is responsible for the activation of encrypted TF (Kunzelmann-Marche *et al*., 2000;

Pendurthi *et al*., 2007). This was supported by the recent finding that PDI enhances TF activity via its chaperone activity and independent of oxidoreductase activity (Versteeg and Ruf, 2007). Contrastingly, the chaperone effect of PDI in activating FX by FVIIa/TF was disapproved (Persson, 2008). Apart from these theories, experimental evidences have shown various other means by which TF can be decrypted, via calcium ionophores, exposure to stimuli like cell lysis, apoptotic signals and TF self-association and association with specialized membrane domains (Greeno *et al*., 1996; Popescu *et al*., 2010). It is still uncertain about the exact mechanism by which TF procoagulant activity is regulated *in vivo*.

1.7.2. Factor VII/VIIa

Factor VII (FVII) is a 50 kDa vitamin K dependent serine protease synthesized in the liver. It circulates as a zymogen at a plasma concentration of 10 nM (Kalafatis *et al*., 1997). Activated FVII (FVIIa) is formed by proteolytic cleavage at Arg^{152}-Ile^{153} bond of FVII mediated through α-thrombin and FXa (Radcliffe and Nemerson, 1975), FIXa (Seligsohn *et al*., 1979), FVIIa (Lawson et al., 1992) and FXIIa (Kisiel *et al*., 1977). The active form of the enzyme has a light chain (152 residues) and a heavy chain (254 residues) linked by a disulfide bond. The light chain is composed of an N-terminal membrane binding γ-carboxyglutamic acid – rich (Gla) domain and two epidermal growth factor (EGF) domains while the heavy chain constitutes the protease domain. The overall structure of Gla-domainless free FVIIa (Pike et al., 1999; Kemball-Cook *et al*., 1999) resembled to that in complex with TF with the protease domain associated with the light chain to form an extended and compact structure (Banner *et al*., 1996). This structural arrangement resembles to that of other coagulation proteases like FXa (Padmanabhan *et al*., 1993), FIXa (Brandstetter *et al*., 1995) and APC (Mather *et al*., 1996). The EGF-1 domain is rotated by 180º around the EGF-

1/EGF-2 linker, compared to TF/FVIIa complex. The two EGF modules are linked closer to each other via a hydrogen bond to provide an extended conformation and exhibit identical conformations in the free and complex structure. Compared to the complex structure, the N-terminal of EGF-1 has a partially formed and unoccupied Ca^{2+} binding site which could be inferred to the absence of Gla domain in the free FVIIa structure.

The active site inhibitor used in the structure of Pike *et al*. was same as that used by Banner *et al*. However, there was difference in the binding, indicating conformation changes in the protease domain upon interaction with cofactor TF. The protease domain of free FVIIa exhibited minor differences specifically in the region (Leu^{305}-Glu^{325}) flanking the active site cleft when compared to FVIIa/TF. In FVIIa/TF, this region has a α-helix (307-312) followed by a loop. The N-terminal part of the helix and Met^{306} preceding the helix form the TF interface. In free FVIIa this helix is distorted. This could be explained by the absence of an N-capping Asp or Glu residue at 306 position that provides a helical conformation as seen in other serine proteases like FXa, thrombin and trypsin. However, in FVIIa this position is occupied by a conserved Met residue. When FVIIa binds to TF, this Met^{306} gets sandwitched in a hydrophobic pocket on the surface of TF with side chains of Glu^{91} and Tyr^{94} capping the 307-312 helix. Also a hydrogen bond formed between Arg^{315} and Gly^{372}, links the helix to the activation domain thereby stabilizing it. This explains how TF enhances catalytic activity when it binds to FVIIa. The conformation of Leu^{305}-Glu^{325} region is also affected by the glycosylated Asn^{322} that follows the α-helix (307-312). However, this N-glycosylation is not attributed to proteolytic activity and TF binding but suggested to play a role in substrate recognition. The active site inhibitor imposed an active conformation for free FVIIa on the protease domain that includes proper insertion of N-terminal Ile^{153} deep into the protease domain. This inturn facilitates the formation of S1 recognition pocket and oxyanion hole. The N-terminal Ile^{153} gets stabilized when it forms a salt bridge with Asp^{343}. These changes are also induced in FVIIa when it binds to TF (Pike *et al*., 1999; Persson, 2000).

The FVII zymogen structure comprising of EGF-2 and protease domain was solved in the presence of an exosite inhibitor A-183 (Eigenbrot *et al*., 2001). Comparing this structure with that of FVIIa/sTF (Banner *et al*., 1996) and FVIIai (Pike et al., 1999), it was concluded that significant differences were found mostly with the second β barrel that contains the activation domain. These include the absence of a calcium binding site and substrate binding cleft. However, differences in the first β barrel were attributed to the binding of A-183. The N-terminus in the activation domain was found to be linked to EGF-2. Major changes were also observed with the TF binding region of the protease domain, which was due to the alteration in the β-strand B2 (Eigenbrot, 2002).

1.7.3. TF/FVIIa: an allosteric pair

The crucial role of FVIIa/TF complex in initiating the blood coagulation has led to the detailed characterization of the complex. The TF/FVIIa complex exemplifies a perfect allosteric pair (**Fig. 1.7**). The light chain of FVIIa constitutes the region with high allosteric influence. This is through the interaction of eight Ca^{2+} to the Gla and EGF-1 domain of FVIIa. The FVIIa protease domain exhibits three allosteric sites: the TF binding region, the active site binding cleft and the macromolecular substrate binding exosite. The macromolecular substrate exosite is located between the calcium binding site and the N-terminal insertion region and mainly composed of acidic residues (Dickinson *et al*., 1996; Ruf and Dickinson, 1998). These three allosteric sites are organized such a way that binding at any one these brings conformational changes to the other two (Dickinson *et al*., 1998). TF binding to FVIIa can enhance both amidolytic (cleavage of small peptidyl substrates) and proteolytic (cleavage of macromolecular protein substrates) activities. This can be explained with the fact that FVIIa bound to membrane TF can enhance the catalytic activity to 10^5 folds while the removal of phospholipids can only enhance the catalytic activity to just 50 folds. These can be well explained through the complex structure (FVIIa/sTF) solved at 2 Å (Banner et al., 1996). The structure shows a fully extended conformation of FVIIa over TF. A characteristic feature of Gla domain was the presence of seven bound Ca^{2+} of which the linear row of six Ca^{2+} interacts with Gla6 and Gla7 to form a 'W' like structure. In this structure, the hydrophobic residues (Phe^4, Leu^5 and Leu^8) protrude down towards the cell membrane and it is speculated that this may help in the insertion of these residues to the phospholipid membrane. By doing so, the row of six calcium ions can be aligned along the phospholipid head groups. The Gla domain interacts with TF2 via hydrophobic interaction. The EGF-1 domain also harbours a calcium ion and it interacts with the interface of TF1/TF2. EGF-1 and EGF-2 are connected through a helix. The EGF-2 and catalytic domain are disulfide bonded and interacts with TF1 of tissue factor. The FVIIai loop Cys^{310}-Cys^{329} also called as methionine loop lies between TF binding region on the protease domain and substrate binding cleft. In the absence of TF, this loop might overlay on the active site or interact with Ile153 so that it cannot get inserted into the protease domain and later make salt bridge with Asp343. It was also suggested that Gla and EGF might also occlude the active site in the absence of TF. The active site in the protease domain of FVIIa/TF complex was aligned 80 Å above the phospholipid membrane.

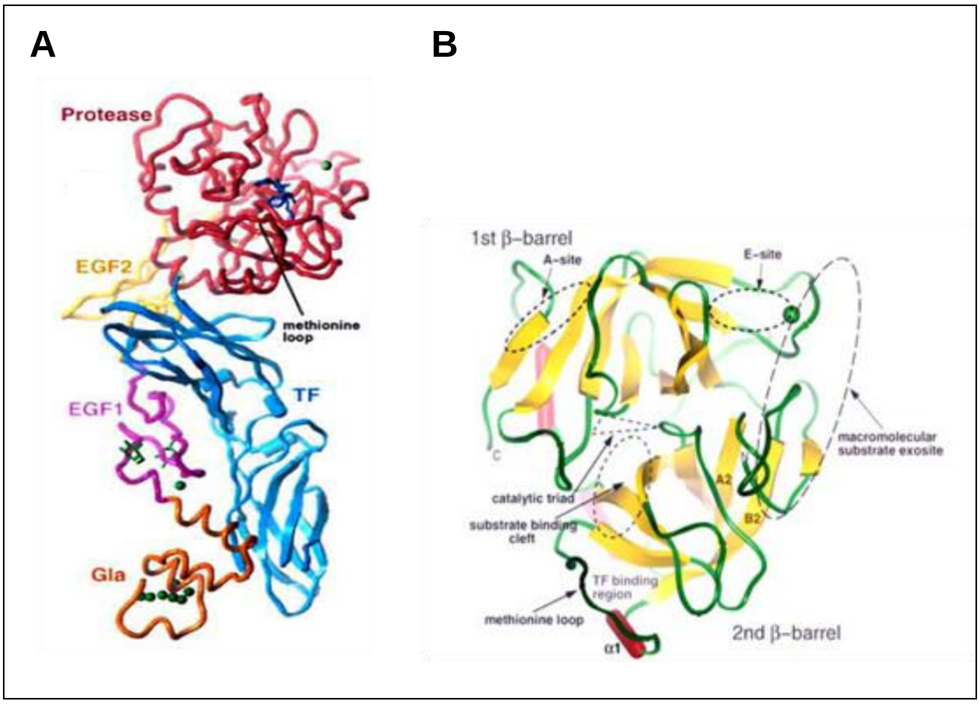


Figure 1.7: TF/FVIIa: an allosteric pair. **A)** The structure of active site inhibited FVIIa/TF (the covalent inhibitor bound to the active site is represented in dark blue). FVIIa with its protease domain and light chain forms an extended and compact structure with its cofactor TF. There are 9 Ca^{2+} bound (represented in green), 7 in the Gla domain, 1 in the EGF-1 domain and 1 in the protease domain. The Gla domain forms a 'w' like structure upon binding of the Ca^{2+} to Gla 6 and Gla 7 residues. This is considered to be important for interacting with the phospholipid membrane. The EGF-1 interacts with the interface of the two fibronectin domains of TF. The EGF-2 and protease domain are disulfide bridged and interacts with the fibronectin domain 1 of TF. **B)** Structure of the FVIIa protease domain showing regions of allosteric control. These include the TF binding region, substrate binding cleft and the macromolecular substrate exosite. The macromolecular substrate exosite is located between the calcium binding site (green sphere) and the N-terminal insertion region. Apart from these three sites, two new allosteric sites have been located on the protease domain. These are A- and E-site present in the first β-barrel. The 'methionine loop' is found between the TF binding region on the FVIIa protease domain and substrate binding cleft [*Adapted from Eigenbrot, Current Protein and Peptide Science, 2002, 3, 287-299]*.

FRET studies with free FVIIa and FVIIa/TF have shown that the active site was positioned 83 Å and 75 Å respectively from the membrane surface. In both the cases, FVIIa was oriented perpendicular to the membrane surface. This orientation of the active site with respect to TF binding could be attributed to the proper alignment of active site to the scissile bond of the macromolecular substrate (Morrissey, 2001). Recently, two exosites named as A-site and E-site was located in the first β-barrel domain of FVIIa protease domain (Eigenbrot, 2002).

1.7.4. FX/Xa

FX constitutes the macromolecular substrate for FVIIa/TF. Human FX is a vitamin K dependent glycoprotein (MW: 59 kDa) synthesized in the liver (Hertzberg, 1994). The circulating FX is activated by both extrinsic tenase (FVIIa/TF) and intrinsic tenase (FIXa/FVIIIa) on the membrane surface during which an activation peptide of 51 residues is removed (Fujikawa *et al*., 1975; Panteleev *et al*., 2004). The FX/FXa is composed of a light chain (139 residues) and a heavy chain (303 residues) linked by a disulfide linkage. The N-terminal Gla domain has eleven Gla residues and can be removed by a cleavage at Tyr^{44}-Lys^{45} via chymotrypsin cleavage (Morita and Jackson, 1986). The Gla domain is followed by two EGF domains. These domains help FX to interact and align with FVIIa/TF and helps in its proteolysis (Ruf et al., 1992; Kirchhofer *et al*., 2000). Mutational studies have shown that EGF-2 interacts with FVIIIa/FVa, the interaction which can also be enhanced by EGF-1(Skogen *et al*., 1984; Hertzberg *et al*., 1992). The N-terminal EGF domain harbours a calcium ion thereby mediating the proper folding of Gla domain as isolated Gla domain have shown to exhibit minimal calcium binding (Persson *et al*., 1989; Persson *et al*., 1991). However the recently solved structure of FX binding protein (X-bp) with Gla 1-44 of FX showed the presence of seven calcium ions bound to the Gla residues. An eighth calcium binding site formed by Gla35 and Gla39 was also observed in the C-terminal helix of Gla domain. These conserved residues are found only in FX and FIX and are suggested to interact with FVIIIa during intrinsic tenase complex formation (Mizuno *et al*., 2001). The protease domain of FXa, like other serine proteases (FVIIa, FIXa and trypsin) harbours a calcium ion binding site (Padmanabhan *et al*., 1993; Brandstetter *et al*., 1996). It also possesses a sodium ion binding site, the first of the monovalent binding site to be identified in FXa (Orthner and Kosow, 1980; Zhang and Tulinsky, 1997). The heparin binding or pro-heparin binding exosite on FXa/FX protease domain has also been characterized. This exosite not only facilitates binding of inhibitors like Ixolaris and NAPc2, but also help in recognizing FVa and prothrombin in the prothrombinase complex (Rezaie, 2000; Monteiro *et al*., 2008; Murakami *et al*., 2007).

1.7.5. Assembly of extrinsic activation complex (FVIIa/TF_{PL}/FX)

The proper alignment of the macromolecular substrate, FX to the FVIIa/TF complex on the membrane surface initiates the activation process. FVIIa can complex with membrane bound TF via interactions that involve both light chain and heavy chain. The Ca^{2+} bound Gla domain helps FVIIa and FX to anchor on to the lipid membrane (Nelsestuen *et al*., 1978; Burnier *et al*., 1981). The cell membrane is assumed to have been occupied by a large number of lipid micro domains rich in phospholipids (Lagerholm et al., 2005) and the Ca^{2+} influences the size and distribution of phospholipid micro domains on the surface (Haverstick and Glacer, 1987; Boettcher *et al*., 2011). It has been hypothesised that the lipid membrane with exposed phosphatidylserine (PS) and phosphatidylethanolamine (PE) plays an important role in holding the coagulations factors on to the surface (Tavoosi *et al*., 2011). PE by itself is a poor enhancer of clotting, but in presence of PS can augment the catalysis by FVIIa/TF. The major portion of the cell membrane is composed of phosphatidylcholine (PC) and sphingomylein. PS and PE are usually confined to the inner leaflets of the membrane. Upon cell rupture and activation, these PS and PE get exposed on to the membrane surface (Neuenschwander *et al.*, 1995; Zwaal *et al.*, 2005). Based on the lipid nanodisc studies with varying concentrations of PS, it has been concluded that the catalytic activity of FVIIa/TF has been enhanced several folds when compared to that incorporated in liposomes. Even FX incorporation on to the nanodisc has been found to increase with increase in PS%. Thus it can be inferred that PS function as a hot spot for the extrinsic activation complex on the membrane surface (Morrissey *et al*., 2011; Tavoosi *et al*., 2011). Using computer simulation studies and solid state NMR experiments with FVIIa Gla domain and nanodiscs, it has been shown that, Ca^{2+} not only stabilizes the Gla domain folding but also helps the Gla residues to interact with the phosphate groups of PS. The study also explained the insertion of Gla domain deep into the membrane surface such that Ca^{2+} can align properly with the phosphate moiety (Morrissey *et al*., 2010). The crystal structure of FVIIa/sTF also clearly shows the Ca^{2+} induced 'W' structure of Gla domain and protrusion of three hydrophobic residues that are believed to get inserted into the membrane (Banner *et al*., 1996). Using mutational studies, it has been found that multiple contact sites are involved in the enzyme complex for macromolecular substrate recognition and proteolysis apart from the enzyme's catalytic cleft. Starting from the Gla domain of FVIIa, Arg^{36} is found to be important for bringing the Gla domain of FX closer to the complex on the membrane surface, site-directed mutagenesis of which has caused an increase in K_M and a decreased K_{cat}. Computational studies have shown that FVIIa Arg^{36} interacts with FX Gla-14 mediated by TF residues Lys^{165} and Lys^{166}. These TF basic residues are in the close proximity of FVIIa Gla Arg36 (Ruf *et al*., 1992; Huang *et al*., 1996; Ruf *et al*., 1999; Kirchhofer et al., 2000). The Ca^{2+} binding site in EGF-1 of FVIIa and FX helps in stabilizing the structural orientation Gla domain with respect to EGF-1 domain. Mutation of two residues Gln^{49} and Asp^{63} in the calcium binding pocket of EGF-1 resulted in reduced TF binding affinity which could be explained by the alteration in FVIIa light chain conformation. The changes either on EGF-1 or Gla domain can indirectly affect FX docking and proteolysis (Kelly *et al*., 1997; Jin *et al*., 1999). The EGF-1 of FVIIa also harbours functional residues important for its interaction with TF (Dickinson *et al*., 1996). Mutational studies has shown that EGF-1 of FX interact with TF which is prerequisite for its activation by the extrinsic tenase complex (Zhong *et al*., 2002; Thiec *et al*., 2003). However, in the protease domain of FVIIa the residues important for FX activation were positioned in three regions: specificity determinants in the substrate binding cleft, TF binding site and a macromolecular substrate exosite (Dickinson *et al*., 1996). During proteolytic activation of FX to FX_{α}, an activation peptide is removed via the cleavage of Arg^{151}-Ile^{152} in the protease domain by FVIIa/TF. FX_{α} is then converted to FX_{β} autoproteolytically by the hydrolysis of a second specific peptide bond (Arg^{290}-Gly^{291}) in the carboxyl terminal region of the protease domain. Both FX_{α} and FX_{β} exhibited similar catalytic activity (Fujikawa *et al*., 1975; Pryzdial and Kessler, 1996) (**Fig. 1.8**).

Apart from the activation by extrinsic tenase complex, FX is also proteolyticaly activated to FXa by the intrinsic tenase complex (FIXa/FVIIIa) (Panteleev *et al*., 2004) and a FX activator from snake venom (Russel’s viper venom FX activator) (Takeya *et al*., 1992).

1.7.6. Inhibitors targeting extrinsic activation complex:

Over the years a number of inhibitors targeting the extrinsic complex (FVIIa/TF/FX) have been characterized. An overview of these inhibitors, specifically inhibiting the proteolysis of FX by FVIIa/TF has been discussed here:

1.7.6.1. Monoclonal antibodies

Monoclonal antibodies (mAbs) targeting the assembly of extrinsic complex can prevent the activation of FX and have potentials in antithrombotic therapy. They can be categorized into (I) mAbs that interfere with substrate binding to TF and (II) mAbs that prevent FVIIa binding to TF. In the first type that affects TF-FX association, mAbs are developed based on TF sequences primarily TF2 that interacts with Gla domain of FX (Fiore *et al*., 1992; Huang *et al*., 1998; Kirchhofer *et al*., 2000; Peng *et al*., 2007). The second type of mAbs is based on FVIIa-TF interaction sites mainly FVIIa light chain-TF and FVIIa methionine loop-TF (Carson *et al*., 1985; Pawashe *et al*., 1994; Kirchhofer *et al*., 2000).

1.7.6.2. Peptides

A cyclic pentapeptide (Cys-Glu-Gln- Tyr-Cys) based on the loop 1 of EGF-2 domain of FVIIa was found to inhibit TF dependent FX activation. It was a non-competitive inhibitor for FX without having any effect on FXa and FVIIa amidolytic activity (Örning *et al*., 2002). Similarly, peptide synthesized based on $TF_{157-167}$ that corresponds to the TF2 region interacting with FX Gla domain was found to inhibit FX proteolysis (Rønning et al., 1996; Örning *et al*., 1998). Peptide based on FX Gla domain was also found to inhbit its activation in FVIIa/TF system (Nawroth *et al*., 1986).

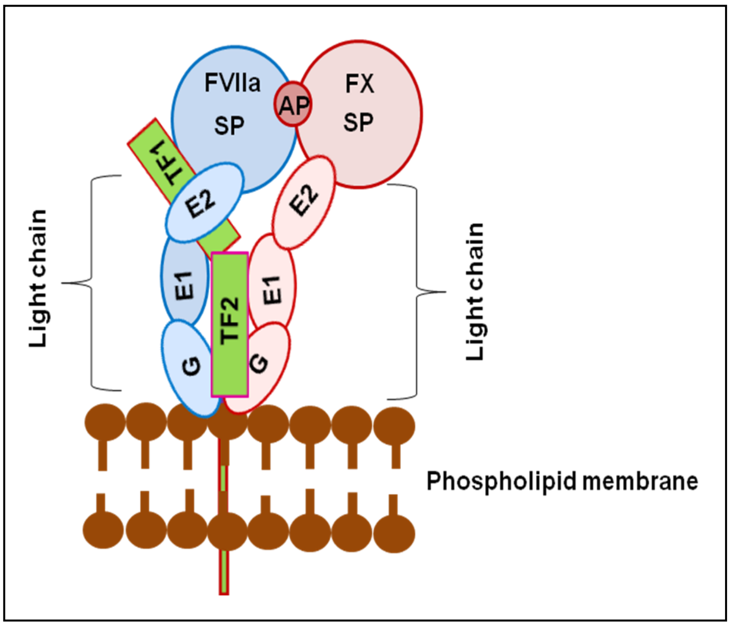


Figure 1.8: Assembly of extrinsic activation complex (FVIIa/TF/FX). The FVIIa/TF constitutes the extrinsic tenase complex that catalytically activates the macromolecular substrate FX to form FXa. This occurs via the cleavage at Arg152-Ile153 bond in the protease domain of FX, to release an activation peptide (AP) of 52 residues. Both the enzyme (FVIIa) and substrate (FX) have similar structure having a light chain and a heavy shain. The light chain is composed of Gla domain (G), EGF-1 (E1) and EGF-2 (E2) domains. Both FVIIa and FX bind to the phospholipid membrane via their Gla domains. The heavy chain consists of the serine protease (SP) domain. Mutational studies have determined residues involved in the interaction of FX with FVIIa/TF. This interaction is essential for the proper alignment of the substrate along the FVIIa/TF in order to enable an efficient catalysis. The interaction site is mainly located in the Gla domain and EGF-1 of FX (*discussed in the section 1.7.5*]

1.7.6.3. Tissue factor pathway inhibitor (TFPI)

Discussed in section 1.2.1

1.7.6.4. Nematode Inhibitors

Nematode anticoagulants characterized from *Ancyclostoma* species are thought to facilitate blood feeding in hookworms by preventing thrombosis. Nematode anticoagulant protein c2 (NAPc2) from *A. caninum* is an 8 kDa peptide that contains two antiparallel β-sheets and a short helix (Cappello *et al.*, 1995; Duggan *et al.*, 1999). Both the natural and recombinant NAPc2 inhibited FVIIa/TF/FX via a unique mechanism resulting in the formation of a quaternary complex (**Fig. 1.9**). It requires initial binding to FX/FXa at an exosite and later binding to FVIIa/TF active site, thus preventing the complete proteolysis of the substrate. Though the inhibition is similar to the physiological inhibitor TFPI, it differs in that TFPI binds to FXa active site. However, NAPc2 binding to exosite moderately affect the amidolytic activity of FXa (Bergum *et al*., 2001; Vlasuk and Rote, 2002; Lee and Vlasuk, 2003). The recently solved crystal structure of FXa-NAPc2 reveals the interaction of the peptide to a novel exosite overlapping with the heparin binding exosite of FXa. NAPc2 is thought to inhibit FVIIa active site via a P1 Arg residue present in its reactive loop (Murakami *et al*., 2007). Other nematode anticoagulants characterized from *A. caninum* are NAPc3 and NAPc4, both exhibiting similar inhibitory mechanism to NAPc2 (Mieszczanek *et al*., 2004).

1.7.6.5. Tick Inhibitors

The well characterized tick inhibitors from the hard tick, Ixodes scapularis are Ixolaris and Penthalaris. Ixolaris is a two-Kunitz domain inhibitor whereas Penthalaris is a five-Kunitz domain inhibitor. Ixolaris exhibit inhibition similar to NAPc2 and TFPI but differ from the latter that like NAPc2 it binds to an exosite on FX/FXa and does not affect the active site of FXa (**Fig. 1.10**). It is postulated that Ixolaris with its second Kunitz domain binds to FX/FXa exosite (proheparin/heparin binding exosite) followed by the docking of its first Kunitz domain of FVIIa/TF active site resulting in a quaternary complex. However in Ixolaris the P1 residue in the reactive site is Glu instead of a basic residue as seen in NAPc2 (Francischetti et al., 2002; Monteiro et al., 2005; Monteiro et al., 2008). Penthalaris also exhibit a similar inhibitory mechanism like that of Ixolaris but its Kunitz specificity is uncertain (Francischetti et al., 2004).

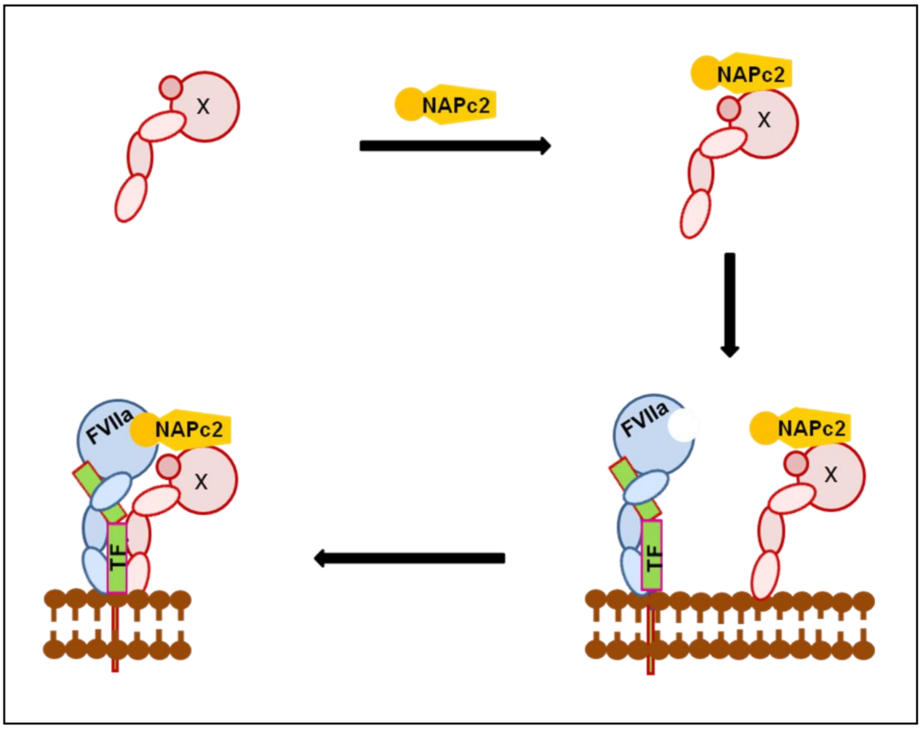


Figure 1.9: Mechanism of action of NAPc2. The nematode anticoagulant NAPc2 from *Ancyclostoma caninum* inhibits the FX activation by FVIIa/TF. It does by binding initially to a proexosite/exosite (proheparin/heparin binding exosite) on FX/Xa. This binary structure then interacts with the active site of FVIIa/TF forming a quaternary inhibited complex. It differs from TFPI in that it does not bind to FXa active site, however partially affects the amidolytic activity of FXa.

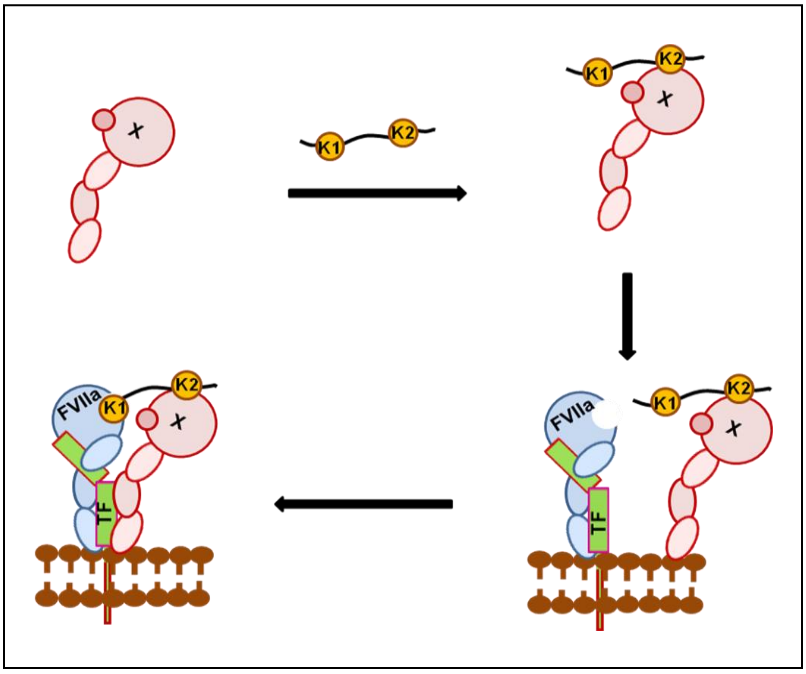


Figure 1.10: Mechanism of action of ixolaris. Ixolaris is a two Kunitz domain inhibitor isolated from the hard tick, *Ixodes scapularis*. It exhibits a similar mechanism to NAPc2 in inhibiting the extrinsic complex. With its second Kunitz domain (K2), it forms a binary complex with FX/FXa by binding to an exosite (proheparin/heparin binding exosite). This mediates it to bind to the active site of FVIIa/TF via its first Kunitz domain (K1), leading to the formation of a quaternary inhibited complex. However compared to NAPc2, it enhances the amidolytic activity of FXa.

1.7.6.6. Snake venom inhibitors

Snake venoms are composed of pharmacologically important peptides and proteins. They are used for paralyzing and digesting the prey (Russell, 1980; Harvey, 1991). Snake venoms can be neurotoxic (Brutto and Brutto, 2011) or hemotoxic (Sajevic, 2011) depending upon their physiological action. Toxins that perturb the hemostatic system of the prey organism can be either procoagulant (Kini *et al*., 2001; Isbister, 2009) or anticoagulant (Kini, 2006; Yamazaki and Morita, 2007, Kini, 2011). Since the current thesis deals with a snake venom protein inhibiting FX activation by extrinsic tenase complex, an over view of snake venom proteins targeting the extrinsic complex has been discussed here:

1.7.6.6.1. Anticoagulant Phospholipiase A_2 enzymes

Phospholipase A_2 (PLA_2) is 13 kDa proteins that specifically cleave glycerophospholipids at the sn-2 position of the glycerol backbone releasing lysophospholipids and fatty acids. They have a typical fold, having a core of three α-helices, a distinctive calcium loop and a β-wing that consist a single loop of antiparallel β-sheet around the calcium loop. In viperid and crotalids, the COOH-terminal segment of PLA_2 forms a semi-circular banister, around the Ca^{2+} binding loop (Arni and Ward, 1996; Kini, 1997). PLA_2s mediate their anticoagulation effect by the hydrolysis of phospholipids or by binding to them (Boffa and Boffa, 1976; Stefansson *et al*., 1990; Mounier *et al.*, 2000). PLA_2s can be categorized to non-anticoagulant, weak and strong based on the concentration at which they can impose their anticoagulant effect (Boffa and Boffa, 1976). Weak anticoagulant PLA_2s targeting the extrinsic tenase complex include superbins I and II or CM-I and CM-II from *Austrelaps supererbus* and *Naja nigicollis* respectively (Stefansson *et al.*, 1989; Subburaju and Kini, 1997). The anticoagulant properties of CM-I and CM-II depended on the presence of phospholipids (Stefansson *et al.*, 1989). The PLA_2 from *B. lanceolatus* was shown to inhibit FX activation by extrinsic tenase via a phospholipid-dependent manner (Lobo de Araujo *et al.*, 2001). However, CM-IV strongly inhibited the extrinsic tenase complex via both enzymatic and non-enzymatic mechanisms (Kini and Evans, 1995). Based on the amino acid sequence analysis of strong, weak and non-anticoagulant PLA_2s as well as chemical modification and site directed mutagenesis studies; an anticoagulant region has been proposed. In strongly anticoagulant PLA_2s, the region that lies between residues 54 and 77 is positively charged and has a pair of lysine residues at both ends which is absent in both weak and non-anticoagulant PLA_2s (Kini and Evans, 1987; Inada *et al.*, 1994; Mounier *et al.*, 2000) (**Fig. 1.11A**).

1.7.6.6.2. Anticoagulant C-type lectin-related proteins

C-type lectin-related proteins are structurally homologous with true C-type lectins but lack carbohydrate binding property (Ebner *et al.*, 2003). FX binding protein (X-bp) has been characterized from *Deinagkistrodon acutus* (Cox, 1993; Atoda *et al.*, 1998) and *A. acutus* (Xu *et al.*, 2000) venoms. However, FIX/FX-binding proteins are characterized from *T. flavoviridis* (Atoda and Morita, 1989; Atoda *et al.*, 1991), *B. jararaca* (Sekiya *et al.*, 1993), *Echis carinatus leucogaster* (Chen and Tsai, 1996), and *A. halys brevicaudus* (Koo *et al.*, 2002) snake venoms. A characteristic feature of these proteins is that they are αβ-heterodimers with a single disulfide linkage. Each subunit of these molecules consists of an extended loop that contributes to a tight interface with the other subunit via domain swapping. The dimeric interface contributes for a concave, binding site for coagulation factor Gla domain (Mizuno *et al.*, 1999). The structure of FIX/FX-bp (Mizuno *et al.*, 1997) and X-bp complexed with Gla domain of FX (Mizuno et al., 2001) has been solved. Ca^{2+} was found to be essential for the activity as replacement with terbium hinders interaction with Gla domain of FX. These proteins prevent FX assembly on membrane surface by tightly binding to its Gla domain with a stoichiometry of 1:1 thereby preventing its activation (**Fig. 1.11B**).

1.7.6.6.3. Anticoagulant three-finger toxins

Three-finger toxins (3FTxs) belong to a family of non-enzymatic polypeptides containing 60-74 amino acid residues. Found in elapids, hydrophids and later in colubrids, 3FTxs are structurally characterized in having four or five disulphide bridges, of which four are conserved in all the members. Members of this family exhibit a similar pattern of protein folding: three β-stranded loops extending from a central core containing the four conserved disulphide bridges. Due to their structural appearance, they are commonly called as three-finger toxins. Inspite of having a common structural mould, they differ very much functionally, making them an interesting target for pharmacological studies (Tsetlin, 1999; Kini, 2002; Kini, 2006). Very little information is known about anticoagulant 3FTxs. The anticoagulant and antiplatelets effects of 3FTxs were first studied in cardiotoxins from *Naja nigricollis crawshawii*, however their mechanism of anticoagulation is not well understood (Kini *et al*., 1988). Recently, from our lab a novel anticoagulant 3FTx hemextin specifically inhibiting FVIIa was characterized (**Fig. 1.11C**). A heterotetramer, the two subunits hemextin A and B synergistically exhibited a non-competitive inhibition towards FVIIa with a Ki of 25 nM. An interesting feature about this inhibitor is that hemextin A itself is a mild anticoagulant whereas hemextin B did not show any activity. However, together they exhibited potent anticoagulation (Banerjee *et al*., 2005; Banerjee *et al*., 2007).

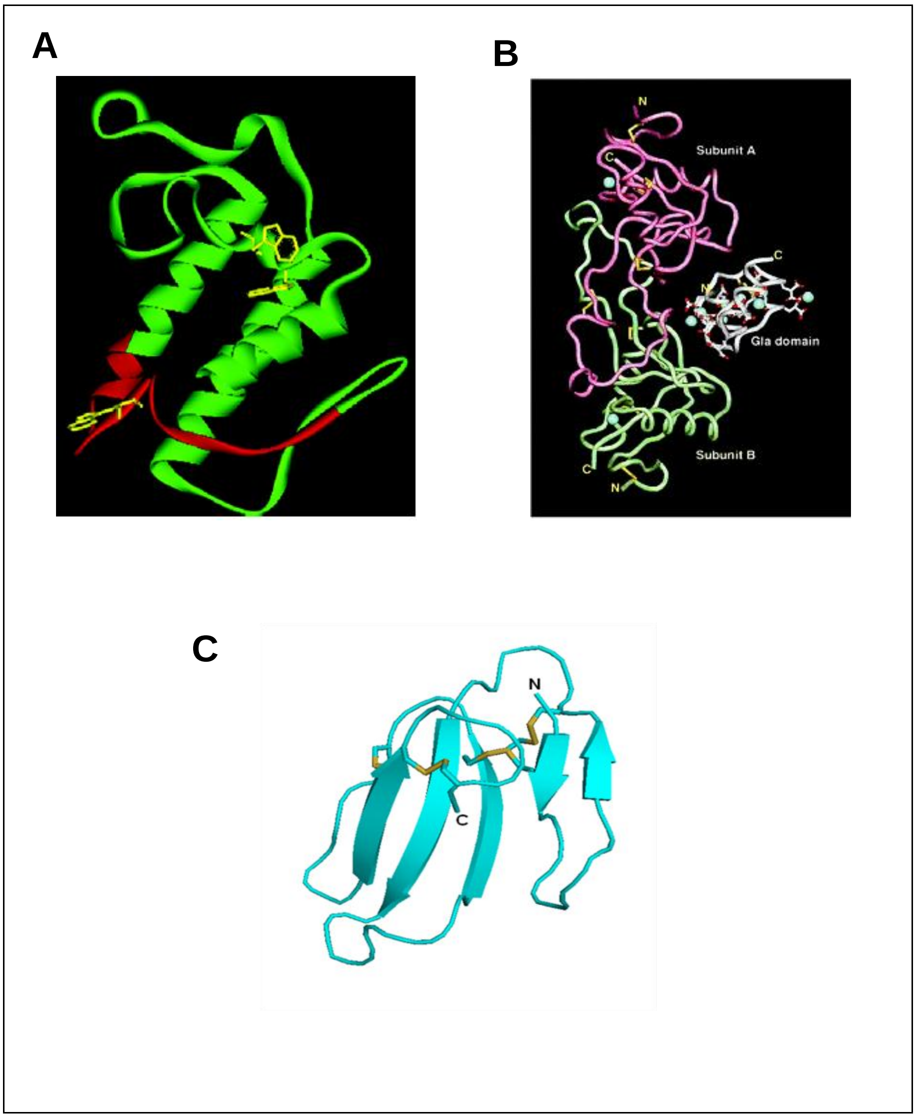


Figure 1.11: Snake venom anticoagulant proteins. **A)** The ribbon model of the anticoagulant PLA_2 from *N. naja*. The fully exposed and easily accessible predicted anticoagulant region is highlighted in red. Tryptophan regions are shown in yellow [*Adapted from Kini, Biochem. J. (2006) 397, 377–387*]. **B)** The complex structure of X-bp with X-Gla domain (1-44 residues). The X-bp, a heterodimer isolated from *Deinagkistrodon acutus* have two subunits represented in magenta and green. The bound Ca^{2+} on Gla domain (1-44 residues) is represented as blue spheres [*Adapted from Mizuno et al., PNAS (2001) 98, 7230-7234*]. **C)** The overall crystal structure of hemextin A, a novel anticoagulant 3FTx from *H. haemachatus* showing the β-strands. The disulfide linkages are shown in yellow [*prepared using PyMOL*].

1.8. Aim and Scope of the thesis

Unwanted blood clots lead to heart attack and stroke that kill a large number of people in developed countries including Singapore. Current anticoagulation therapy has some drawbacks due to their non-specific actions. Therefore, pharmaceutical industries are searching for new drugs that specifically block individual clotting proteins. Snake venoms are complex mixtures of protein toxins that bind to particular human proteins and influence their biological effects. They can distinguish closely related protein receptors, ion channels and blood clotting proteins and hence have potential medical applications. Over the years toxins have provided impetus for the design and development of numerous therapeutic agents useful in the treatment of human diseases. For example, Captopril (for lowering blood pressure) and Integrillin (for reducing unwanted clot formation in heart attack and stroke patients) were developed based on snake toxins. Previously, from our lab we have characterized snake venom 3FTxs targeting two different stages of blood coagulation (hemextin targeting FVIIa and naniproin targeting the prothrombinase complex). This has given us the urge to search for novel anticoagulants especially targeting the extrinsic activation complex. Here we report a novel anticoagulant protein exactin from the venom of *Hemachatus haemachatus* that specifically inhibits FX activation by the extrinsic tenase complex without affecting FVIIa and FXa amidolytic activities, signifying its unique mechanism when compared to TFPI, NAPc2 and Ixolaris. To understand its usefulness as the anticoagulant lead, we also studied its *in vivo* toxicity and was found to be a weak reversible, post-synaptic neurotoxin in i*n vivo* and *ex vivo* animal studies. This is the first report of a 3FTx with a dual function of anticoagulant and neurotoxic effects.

Specifically the objectives were:

1. To isolate and purify exactin from *H. haemachatus* crude venom.
2. To structurally characterize exactin, in terms of its complete amino acid sequence and secondary structure determination.
3. To understand the mechanism and kinetics by which exactin specifically inhibits FX activation by the extrinsic tenase complex.
4. To evaluate the toxicity of exactin through *in vivo* and *ex vivo* animal studies.
5. To identify the condition(s) for crystallizing exactin in order to solve its three-dimension structure.

   On the whole the above mentioned objectives were all covered within the scope of this thesis. Objectives 1, 2 and 3 have been explained in detail in the chapter 2 while objective 4 and 5 are covered in chapters 3 and 4, respectively.

Chapter 2

Anticoagulant properties of exactin

2.1. Introduction

Protein and peptides accounts for 90-95 % of the dry weight of the crude snake venom. Size-exclusion chromatography (SEC) can be used as an initial step for purifying proteins from the crude venom. This separation technique based on molecular size can segregate proteins of various masses present in the crude venom like enzymes (>40 kDa), phospholipases (12-15 kDa), 3FTxs and serine protease inhibitors (6-8 kDa). SEC can be followed by reverse-phase high performance liquid chromatography (RP-HPLC) where proteins will be separated on the basis of hydrophobicity. As a final step in purification, RP-HPLC can yield purified proteins by optimizing parameters like choice of the column, ion-pairing agent, mobile-phase and slope of the eluting gradient. The mass and homogeneity of purified protein can be determined by electrospray ionization-mass spectrometry (ESI-MS).

With the interest in developing the treatment for thromboembolic disorders, the search for novel anticoagulants, particularly three-finger toxins targeting the extrinsic complex, is carried out. Due to their small size, stable fold, non-enzymatic nature and insufficient exploration in anticoagulation studies, 3FTxs have become an exciting target. For the current study, *Hemachatus haemachatus* crude venom was selected. Though the venom contain both neurotoxic and cytotoxic components (Strydom and Botes, 1971; Fryklund and Eaker, 1973; Bengis and Noble, 1976), anticoagulant property for the first time has been attributed to a novel synergistically acting 3FTx, hemextin that mediate its effect through binding to an exosite on FVIIa (Banerjee *et al*., 2005, Banerjee *et al*., 2007). This has led us to screen for anticoagulants specifically targeting the extrinsic activation complex (FVIIa/TF_{PL}/FX). The mechanism of action of the purified anticoagulant protein, exactin is determined using both clot-based and chromogenic assays (Pratt and Monroe, 1992; Walenga and

Hoppensteadt, 2005). A ‘Dissection Approach’ is carried out to delineate the target of exactin on the blood coagulation cascade (**Fig. 2.1**). It utilizes three clotting times: prothrombin time, Stypven time and thrombin time. “It is based on the simple principle that initiating the cascade upstream from the inhibited step will result in elevated clotting times, while initiating the cascade downstream from the inhibited step will not affect the clotting time” (Kini and Banerjee, 2005). The effect of an anticoagulant on extrinsic tenase (FVIIa/TF_{PL}), prothrombinase complex (FXa/FVa) and the fibrin formation can be evaluated through prothrombin time while Stypven time circumvents extrinsic tenase complex, measures its effect on prothrombinase complex and fibrin formation. Thrombin time monitors its effect on fibrinogen conversion to fibrin. Chromogenic assays however, are based on enzyme specific peptidyl substrates on to which a chromophore has been linked. When the enzyme cleaves the substrate, it releases a chromophore which can be measured at a particular wavelength using a spectrophotometer.

Overall this chapter deals with the purification and structural characterization of exactin along with the mechanism by which it specifically inhibits FX activation by the extrinsic tenase complex.

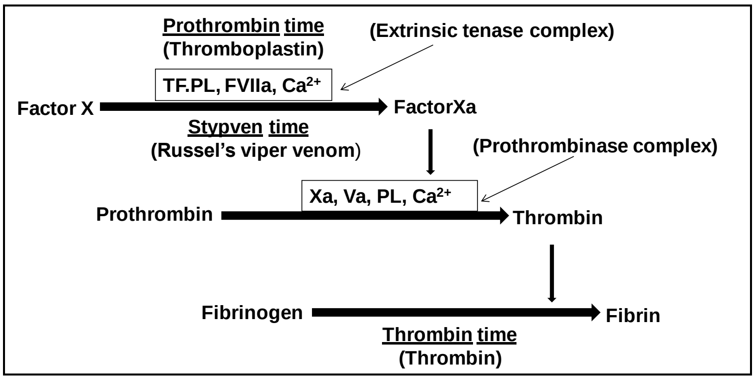


Figure 2.1: Dissection approach. The sequential use of the three clotting times – prothrombin, Stypven and thrombin time can assign the anticoagulant site of an inhibitor in the coagulation cascade. Prothrombin time involves the initiation of clotting by the addition of thromboplastin (lipidated TF) with Ca^{2+}. Stypven time involves the activation of plasma FX by a snake venom FX activator, RVV-X. Activated FXa along with FVa can complex to form prothrombinase complex and in presence of Ca^{2+} can convert prothrombin to thrombin. Thrombin time on the other hand measures the conversion of fibrinogen to fibrin by thrombin.

2.2. Materials and Methods

2.2.1. Materials

Lyophilized *H. haemachatus* crude venom was purchased from South African Venom Suppliers (Louis Trichardt, South Africa). Reagents for thromboplastin time, thrombin time and activated partial thromboplastin time (APTT) were from Helena Laboratories (Texas, USA). Reagents for N-terminal sequencing were from Applied Biosystems (California, USA). Acetonitrile was from Merck KGa (Darmstadt, Germany). Triflouroacetic acid and 4-vinylpyridine were from Sigma-Aldrich (Missouri, USA). β-Mercaptoethanol was from Nacalai Tesque (Kyoto, Japan). The chromogenic substrates, S-2222 (benzoyl-Ile-Glu (Glu-γ-methoxy)-Gly-Arg-*p*NA_HCl), S-2288 (H-D-Ile-Pro-Arg-pNA·2HCl) and S-2238 (H-D-Phe-Pip-Arg-pNA·2HCl) were from Chromogenix (Milano, Italy). Spectrozyme® FIXa was from American Diagnostica Inc (Connecticut, USA). Phosphatidyl choline: phosphatidyl serine (PCPS) (7:3) was purchased from Avanti Polar Lipids Inc (Alabama, USA). Human plasma was obtained from healthy volunteers through Tissue Repository (National University Hospital, Singapore). Superdex 30 HiLoad (16/60) column and Jupiter C_{18} (5 μ, 300 Å, 4.6 × 250 mm) were purchased from GE Healthcare (Uppsala, Sweden) and Phenomenex (City, California, USA), respectively. All other chemicals and reagents used were of the highest purity.

2.2.2. Purified blood coagulation proteins

The blood coagulation factors human FVIIa, FX, FXa, FVa, FIXa, prothrombin, α-thrombin and Russell’s Viper Venom Factor X activator (RVV-X) were from Haemtech (Vermont, USA). Human FVIII was purchased from American Diagnostica Inc (Connecticut, USA). Recombinant human tissue factor (TF_{PL}) (Innovin) was purchased from Dade Behring (Marburg, Germany). Recombinant human soluble TF

($sTF_{1–218}$) was a gift from Dr. Toshiyuki Miyata (National Cardiovascular Center, Suita, Japan).

2.2.3. Preparation of phospholipid vesicles

The single unilamellar vesicles (SUV) of PCPS (7:3) in 50 mM HEPES, pH 7.4 was prepared with minor modifications by sonication as per the procedure of Huang (Huang, 1969). Briefly, PCPS in the vial subjected to a stream of N_2 gas stream was weighed out to make a final concentration of 5 mg/ml in 50 mM HEPES, pH 7.4. The cloudy suspension due to large, multilamellar vesicles formed was subjected to sonication on a bath sonicator for 1 h. The clear solution thus obtained having SUV was stored at 4ºC for further studies.

2.2.4. Purification of exactin

H. haemachatus crude venom (100 mg in 1 ml of distilled water) was size-fractionated by gel-filtration chromatography using a Superdex 30 column (1.6 × 60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer using an ÄKTA purifier system (GE Healthcare, Uppsala, Sweden). Fractions under each peak were pooled and its effect on PTT time was analyzed (discussed below). Peak 3 from gel-filtration chromatography that exhibited prolongation of PTT was sub-fractionated by RP-HPLC on a Jupiter C_{18} column (4.6 × 250 mm) equilibrated with 0.1% TFA. The bound proteins were eluted using a linear gradient of 80% acetonitrile in 0.1% TFA at a flow rate of 2 ml/min. The elution was monitored at 215 nm. The individual fractions were collected, lyophilized and reconstituted in activation buffer (50 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA). The inhibitory effects of these fractions were examined on FX activation by the reconstituted extrinsic tenase complex (discussed below). The peak corresponding to exactin was re-chromatographed using a shallow gradient on the same column. The homogeneity and mass of exactin were checked using ESI-MS performed on API-300 LC/MS/MS system (Perkin-Elmer Sciex, Connecticut, USA). The samples were delivered by direct injection. LC-10AD liquid chromatography system (Shimadzu, Kyoto, Japan) was used for solvent delivery (50% acetonitrile in 0.1% FA) at a flow rate of 40 µl/min. Ionspray, orifice and ring voltages were typically set at 4600 V, 50 V and 350 V, respectively. Nitrogen was used as nebulizer and curtain gas. Analyst software 1.4.1 was used to analyze and deconvolute the raw mass data. Purified exactin fractions were thus pooled and freeze-dried.

2.2.5. N-terminal sequencing

N-terminal sequencing of the native protein was performed by automated Edman degradation using an ABI Procise 494 protein sequencer (Applied Biosystems, California, USA) with an on-line 785A phenylthiohydantoin derivative analyzer. Samples were loaded onto a glass filter pre-coated with Biobrene resin (Applied Biosystem, California, USA) dried off under argon and introduced into the sequencer. The phenylthiohydantoin amino acids were sequentially identified by mapping the respective separation profiles with the standard chromatogram. The number of cysteine residues in the native protein was identified by reduction and pyridylethylation of the protein and examining its modified mass in ESI-MS.

2.2.6. Reduction and pyridylethylation

In order to determine the number of cysteine residues in the native exactin, the protein was reduced and pyridylethylated using procedure described previously (Joseph *et al.*, 1999). Briefly, 0.8 mg of exactin was dissolved in 350 µl of the denaturant buffer (0.13 M Tris-HCl, pH 8.5, 1 mM EDTA, 6 M guanidine hydrochloride). After the addition of 0.74 µl of β-mercaptoethanol, the mixture was incubated under stream of N_2 gas for 3 h at 37ºC. 4-Vinylpyridine (3.4 µl) was added to the mixture and kept at room temperature for 2 h under N_2. S-pyridylethylated protein was purified on an analytical Jupiter C18 column (4.6 × 250 mm) using a linear gradient of 80 % acetonitrile at a flow rate of 1 ml/min. The modified mass of exactin was examined in ESI-MS. Cysteine residues in the amino acid sequence were identified by similarity with other 3FTxs. [*Note: the amount of β-mercaptoethanol and 4-vinylpyridine were calculated assuming that the native protein contains 5 disulfide linkages*]

2.2.7. CD spectroscopy

Far-UV CD spectra (260–190 nm) of exactin, haditoxin and β-cardiotoxin (0.5 mg/ml) were recorded using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). All the proteins were dissolved in 5 mM phosphate buffer, pH 7.4 and the measurements were carried out at room temperature using a 0.1 cm path length stoppered cuvette. The instrument optics was flushed with 30 liters of nitrogen gas/min. The spectra were recorded using a scan speed of 50 nm/min, a resolution of 0.2 nm, and a bandwidth of 2 nm. A total of four scans were recorded and averaged for each spectrum, and the base line was subtracted.

2.2.8. Effect of exactin on plasma clotting times

To identify the specific steps in coagulation cascade that are inhibited, the dissection approach (Kini and Banerjee, 2005) was used and the effects of exactin on the four clotting times were examined. The fibrin clot formation was monitored using a microplate coagulation test method (Pratt and Monroe, 1992). All the experiments were done at 37 ºC and the fibrin clot formation was monitored using a 96-well microplate reader (Tecan Sunrise, Männedorf, Switzerland) for 10 min at 650 nm (unless otherwise stated). The effect of various concentrations of exactin (1 µM to 300 µM) dissolved in 50 mM Tris-HCl buffer, pH 7.4 were studied on prothrombin time (Quick, 1935), Stypven time (Hougie, 1956), thrombine time (Jim, 1957) and activated partial thromboplastin time (APTT) (Proctor and Rapaport, 1961).

2.2.8.1. Prothrombin time

Briefly, 100 µl of plasma, 25 µl of 50 mM Tris-HCl, pH 7.4 and 50 µl of various concentrations of exactin were incubated for 5 min. The clotting was initiated by the addition of 25 µl pre-warmed thromboplastin (with calcium) reagent and the fibrin clot formation was monitored.

2.2.8.2. Stypven time

In a reaction well, 50 µl of plasma was incubated with 50 µl of various concentrations of exactin for 3 min. 50 µl of RVV-X (10 ng/ml) was added and incubated for another 2 min. The clotting was initiated by the addition of 50 µl of 25 mM pre-warmed $CaCl_2$ and the fibrin formation was monitored.

2.2.8.3. Thrombin time

Equal volumes of plasma, 50 mM Tris-HCl, pH 7.4 and various concentrations of exactin (50 µl) were incubated together for 5 min followed by the addition of 50 µl pre-warmed thrombin time reagent (0.15 NIH units) and the fibrin formation was monitored.

2.2.8.4. Activated partial thromboplastin time (APTT)

The effect of exactin on APTT was studied by incubating equal volumes (50 µl) of plasma and various concentrations of exactin for 3 min. Pre-warmed APTT reagent (50 µl) was added and further incubated for 2 min after which the clotting was initiated by the addition of 50 µl pre-warmed 25 mM $CaCl_2$ and the fibrin clot formation was monitored.

2.2.9. Effect of exactin on FX activation by extrinsic tenase complex

All the reactions were carried out at 37ºC. In a reaction volume of 200 µl, the extrinsic tenase complex was reconstituted by incubating 10 pM of human FVIIa with Innovin in an activation buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA) for 15 min. After 15 min incubation with various concentrations of exactin (25 µl of 300 pM to 10 µM), 25 µl of FX was added to make a final concentration of 30 nM. The activation was quenched after 15 min by adding 50 µl of quench buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA). The initial reaction velocity of S-2222 cleavage by FXa formed in the reaction mixture was measured by the hydrolysis of 50 µl of 500 µM of S-2222 in a microplate reader at 405 nm. The amount of FXa generated at each concentration of exactin was measured from a FXa standard curve. FXa formed in the absence of inhibitor was considered as 100% and the IC_{50} concentration was determined.

2.2.10. Effect of exactin on FX activation by intrinsic tenase complex

The intrinsic tenase complex $(FIXa/FVIIIa)_{PL}$ was reconstituted according to the method of Zhang *et al.* with minor modifications (Zhang *et al*., 1998). Briefly, all the reactions were carried out in 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA having 67 µM of reconstituted PCPS at 37 °C. In a reaction volume of 200 µl, FVIII (5 nM) was incubated for 10 min with thrombin (500 pM) and the reaction was quenched by the addition of 25 µl hirudin (115 units/ml/well), a thrombin inhibitor. To this 25 µl FIXa was added to make a final concentration of 1 nM. After 10 min, varying concentration of exactin (25 µl of 30 nM to 300 µM) was added and incubated for 15 min. The substrate, 25 µl FX was added to make a final concentration of 25 nM. The reaction was stopped after 15 min by adding the quench buffer 50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA. The hydrolysis of 25 µl of 500 µM S-2222 by the FXa formed was measured at 405 nm. The IC_{50} value was calculated similar to that mentioned in section 2.2.9.

2.2.11. Effect of exactin on FX activation by RVV-X

All the reactions were carried out in 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA at 37 ºC. 50 µl of exactin (300 pM -10 µM) was incubated with 25 µl of RVV-X (100 pM) in a 200 µl 96-well plate for 15 min. The FX activation was initiated by the addition of 25 µl FX (12.5 nM). The FXa formation was quenched after 15 min by the addition of 50 µl of quench buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA) and the hydrolysis of 50 µl of 500 µM S-2222 by FXa generated was measured at 405 nm. The IC_{50} value was calculated similar to that mentioned in section 2.2.9.

2.2.12. Effect of exactin on prothrombin activation by prothrombinase complex

The assay was carried out in 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA at 37ºC. The PCPS concentration used was 67 µM. In a 200 µl 96-well plate, varying concentration of exactin (50 µl of 30 nM to 300 µM) was added to a reconstituted FXa (10 pM) – FVa (1 nM) complex (prothrombinase complex) and incubated for 15 min. Prothrombin (25 µl of 12.5 nM) was added and incubated for another 15 min. The reaction was quenched by adding 50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA. The hydrolysis of 50 µl of 250 µM S-2238 by thrombin generated in the reaction mix was measured in a multiplate reader at 405 nm. The IC_{50} value was calculated by determining thrombin generated from a standard curve, considering that thrombin generated in the absence of inhibitor is 100%.

2.2.13. Effect of exactin on FIX activation by extrinsic tenase complex

The dose-response effect of exactin (0.01 µM to 300 µM) on FIX activation by extrinsic tenase complex was examined at 37 °C in an activation buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA). Briefly, the extrinsic tenase complex was reconstituted from 1 nM FVIIa and Innovin. The tenase complex was incubated with various concentrations of exactin (25 µl). FIX was then added to the reaction mix to make a final concentration of 600 nM (25 µl). After 15 min of incubation, the reaction was quenched by adding EDTA buffer and the hydrolysis of the chromogenic substrate, 100 µl of Spectrozyme FIXa (1 mM) by FIXa generated in the reaction mixture was measured at 405 nm. From a FIXa standard curve, the amount of FIXa formed at each concentration of exactin was measured. This was used to determine the IC_{50} value, considering FIXa generated in the absence of inhibitor as 100%.

2.2.14. Mechanism of inhibition of the extrinsic activation complex

To understand the mechanism of inhibition, we studied the effects of exactin (30 nM to 300 µM) on the activation of FX by FVIIa/sTF complex and by FVIIa (in the presence or absence of PLs). We also examined the kinetics of inhibition when appropriate. An activation buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA) and a quench buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA) was used for all the experiments. All the incubations were at 37ºC.

To examine the role of PLs, FVIIa (10 nM) and sTF (30 nM) in activation buffer containing 2.5 mM $MgCl_2$ (Persson and Østergaard, 2007) was incubated with various concentrations of exactin (50 µl) for 15 min before the addition of 25 µl FX (2 µM final concentration). To examine the role of TF, 25 µl of FVIIa (10 nM and 20 nM in activation buffer with or without PCPS [67 µM] in the absence of TF, respectively) was incubated with various concentrations of exactin (50 µl) for 15 min. FX (25 µl) was added to make a final concentration of 640 nM. In all experiments, the reaction was quenched after 15 min of FX activation by the addition of EDTA buffer and the amount of FXa formed was measured similar to that mentioned in section 2.2.9.

2.2.15. Effect of exactin on FVIIa, FXa, FIXa and thrombin amidolytic activity

The amidolytic activities of various serine proteases with exactin were studied at 37ºC in the presence of the activation buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA). In a total volume of 200 µl in the individual wells of a 96-well plate, the effect of exactin (0.03 µM to 300 µM) on final concentrations of FVIIa [in presence of sTF (FVIIa- 10 nM, sTF-30 nM), without sTF (FVIIa-300 nM)], FXa (1 nM), FIXa (300 nM) and α-thrombin (3 nM) were examined. The chromogenic substrates S-2288 (500 µM), S-2222 (1 mM), Spectrozyme FIXa (1 mM) and S-2238 (250 µM) were used respectively. The rate of substrate hydrolysis was measured at 405 nm.

2.2.16. Kinetics of inhibition of extrinsic complex mediated FX activation

The inhibitory kinetics (with exactin) of extrinsic tenase complex, $FVIIa_{PL}$ and FVIIa/sTF mediated FX activation was determined. All the reactions were carried out in an assay buffer of 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA (except for kinetic studies with FVIIa/sTF where the activation buffer contained 2.5 mM $MgCl_2$) at 37 ºC and the quench buffer used was 50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA. The inhibitory action of exactin was examined over a wide range of substrate concentrations. For FX activation by extrinsic tenase complex, varying concentration of FX (0.58 nM – 50 nM) were added to individual wells of a 96-well plate containing FVIIa (10 pM) in complex with Innovin and exactin (0 nM, 30 nM, 100 nM and 300 nM). After 15 min, FXa generation was quenched and the rate of hydrolysis of 500 µM S-2222 was measured at 405 nm over 5 min.

For kinetic studies in the absence of PLs, the effect of exactin (0 µM, 100 µM and 300 µM) on FX (0.025 µM to 5 µM) activation by FVIIa (10 nM) and sTF (30 nM) was examined. In case of FX activation by FVIIa in the presence of PLs and absence of TF, FX (11.4 nM to 1 µM) with 10 nM FVIIa and 67 µM PCPS was used. The exactin concentrations used were 0 nM, 30 nM, 100 nM and 300 nM. The experimental design was same as the above. The rate of FXa formed at each concentration of FX was measured from a standard curve.

2.2.17. Kinetics of inhibition of intrinsic tenase mediated FX activation

All the reactions were carried out in an assay buffer of 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA at 37 ºC and the quench buffer used was 50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA. The inhibitory action of exactin (0 µM, 3 µM, 5 µM and 10 µM) was examined over a wide range of substrate (FX) concentrations (1 nM - 1 µM). The experimental procedure was similar to that mentioned in 2.2.10

2.2.18. Kinetics of inhibition of RVV-X mediated FX activation

All the reactions were carried out in an assay buffer of 50 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 1% BSA at 37 ºC and the quench buffer used was 50 mM HEPES pH 7.4, 140 mM NaCl, 50 mM EDTA, 1% BSA. The inhibitory action of exactin (0 µM, 3 µM, 5 µM and 7 µM) was examined over a wide range of substrate (FX) concentrations (1 nM - 1 µM). The experimental procedure was similar to that mentioned in 2.2.11.

2.2.19. Kinetics of inhibition of extrinsic tenase mediated FIX activation

Inhibitory kinetics (with exactin) of extrinsic tenase mediated FIX activation was determined. The experimental procedure was similar to section 2.2.13. Briefly, exactin (0 µM, 10 µM, 30 µM and 100 µM) was incubated with reconstituted extrinsic tenase complex formed from 1 nM FVIIa and Innovin. Varying concentrations of FIX (0.025 µM to 5 µM) was then added to the reaction mixture.

After 15 min of incubation, the reaction was quenched and the rate of hydrolysis of 1 mM Spectrozyme FIXa was monitored at 405 nm over 5 min. The rate of FIXa formed at each concentration of FIX was measured from a standard curve.

2.2.20. Data analysis with kinetics of inhibition

Data obtained from the inhibition studies with exactin were fitted to the following equation for mixed-type inhibition (Silverman, 2002) to determine the Ki and Ki' values.

$$\frac{1}{v}=\frac{Km}{V\max}\left(\frac{1}{[S]}\right)\left(1+\frac{[I]}{Ki}\right)+\frac{1}{V\max}\left(1+\frac{[I]}{Ki'}\right)$$

To determine the Ki and Ki' values, the data from the Lineweaver-Burk plot were re-plotted as *Km/Vmax {1+ [I]/Ki}* (the slope) versus [I] or as *1/Vmax app.* (the y-axis intercept) versus [I] respectively. The x-axis intercept for the respective plots would give Ki and Ki'. Ki denotes the affinity of inhibitor towards the enzyme while Ki' denotes the affinity of the inhibitor towards enzyme-substrate complex.

2.3. Results

2.3.1. Purification of exactin

The crude venom was size-fractionated by gel-filtration chromatography into five peaks (**Fig. 2.2**). The anticoagulant property of each peak was analyzed by evaluating its effect on the prolongation of plasma prothrombin time. Peak 2 and 3 exhibited a significant dose-dependent prolongation of prothrombin time (*inset*). The peak 3 (from SEC) was further fractionated on a C_{18} RP-HPLC column. **Fig. 2.3** represents the RP-HPLC chromatogram of peak 3 on to which the inhibitory effect of each fraction on FX activation by the extrinsic tenase complex was overlaid. The elution profile indicated the presence of several inhibitors of the extrinsic activation complex. Some of these inhibitors were partially characterized, an account of which has been described in this chapter. In the current study, focus was given for the purification and characterization of exactin (Extrinsic Activation Inhibitor) (*peak highlighted by a black arrow*). This peak was further re-chromatographed using a shallow gradient on the same column (**Fig. 2.4A**). The homogeneity and mass of the purified anticoagulant protein exactin was determined by ESI-MS. The mass spectra showed 3 peaks of mass/charge (*m/z*) ratio ranging from +4 to +6 charges (**Fig. 2.4B**) and the mass was calculated to be 6621.12 ± 0.22 Da (*inset*).

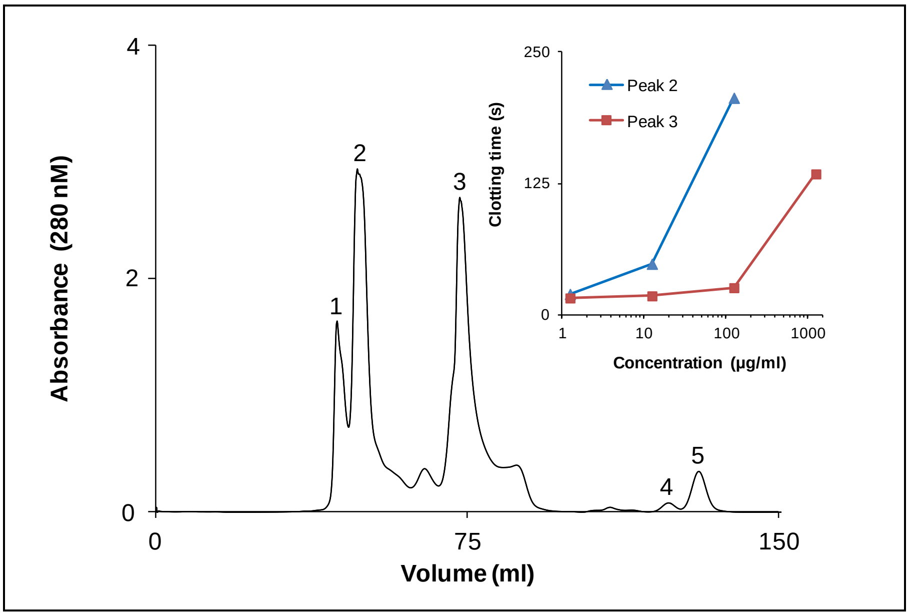


Figure 2.2: Purification of exactin: SEC. The crude venom of *H. haemachatus* was size-fractionated into five peaks. Peak 2 and 3 significantly prolonged the plasma clotting time (*inset*) as determined by prothrombin time. Peak 2 mainly corresponds to snake venom PLA_2's and peak 3 corresponds to non-enzymatic three-finger toxins.

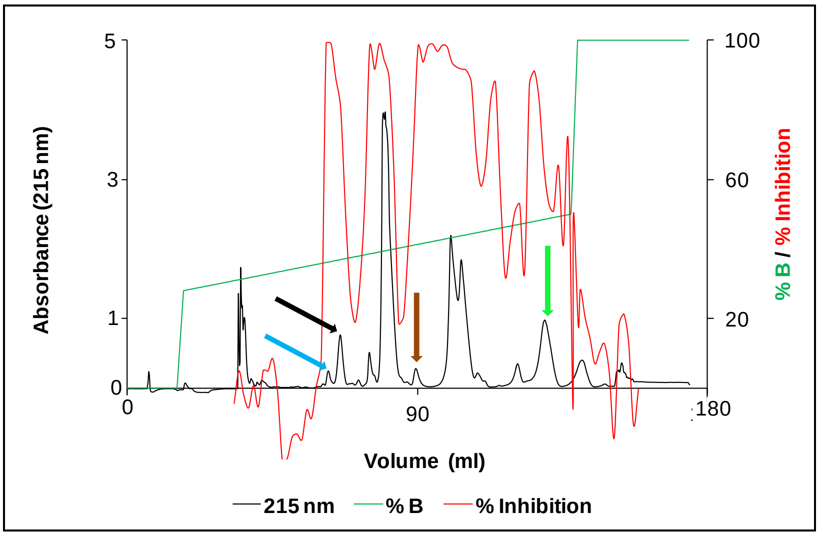
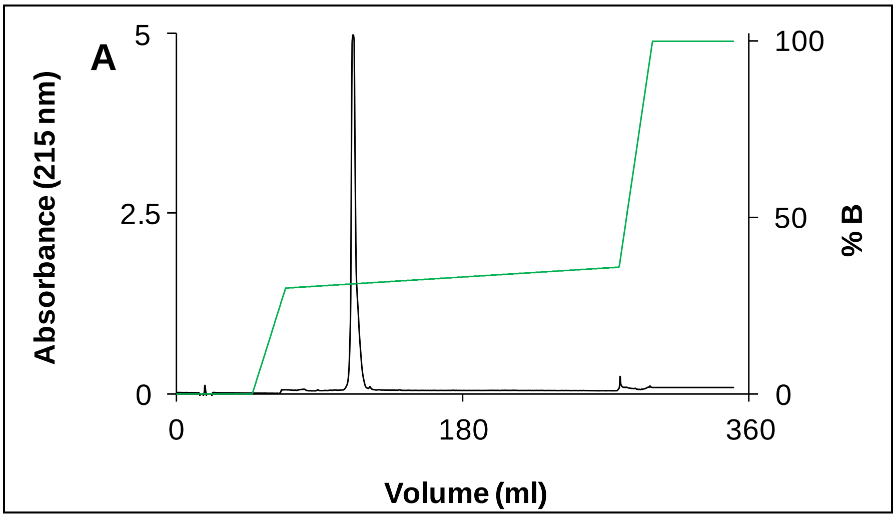


Figure 2.3: Purification of exactin: RP-HPLC. The peak 3 of SEC corresponding to the non-enzymatic 3FTXs, were subjected to RP-HPLC on a Jupiter C_{18} column (4.6 × 250 mm). A linear gradient of 28-50% of solvent B (green line) was used. Each fraction collected were freeze-dried and reconstituted in a calcium buffer. The inhibitory effect of each fraction towards FX activation by the extrinsic tenase complex was evaluated. The red line corresponds to % inhibition of FX activation that is overlaid on to the chromatogram. A number of inhibitors have been found, but the purification and functional characterization of exactin (black arrow) was carried out. We also partially characterized other anticoagulants, highlighted as blue arrow (7277 Da), brown arrow (7438 Da) and green arrow (peak 10).



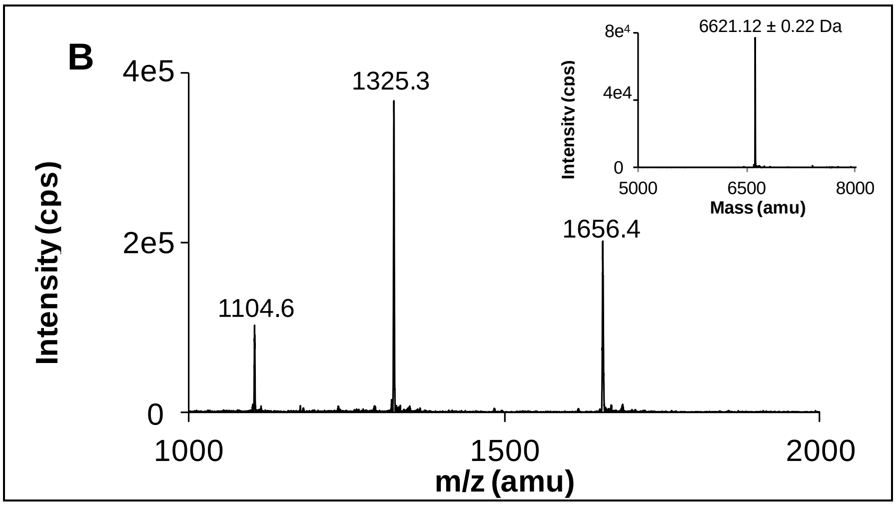


Figure 2.4: Purification of exactin to homogeneity. **A)** Re-chromatography of exactin. Exactin was purified on a shallow gradient of 28%-35% of solvent B (green line) on a Jupiter C_{18} column (4.6 × 250 mm). **B)** ESI-MS profile of exactin. The homogeneity and mass of the purified protein was determined in ESI-MS. The molecular mass of exactin was determined as 6621.12 ± 0.22 Da.

2.3.2. N-terminal sequence determination

The complete amino acid sequence of exactin was determined by automated Edman degradation. Exactin had 57 amino acid residues. The calculated mass of 6621.5 Da from the sequence of native exactin coincides well with the observed molecular mass of 6621.12 Da. Since the "native" exactin was sequenced, the number of cysteinyl residues in exactin was determined via reduction and pyridylethylation and determining the mass in ESI-MS. An observed mass of 7470.91 ± 1.6 Da (**Fig. 2.5**) for the reduced and pyridylethylated exactin denotes the presence of eight cysteinyl residues. The amino acid sequences of the proteins were subjected to a similarity search using BLAST (Altschul *et al.*, 1997). Multiple sequence alignment was carried out. The sequence data of the homologus proteins were obtained from PDB (Protein Data Bank) database. Sequence alignment showed that exactin belongs to the 3FTx family based on the number and position of cysteine residues. It showed 82% identity to weak toxin CM1b isolated from *H. haemachatus* venom (**Fig. 2.6A**). It also showed identity (>58%) to a number of *O. hannah* (king cobra) neurotoxins. Interestingly, exactin showed low identity (< 35 %) to other well characterized anticoagulant 3FTxs (naniproin, hemextin and siamextin) **(Fig. 2.6B)**.

2.3.3. CD spectroscopy

Exactin showed intense minima at 212 nm and 194 nm and maxima at 200 nm (**Fig. 2.7**). Like other 3FTxs - β–cardiotoxin (Rajagopalan *et al*., 2007) and haditoxin (Roy *et al*., 2010), exactin also have predominantly β–sheet structure.

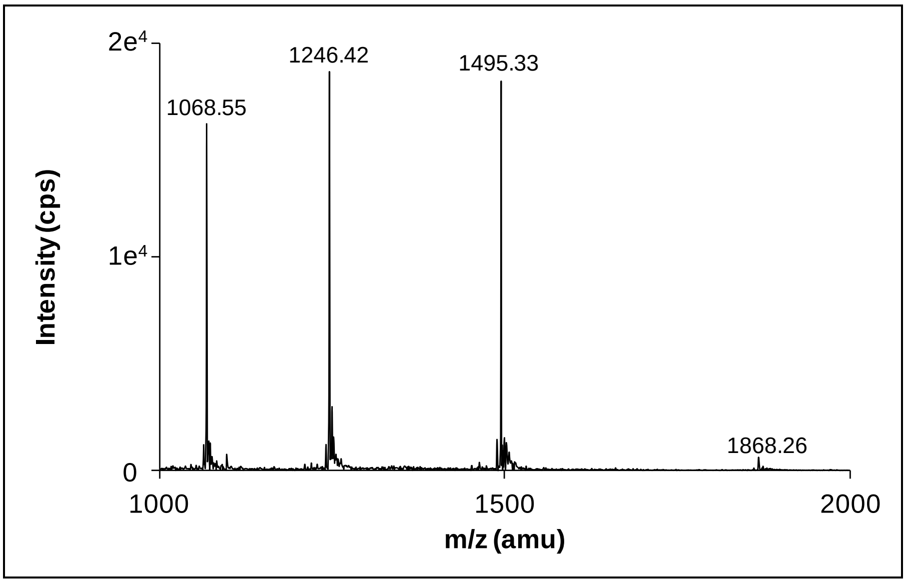


Figure 2.5: Determination of number of cysteinyl residues in exactin. Since the complete sequence of exactin was derived by direct sequencing of the native protein, number of cysteinyl residues were determined from the mass of reduced and pyridylethylated exactin. A mass of 7470.91 ± 1.6 Da confirms the presence of 8 cysteinyl residues.

A

```
                                                                       % Id
Exactin     LECYQKSKVVTCQPEQKFCYSDTTMFFPNHPVYLSGCTFSCTEEGN-RRCCTTDKCNR----
CM-1b       LECYQKSKVVTCQPEQKFCYSDTMTFFPNHPVYLSGCTF-CRTDESGERCCTTDRCNK----     82
CM-2a       LECYQMSKVVTCKPEEKFCYSDVFMPFRNHIVYTSGCSSYCR-DGTGEKCCTTDRCNGARGG     65
CM-3        LECYQMSKVVTCKPEEKFCYSDVFMPFRNHVVYTS-GCSSYCRDGTGEK-CCTTDRCNGARGG    65
Oh9-1       LICHRVHGLQTCEPDQKFCFRKTTMFFPNHPVLLMGCTSSCPTEKYSV-CCSTDKCNK----     58
Oh-26       LICHQRHGLQTCEPAQKFCFAQTVMPFPNHPLTLMGCTYSCPTEKNAV-CCSTDKCNR----     58
Oh-5        LICHRVHGLQTCEPDQKFCFRKTTMFFPNHPVLLMGCTYSCPTEKYSV-CCSTDKCNK----     58
sntx11      LICHRVHGLQTCEPDQKFCFRKTTMFFPNHPVLLMGCTYSCPTEKYSV-CCSTDKCNK----     58
sntx14      LICHQLHGLQTCEPAQKFCQKRTTMFFPNHPVLLMGCTYNCPTERYSV-CCSTDKCNK----     58
sntx26      LICHQVHGLQTCEPAQKFCQKRTTMFFPNHPVLLMGCTYNCPTERYSV-CCSTDKCNK----     58
Oh-46       LICHQVHGLQTCEPAQKFCQIRTTMFFPNHPVLLMGCTYNCPTERYSV-CCSTDKCNK----     58
```

B

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Exactin       LECYQKSKVVT---CQPEQKFCYSDTTMFFPNHPVYLSGCTFSCTEEGN---RRCCTTDKCNR
Naniproin     LKCNRLIPPFWKT-CPEGKNLCYKMTMRLAPKVPVKRG-CIDVCPKSSLLIKYMCCTNDKCN     35
Hemextin A    LKCHNKLVPFLSKTCPEGKNLCYKMTMLKMPKIPIKRG-CTDACPKSSLLVKVVCCNKDKCN     35
Siamextin     LKCNKLVPLFYKT-CPAGKNLCYKMFMVATPKVPVKRG-CIDVCPKSSXLVKYVCCNQDRCX     28
```

Figure 2.6: Sequence alignment of exactin. The homology of each of the sequence in (% Id) identity is given. The conserved eight cysteine residues as well as the identical residues among the sequences are highlighted in black. **A)** Comparison was made with the following sequences: CM-1b from *H. haemachatus*; CM-2a and CM-3 from *N. haje annulifera*; Oh9-1, Oh-26, Oh-5, Oh-46, short neurotoxin (sntx) 11, sntx14 and sntx26 were from *O. hannah*. **B)** Sequence comparison of exactin with other anticoagulant 3FTxs. The comparison was made with hemextin A from *H. haemachatus*; naniproin from *N. nigricollis* and siamextin from *N. siamensis*.

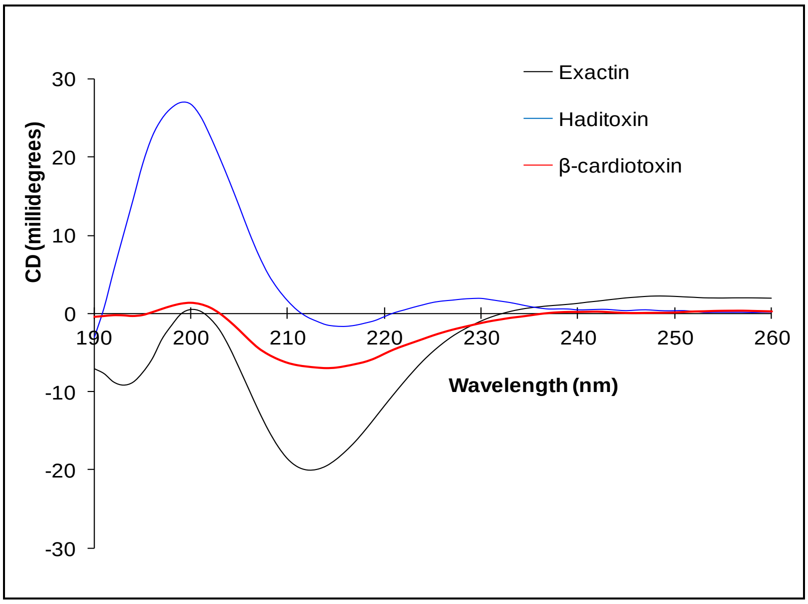


Figure 2.7: CD spectra of exactin. The CD spectra of exactin were compared with that of haditoxin and β-cardiotoxin (0.5 mg/ml) from *O. hannah*. Exactin showed predominantly β-sheet structure with intense minima at 212 nm and 194 nm and a maximum at 200 nm.

2.3.4. Anticoagulant site of exactin

A ‘dissection approach’ (Kini and Banerjee, 2005) was used to understand exactin’s target in the blood coagulation cascade. Exactin significantly prolonged prothrombin time compared to Stypven time and APTT (**Fig. 2.8A**). However, it did not have any effect on thrombin time. The results suggest that exactin specifically targets the extrinsic complex. To further validate the target complex, we determined the effects of exactin on the reconstituted extrinsic tenase, intrinsic tenase and prothrombinase complexes as well as thrombin (**Fig. 2.8B**). Exactin specifically inhibited FX activation by reconstituted extrinsic tenase compared to intrinsic tenase complex with IC_{50} values of 116.49 ± 3.28 nM and 4.05 ± 0.32 µM, respectively. It also inhibited prothrombin activation by prothrombinase complex with an IC_{50} value of 17.66 ± 0.58 µM but it did not affect thrombin amidolytic activity. The thus results suggest that exactin specifically targets the extrinsic activation complex in the coagulation cascade.

2.3.5. Mechanism of action of exactin

To understand the molecular mechanism, the effect of exactin was examined in various assays where each part of the extrinsic tenase complex was removed sequentially. In the first step, TF was removed and the effect of exactin on the activation of FX by FVIIa in the presence of PL was studied. The removal of TF did not affect the inhibitory potency of exactin (IC_{50} value 102.70 ± 11.71 nM compared to 116.49 ± 3.28 nM for the complete complex) (**Fig. 2.9A**). However, with exactin a very low percent activation of FX by FVIIa was observed in the absence of both TF and PL (**Fig. 2.9A** *inset*). In the second step, we examined the effect of exactin on the FX activation by FVIIa-sTF complex in the absence of PLs.

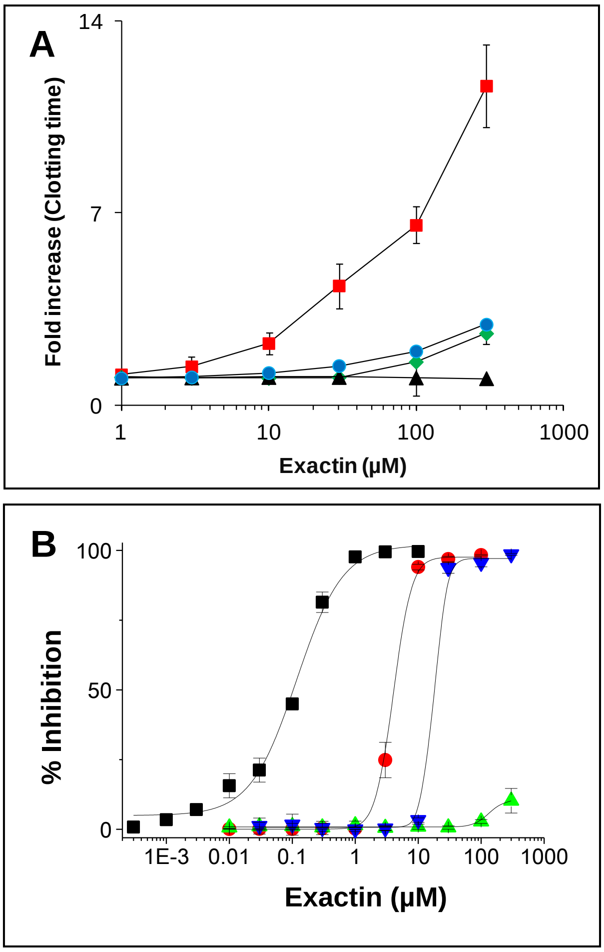


Figure 2.8: Anticoagulant site of exactin. **A)** The effect of exactin on the four blood clotting times was evaluated. These were prothrombin time (■), APTT (●), Stypven time (◆) and thrombin time (▲). Exactin prolonged prothrombin time more significantly compared to others suggesting its target as extrinsic activation complex. **B)** The effect of exactin was evaluated on the reconstituted blood coagulation complexes as well as thrombin. The IC_{50} values were determined for the following: inhibition of FX activation by extrinsic tenase complex (■) was found to be 116.49 ± 3.28 nM; inhibition of FX activation by intrinsic tenase complex (●) was found to be 4.05 ± 0.32 μM; inhibition of PT activation by prothrombinase complex (▼) was found to be 17.66 ± 0.58 μM and finally the effect on thrombin amidolytic activity (▲). Exactin exhibited >30 and >150-folds inhibitory activity to extrinsic tenase when compared to intrinsic and prothrombinase complex activity, respectively. Also exactin did not inhibit thrombin, suggesting its preferable site of anticoagulant activity as 'extrinsic activation complex'. All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

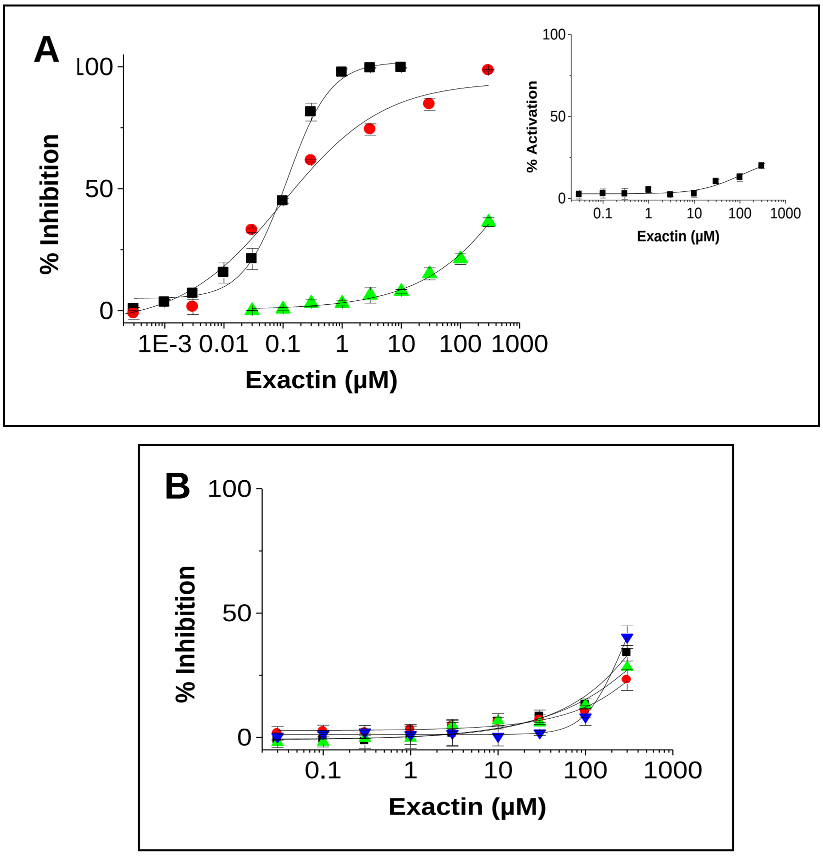


Figure 2.9: Mechanism of action. **A)** The effect of exactin on the sequential removal of each component of the extrinsic activation complex was analysed. Removal of TF did not alter the inhibition of exactin towards FX activation. The IC_{50} value of 102.70 ± 11.71 nM obtained with FX activation by $FVIIa_{PL}$ (●) was comparable to that with extrinsic tenase complex (■) (IC_{50} - 116.49 ± 3.28 nM). Removal of PLs drastically reduced the inhibition by > 1000-folds (▲). A low percent activation was observed when exactin's effect was evaluated on FX activation by FVIIa in the absence of both TF and PLs (*inset*). **B)** The effect of exactin on FVIIa and FXa amidolytic activity: FVIIa/sTF (■); FVIIa (●); $FVIIa_{PL}$ (▲) and FXa (▼). Exactin failed to inhibit the amidolytic activities of the serine proteases FVIIa and FXa, suggesting its full potency towards the entire extrinsic activation complex. All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

The inhibitory potency of exactin was drastically reduced by >1000-fold (**Fig. 2.9A**). We also examined the effect of exactin on the amidolytic activity of FVIIa (with sTF, with PL and in the absence of both sTF and PLs) and FXa. As shown in **Fig. 2.9B**, exactin is a poor inhibitor of both these enzymes (IC_{50} values >300 µM). Taken together, exactin preferably inhibits the macromolecular substrate complex with FVIIA/TF_{PL} (extrinsic activation complex).

2.3.6. Kinetics of inhibition of extrinsic activation complex by exactin

To further understand the interactions, the inhibition kinetics of exactin on FX activation by the complete extrinsic tenase complex, FVIIa in the presence of PL and FVIIa in the presence of sTF were examined. In all the kinetic studies the kinetic constants Ki', Ki as well as the kinetic parameters, K_M and K_{cat} (number of moles of substrate converted to product per mole of enzyme per min) were determined (**Table 2.1, 2.2 and 2.3)**. Exactin exhibited mixed-type inhibition of FX activation by the complete extrinsic tenase complex (**Fig. 2.10B**) as well as FVIIa in the presence of PL (**Fig. 2.11B**). Lineweaver-Burk plots exhibited a decrease in both K_M and V_{max} with increase in inhibitor concentration, typical of mixed-type inhibition. The kinetic constants, Ki' and Ki derived from the secondary plot were found to be 30.62 ± 7.73 nM and 153.75 ± 17.96 nM for FX activation by extrinsic tenase complex (**Fig. 2.10C,D**). The Ki' and Ki for FX activation by FVIIa in the presence of PL was 103 ± 13.49 nM and 184.25 ± 6.13 nM, respectively (**Fig. 2.11C,D**). The affinity of the inhibitor towards the enzyme-substrate complex (FVIIa/TF/FX/PL, Ki' 30.62 ± 7.73 nM) was 5-fold higher compared to the enzyme complex (FVIIa/TF/PL, Ki 153.75 ± 17.96 nM) suggesting its preference to [ES] complex. In the absence of TF, Ki' dropped 3-fold to 103 ± 13.49 nM with a slight decrease in Ki of 184.25 ± 6.13 nM. Thus, exactin appears to bind to the complete complex better than FVIIa/FX complex.

Exactin weakly inhibited the extrinsic activation complex in the absence of PL and exhibited a mixed-type inhibition as from the Lineweaver-Burk plot (both K_M and V_{max} decreased with increase in inhibitor concentration) (**Fig. 2.12B**) with the kinetic constants Ki' and Ki of 158.66 ± 28 μM and 1153.33 ± 49.32 μM respectively (**Fig. 2.12C,D**). Thus, as expected, the affinity towards the enzyme-substrate complex (FVIIa/sTF/FX) and enzyme (FVIIa/sTF) decreased by >1000-fold, suggesting its preference towards membrane-bound complex. As FX activation by FVIIa in the absence of TF and PL was too low, the effect of exactin on FX activation by FVIIa alone was not studied.

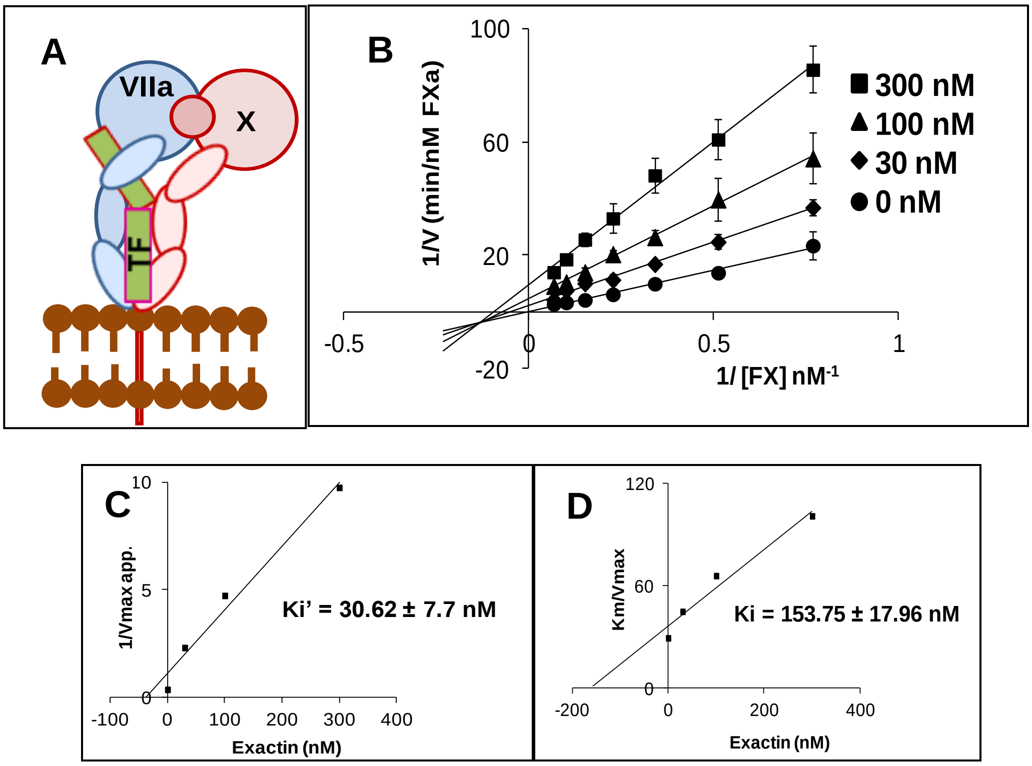


Figure 2.10: Kinetics of inhibition of FX activation by extrinsic tenase complex. **A)** Schematic representation of the extrinsic activation complex (FVIIa/TF/FX on phospholipid membrane). **B)** The Lineweaver-Burk plot for the kinetic activity of FX activation by FVIIa/TF_{PL} in the presence of exactin (0 nM, 30 nM, 100 nM and 300 nM). Both V_{max} and K_M decreased with increase in inhibitor concentration, characteristic of mixed-type inhibition. **C and D)** Corresponding secondary plots depicting kinetic constants Ki’ and Ki for inhibition. Ki’ of 30.62 ± 7.7 nM was obtained towards the enzyme-substrate complex (FVIIa/TF_{PL}/FX) where as Ki of 153.75 ± 17.96 nM was obtained towards the enzyme (FVIIa/TF_{PL}). All the experiments were conducted n=5 and the results were expressed as mean ± SD.

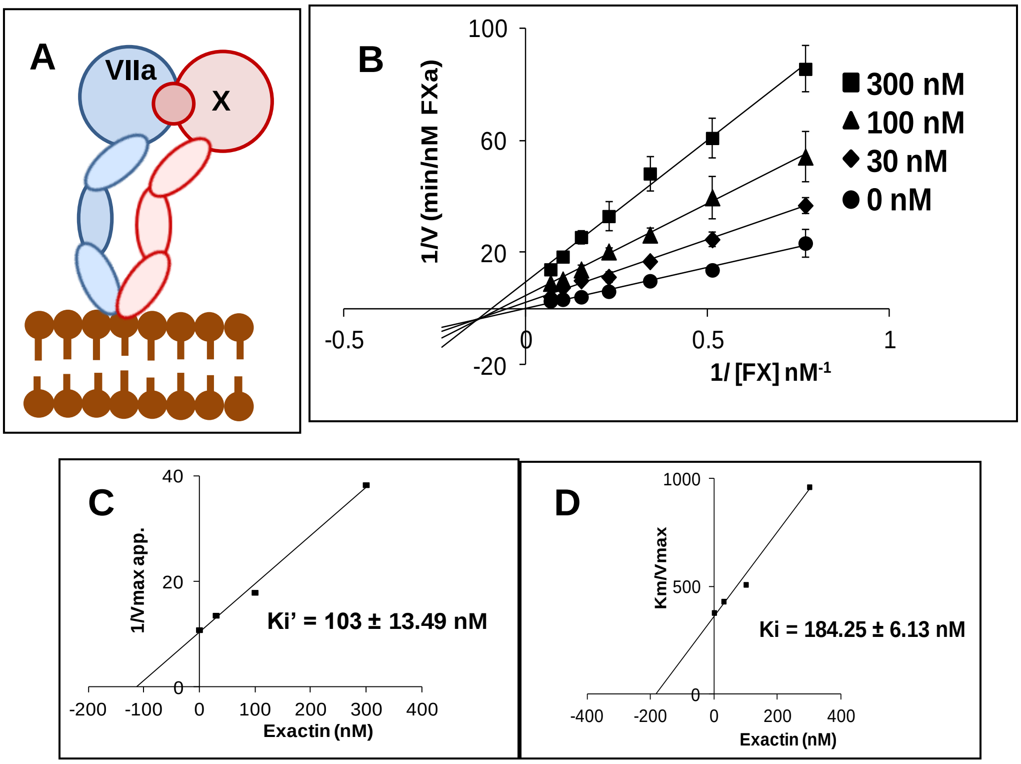


Figure 2.11: Kinetics of inhibition of FX activation by FVIIa in the presence of phospholipids. **A)** Schematic representation of the complex (FVIIa/FX on phospholipid membrane). **B)** The Lineweaver-Burk plot for the kinetic activity of FX activation by $FVIIa_{PL}$ in the presence of exactin (0 nM, 30 nM, 100 nM and 300 nM). Both V_{max} and K_M decreased with increase in inhibitor concentration, characteristic of mixed-type inhibition. **C)** and **D)** Corresponding secondary plots depicting kinetic constants Ki' and Ki for inhibition. Ki' of 103 ± 13.49 nM was obtained towards the enzyme-substrate complex $(FVIIa/FX)_{PL}$ where as Ki of 184.25 ± 6.13 nM was obtained towards the enzyme ($FVIIa_{PL}$). All the experiments were conducted in n=5 and the results were expressed as mean ± SD.

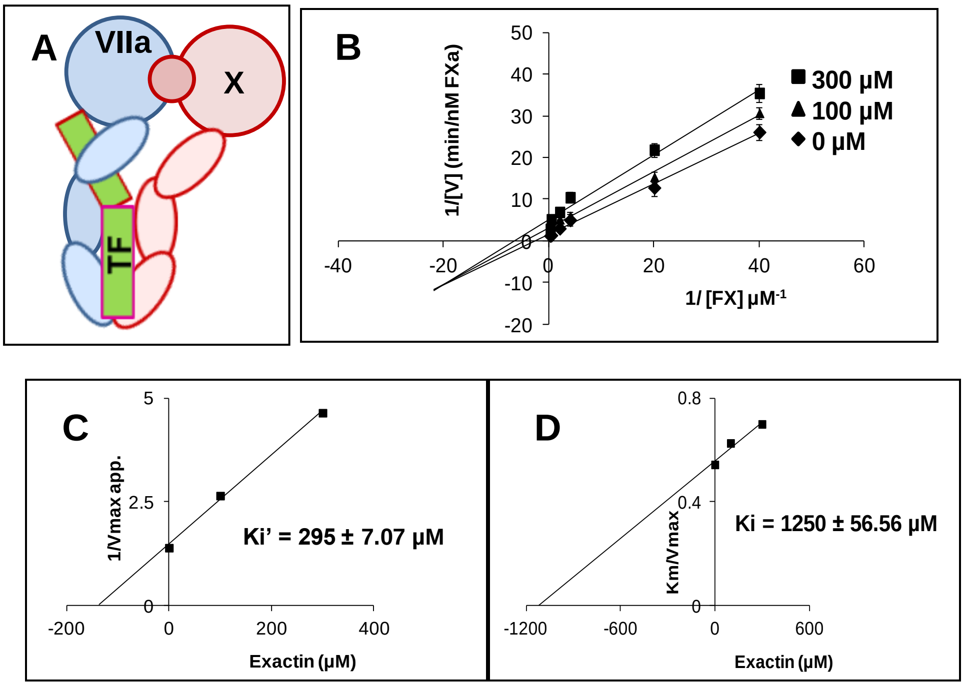


Figure 2.12: Kinetics of inhibition of FX activation by FVIIa/sTF. **A)** Schematic representation of FVIIa/sTF/FX complex. **B)** The Lineweaver-Burk plot for the kinetic activity of FX activation by FVIIa/sTF in the presence of exactin (0 µM, 100 µM and 300 µM). Both V_{max} and K_M decreased with increase in inhibitor concentration, characteristic of mixed-type inhibition. **C and D)** Corresponding secondary plots depicting kinetic constants Ki' and Ki for inhibition. Ki' of 295 ± 7.07 µM was obtained towards the enzyme-substrate complex (FVIIa/sTF/FX) where as Ki of 1250 ± 56.56 µM was obtained towards the enzyme (FVIIa/sTF). All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

Table 2.1: Kinetic parameters for FX activation by extrinsic tenase complex

Exactin (nM)	Km (nM)	Kcat (s^{-1})	Kcat/Km (s^{-1} nM^{-1})
0	14.57 ± 0.87	1.7 ± 0.017	0.117 ± 0.055
30	8.31 ± 0.72	0.95 ± 0.98	0.114 ± 0.008
100	6.46 ± 1.98	0.623 ± 0.2	0.095 ± 0.012
300	5.66 ± 1.9	0.32 ± 0.124	0.057 ± 0.013

Table 2.2: Kinetic parameters for FX activation by $FVIIa_{PL}$

Exactin (nM)	Km (nM)	Kcat (s^{-1})	Kcat/Km (s^{-1} nM^{-1})
0	36.56 ± 2.76	0.0558 ± 0.0046	0.0014 ± 0.00014
30	23.75 ± 2.71	0.0346 ± 0.0026	0.0014 ± 0.0002
100	18.37 ± 1.49	0.0262 ± 0.0035	0.0014 ± 0.0002
300	12.22 ± 1.029	0.0151 ± 0.0031	0.0013 ± 0.00026

Table 2.3: Kinetic parameters for FX activation by FVIIa/sTF

Exactin (µM)	Km (µM)	Kcat (s^{-1})	Kcat/Km (s^{-1} μM^{-1})
0	1.03 ± 0.089	0.0017± 6.13 E-05	0.0016 ± 0.0001
100	0.76 ± 0.126	0.00065 ± 6.01 E-06	0.0009 ± 0.0002
300	0.42 ± 0.022	0.0006 ± 6.66 E-05	0.0014 ± 0.0002

2.3.7. Macromolecular specificities of exactin

Inorder to evaluate the specificity of exactin, the inhibitory potencies towards the macromolecular complexes: enzyme (FVIIa/TF_{PL}) and substrate (FX) separately were examined.

2.3.7.1. Specificity of exactin towards the extrinsic tenase complex

The kinetic studies have shown that exactin exerts its full inhibitory potency towards the entire extrinsic activation complex. Inorder to evaluate the inhibitory specificity of exactin towards the FX activator, FX activation by extrinsic tenase complex, intrinsic tenase complex and RVV-X was studied (**Fig. 2.13**). Compared to the FX activation by the extrinsic tenase complex (IC_{50} - 116.49 ± 3.28 nM), exactin exhibited < 30 folds inhibition to FX activation by both the intrinsic tenase complex and RVV-X (IC_{50} values of 4.05 ± 0.32 µM and 6.1 ± 2.9 µM, respectively). Thus, the results confirm the specificity of exactin towards the extrinsic tenase complex in activating FX. To further understand this, the inhibitory kinetics of exactin on FX activation by both intrinsic tenase complex as well as the snake venom FX activator (RVV-X) was studied. Exactin exhibited a non-competitive inhibition towards FX activation by both intrinsic tenase complex (**Fig. 2.14B**) and RVV-X (**Fig. 2.15B**). The kinetic parameters (K_M and K_{cat}) determined (**Table 2.4, 2.5**) showed that the K_M remains unchanged whereas Vmax decreasaed with increase in inhibitor concentration, typical of non-competitive inhibition. The kinetic constants Ki for FX activation by intrinsic tenase complex (**Fig. 2.14C**) and RVV-X (**Fig. 2.15C**) were determined to be 1.67 ± 0.35 µM and 2.79 ± 0.29 µM, respectively.

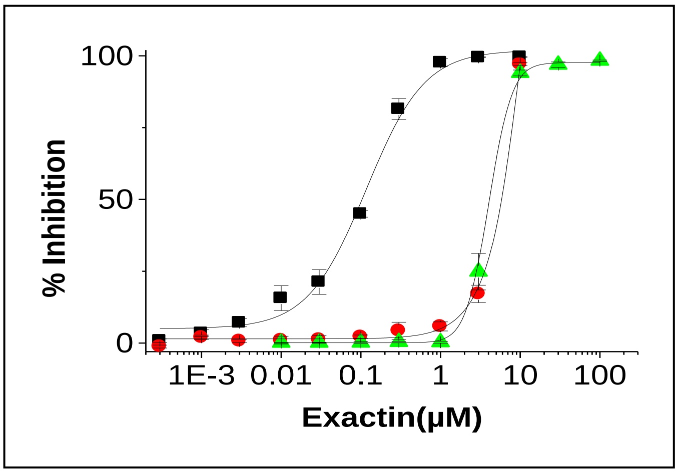


Figure 2.13: Specificity of exactin towards the extrinsic tenase complex. The inhibition of FX activation in the presence of exactin was compared among the three FX activators: extrinsic tenase complex (■) (IC_{50} - 116.49 ± 3.28 nM); intrinsic tenase complex (●) (IC_{50} - 4.05 ± 0.32 μM) and RVV-X (▲) (IC_{50} - 6.1 ± 2.9 μM). Exactin inhibited FX activation by the extrinsic tenase complex with high specificity. All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

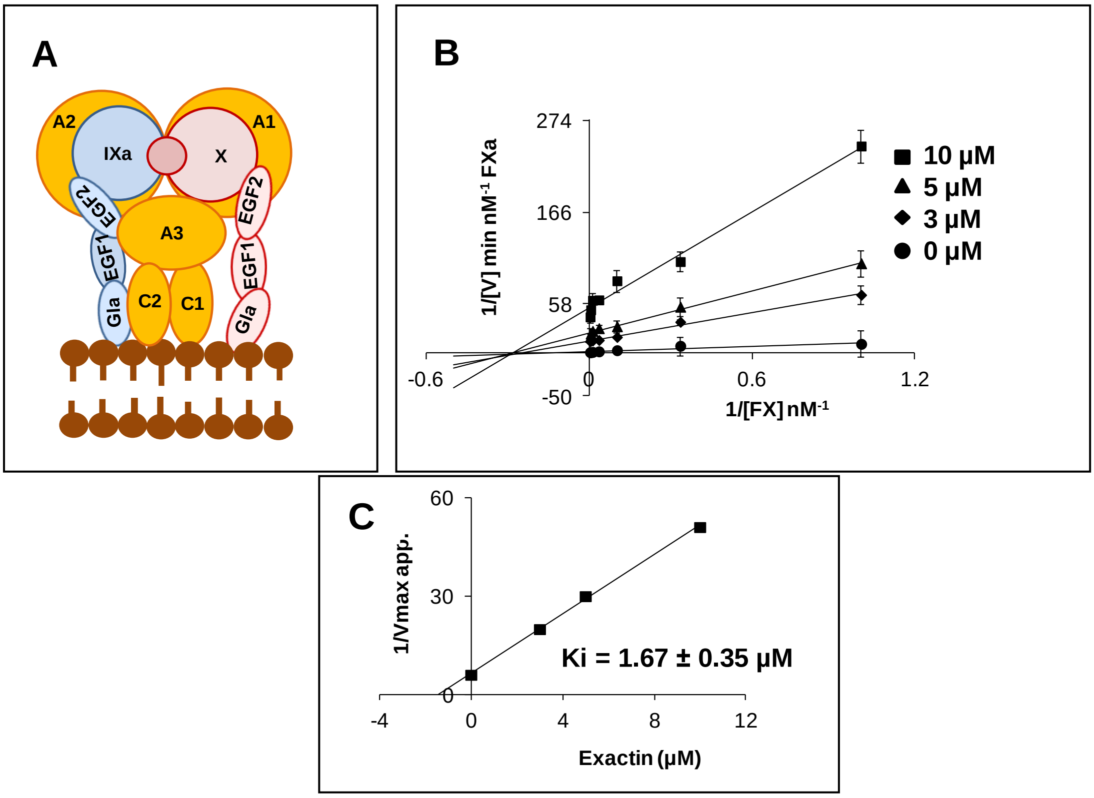


Figure 2.14: Kinetics of inhibition of FX activation by intrinsic tenase complex. **A)** Schematic representation of the intrinsic activation complex (FIXa/FVIIIa/FX). **B)** The Lineweaver-Burk plot for the kinetic activity of FX activation by FIXa/FVIIIa in the presence of exactin (0 µM, 3 µM, 5 µM and 10 µM). K_M remains unchanged while Vmax decreased with increase in inhibitor concentration, characteristic of non-competitive inhibition. **C)** The corresponding secondary plot depicting kinetic constant Ki of 1.67 ± 0.35 µM. All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

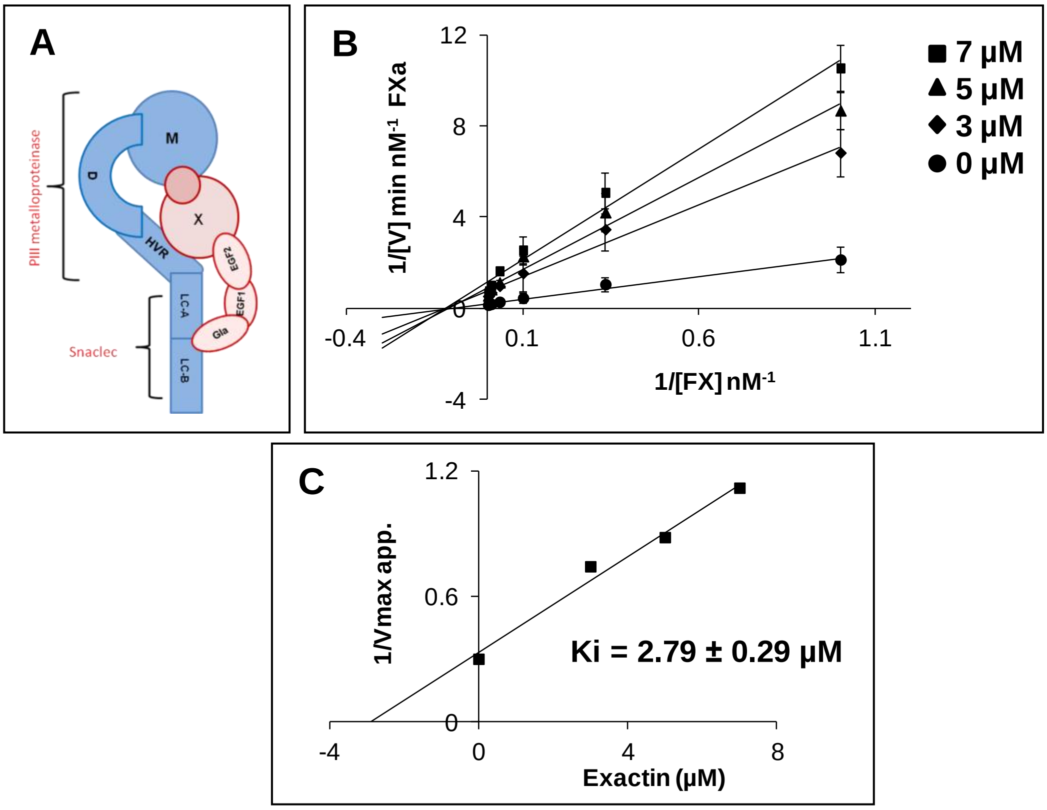


Figure 2.15: Kinetics of inhibition of FX activation by RVV-X. A) Schematic representation of RVV-X bound to FX. **B)** The Lineweaver-Burk plot for the kinetic activity of FX activation by RVV-X in the presence of exactin (0 µM, 3 µM, 5 µM and 7 µM). K_M remains unchanged while Vmax decreased with increase in inhibitor concentration, characteristic of non-competitive inhibition. **C)** The corresponding secondary plot depicting kinetic constant Ki of 2.79 ± 0.29 µM. All the experiments were conducted in n=4 and the results were expressed as mean ± SD.

Table 2.4: Kinetic parameters for FX activation by intrinsic tenase complex

Exactin (μM)	Km (nM)	Kcat (s^{-1})	Kcat/Km (s^{-1} nM^{-1})
0	4.31 ± 0.62	0.48 ± 0.064	0.11 ± 0.012
3	3.26 ± 0.46	0.07 ± 0.0024	0.021 ± 0.002
5	3.21 ± 0.24	0.042 ± 0.004	0.013 ± 0.002
10	3.23 ± 0.24	0.022 ± 0.004	0.007 ± 0.002

Table 2.5: Kinetic parameters for FX activation by RVV-X

Exactin (μM)	Km (nM)	Kcat (s^{-1})	Kcat/Km (s^{-1} nM^{-1})
0	8.48 ± 1.06	0.85 ± 0.027	0.101 ± 0.01
3	8.49 ± 0.55	0.23 ± 0.044	0.0272 ± 0.006
5	9.19 ± 0.53	0.19 ± 0.023	0.021 ± 0.002
7	8.56 ± 0.32	0.15 ± 0.018	0.017 ± 0.0015

2.3.7.2. Specificity of exactin towards FX

We have shown that exactin is specific towards the extrinsic tenase complex in activating FX when compared to other two activators (intrinsic tenase complex and RVV-X). Inorder to understand the macromolecular substrate specificity of exactin, the inhibitory potency of exactin towards the activation of FX and FIX by extrinsic tenase complex was compared. Both FX and FIX serves as macromolecular substrates for the extrinsic tenase complex (Fujikawa *et al.*, 1974; Zögg and Brandstetter, 2011). Exactin inhibited FIX activation with an IC_{50} value of 29.66 ± 5.27 µM. However, when compared to FX activation it was >100-fold lower (**Fig. 2.16A**). Exactin also exhibited a mixed-type inhibition towards FIX activation by the extrinsic tenase complex (**Fig. 2.17B**). The kinetic constants Ki', Ki as well as the kinetic parameters (K_M and K_{cat}) (**Table 2.6**) were determined. The K_M and Vmax decreased with increase in inhibitor concentration, typical for mixed type inhibition. The kinetic constants Ki' and Ki values were 38.66 ± 10.27 µM and 128.6 ± 12.55 µM (**Fig. 2.17 C,D**), respectively. The effect of exactin on the amidolytic activity of FIXa was evaluated and found to be significantly lower (IC_{50} > 300 µM) (**Fig. 2.16B**). The results further support the findings that exactin is a highly specific inhibitor of FX activation by the extrinsic tenase complex.

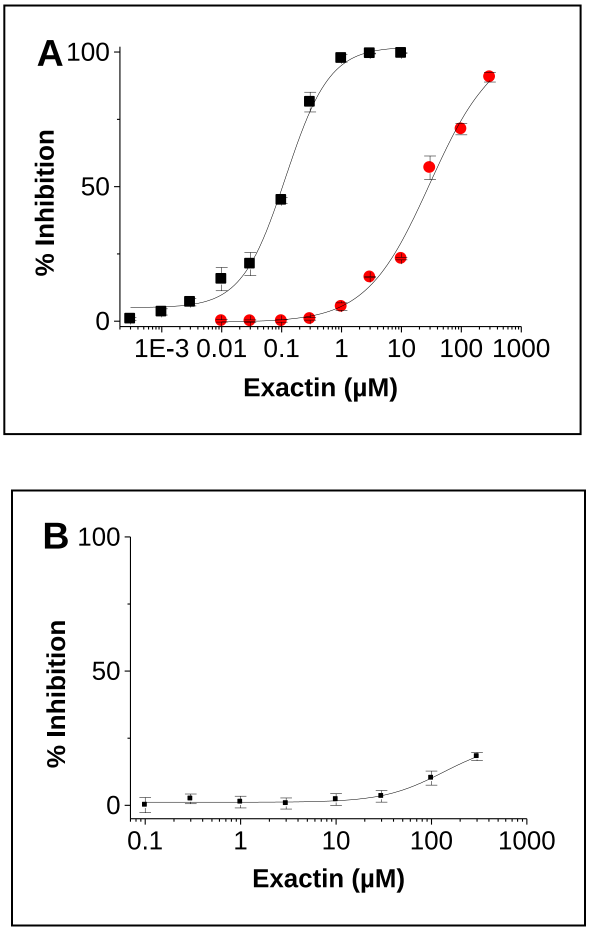


Figure 2.16: Specificity of exactin towards FX. **A)** The inhibitory potency of exactin towards the activation of the two macromolecular substrates (FX and FIX) of extrinsic tenase was evaluated. IC_{50} values of 116.49 ± 3.28 nM and 29.66 ± 5.27 µM for FX (■) and FIX activation (●) were determined, respectively. **B)** The effect of exactin on FIXa amidolytic activity was studied. Exactin was a poor inhibitor of FIXa. All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

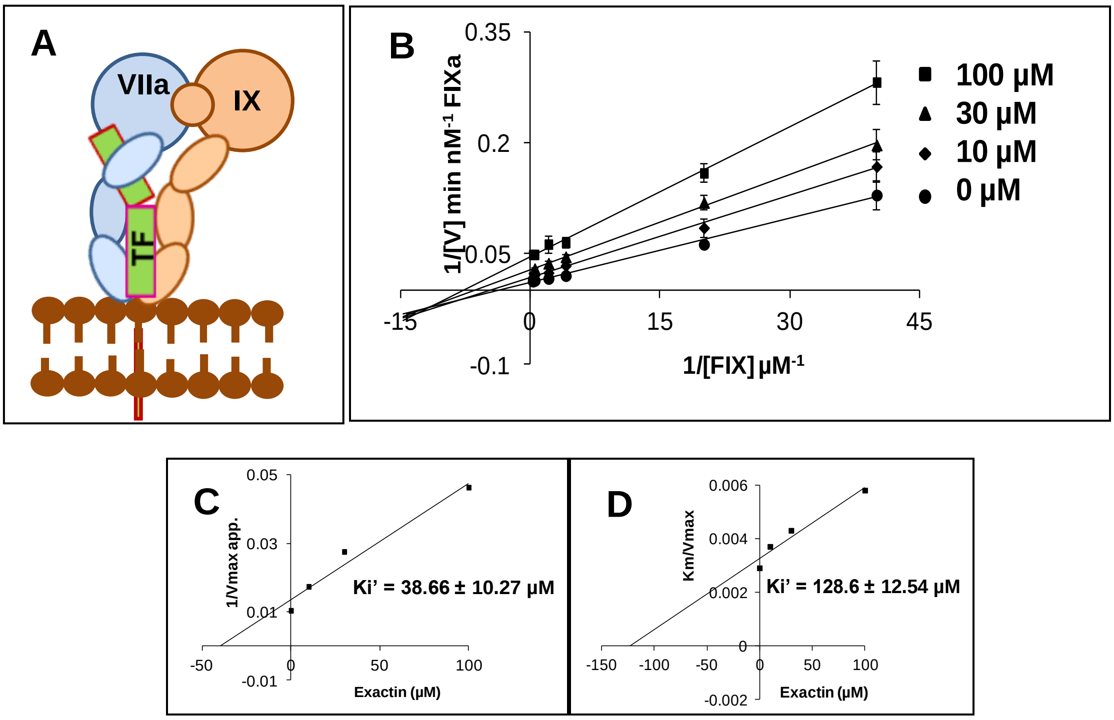


Figure 2.17: Kinetics of inhibition of FIX activation by extrinsic tenase complex. **A)** Schematic representation of FVIIa/TF/FIX complex on the phospholipid membrane). **B)** The Lineweaver-Burk plot for the kinetic activity of FIX activation by FVIIa/TF_{PL} in the presence of exactin (0 µM, 10 µM and 100 µM). Both V_{max} and K_M decreased with increase in inhibitor concentration, characteristic of mixed-type inhibition. **C and D)** Corresponding secondary plots depicting kinetic constants Ki' and Ki for inhibition. Ki' of 38.66 ± 10.27 µM was obtained towards the enzyme-substrate complex (FVIIa/TF_{PL}/FIX) where as Ki of 128.6 ± 12.54 µM was obtained towards the enzyme (FVIIa/sTF). All the experiments were conducted in n=3 and the results were expressed as mean ± SD.

Table 2.6: Kinetic parameters for FIX activation by extrinsic tenase complex

Exactin (μM)	**Km (nM)**	**Kcat (s^{-1})**	**Kcat/Km**
0	299.13 ± 7.84	1.34 ± 0.012	0.0044 ± 0.00015
10	231.83 ± 6.83	0.94 ± 0.0007	0.004 ± 0.0001
30	189± 11.72	0.744 ± 0.018	0.0039 ± 0.00017
100	156.2 ± 2.74	0.353 ± 0.01	0.0022 ± 0.00006

2.3.8. Partial characterization of other extrinsic activation inhibitors

Initial screening for anticoagulants from the crude venom of *Hemachatus* has yielded many inhibitors targeting the FX activation by the tenase complex (**Fig. 2.3**). A few of the inhibitors were purified and IC_{50} values determined. Currently, the structural and functional characterizations of these inhibitors are being carried out. Since these anticoagulants are not named, they will be denoted by their respective masses. Anticoagulant proteins from Peak 2 (blue arrow), peak 6 (brown arrow) and peak 10 (green arrow) were partially characterized.

2.3.8.1. Characterization of anticoagulant protein with mass 7279 Da

The protein with mass of 7279.66 ± 0.98 Da was purified from peak 2 indicated by blue arrow (**Fig. 2.3, 2.18A,B**). The dose-response effect of 7277 on FX activation by extrinsic tenase complex was examined and the IC_{50} value of 17.37 ± 1.91 nM was determined (**Fig. 2.18c**). We have not determined the amino acid sequence of this protein.

2.3.8.2. Characterization of anticoagulant protein with mass 7438 Da

[*Note: The work on this protein was continued by Bhaskar Barnwal, a graduate student from my lab. The partial amino acid sequence was determined by both of us.*]

The peak 6 (red arrow) was sub-chromatographed to purify the anticoagulant protein with mass 7438.63 ± 1.7 Da (**Fig. 2.3, 2.19A,B**). The protein inhibited FX activation by extrinsic tenase complex with an IC_{50} value of 52.6 ± 3.7 nM (**Fig. 2.19C**). The partial sequence of the native protein was determined through Edman degradation. Sequence comparison showed its high identity towards 3FTxs, especially to a neurotoxin like protein from *Naja naja* (**Fig. 2.19D**).

2.3.8.3. Characterization of anticoagulant proteins from peak 10

[*Note: The partial amino acid sequence of proteins* $P10_b$ *and* $P10_d$ *were determined by me and Dr. Guna Shekhar, who continued with the characterization of these proteins*]

The peak 10 (**Fig. 2.3**) contained two major proteins 6770 Da and 6800 Da as determined by ESI-MS, so we went on to separate the proteins by cation exchange chromatography on a Shodex SP-825 column. The column was equilibrated with 25 mM citrate buffer pH 5 and the proteins were eluted with 1M NaCl in 25 mM citrate buffer pH 5. Peak 10 was fractionated into four peaks (P10a, P10b, P10c and P10d) (**Fig. 2.20A**). Each peak was run on a C-18 reverse-phase column. All the four proteins showed similar elution times in RP-HPLC (**Fig. 2.20B**). The mass of the individual proteins were determined by ESI-MS. Proteins P10a and P10c differed in 1 Da mass with P10a as 6771.10±0.028 Da and P10c as 6770.08±0.02 Da. Similarly, the proteins P10b and P10d also differed in 1 Da mass with P10b as 6801.31±0.05 Da and P10d as 6800.3±0.06 Da. The similar elution profile of the four proteins from peak 10 in RP-HPLC as well as the mass difference of 1 Da suggests that the four proteins might be isoforms. The peak 10 with all the four isoforms exhibited a dose-dependent inhibition of FX activation by extrinsic tenase complex ($IC_{50} = 1.11 \pm 0.27$ µg/ml) (**Fig. 2.20C**), however the individual isoforms failed to exhibit any inhibition at concentration of 1 µM, suggesting a synergism among the isoforms for mediating anticoagulant property (*data not shown*). The partial amino acid sequence for all the four isoforms were determined and showed a difference of one residue. P10a, P10c and P10b, P10d differed in one residue at position 17 (D to N) explaining the 1 Da difference. Also, P10a, P10b and P10c, P10d differed in positions 10 and 33, explaining the 30 Da difference among them. This suggests that both proteins might be identical in sequence towards their C-terminal. The sequence alignment showed that the proteins shared similarity to other three-finger toxins especially the cytotoxins (**Fig. 2.20D,E**).

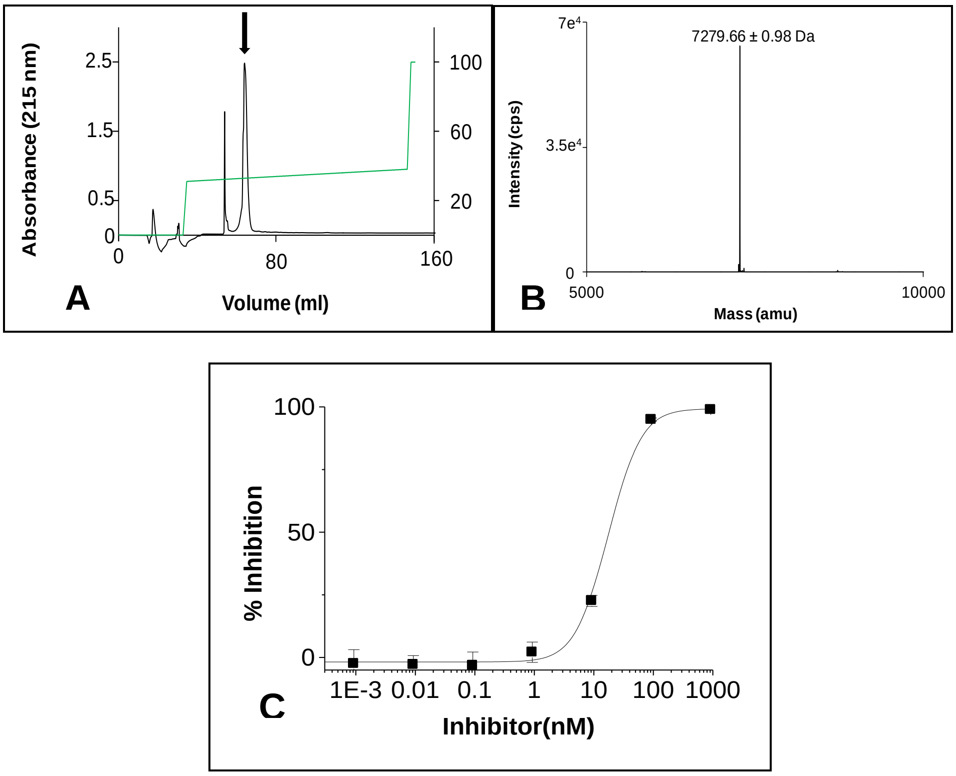
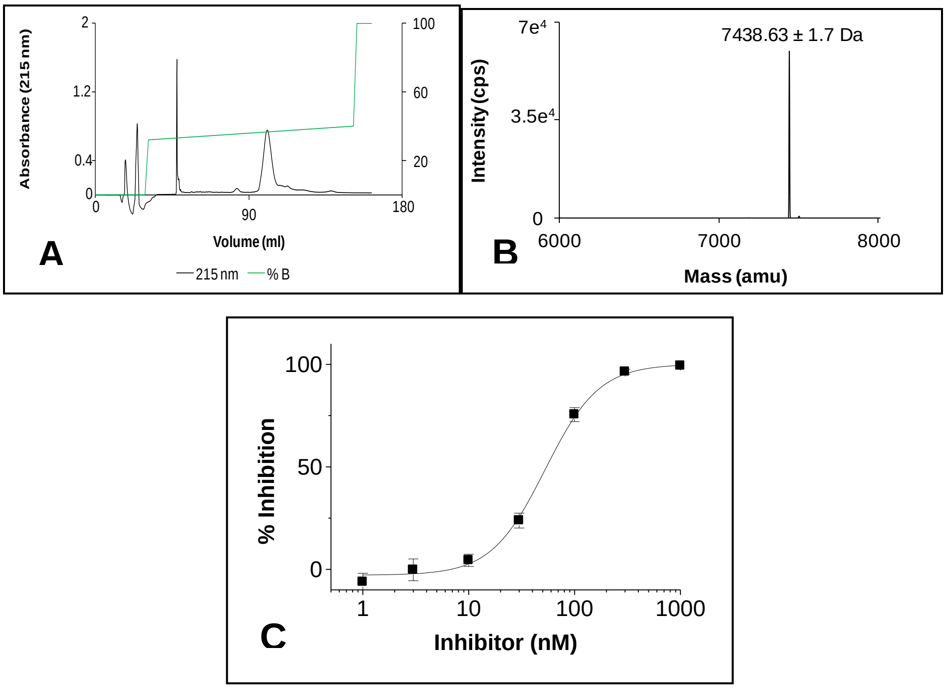


Figure: 2.18. Characterization of the anticoagulant protein with mass 7279 Da. **A)** The purification of anticoagulant protein with mass 7279 Da on RP-HPLC using a Jupiter C_{18} column (4.6 X 250 mm). **B)** The mass of the purified protein as determined by ESI-MS was found to be 7279.66 ± 0.98 Da. **C)** The anticoagulant property of the protein as determined on FX activation by the extrinsic tenase complex. An IC_{50} value of 17.37 ± 1.91 nM was obtained. The enzyme assays were done with n=3 and data expressed as mean ± SD.



D

		% Id
7439	RLCLSDYSIFSETIEICPEGHNYCFKKFPKGITRLPWVI	
NTL2	RLCLSDYSIFSETIEICPDGHNFCFKKFPKGITRLPWVI	95
D2A	LLCLQKYSPGRETSQTCLAGENICFKKWKKGEKTSRXGC	43
Toxin 7	RKCLTKYSRDNESSKTCPSGQNVCFKKWEMGNSSEKNAK	38
MT1	LTCVTSKSIFGITTENCPDGQNLCFKKWYYIVPRYSDIT	38
MTLP-2	LTCVKEKSIFGVTTEDCPDGQNLCFKRWHMIVPGRYKKT	35

Figure: 2.19. Characterization of the anticoagulant protein with mass 7438 Da. **A)** The purification of anticoagulant protein with mass 7438 Da on RP-HPLC using a Jupiter C_{18} column (4.6 X 250 mm). **B)** The mass of the purified protein as determined by ESI-MS was found to be 7438.63 ± 1.7 Da. **C)** The anticoagulant property of the protein as determined on FX activation by the extrinsic tenase complex. An IC_{50} value of 52.6 ± 3.7 nM was obtained. **D)** The partial amino acid sequence of 7438 as determined by Edman degradation. The conserved cysteine residues and identical residues are highlighted in black. For sequence comparison, the following sequences were used: neurotoxin like protein NTL2 from *N. naja*; short neurotoxin D2A from *M. pyrrhocryptus*; toxin 7 from *B. flaviceps*; muscarinic toxin 1 MT1 from *D. angusticeps* and muscarinic toxin like protein 2 MTLP 2 from *N. kaouthia*. The enzyme assays were done with n=3 and data expressed as mean ± SD.

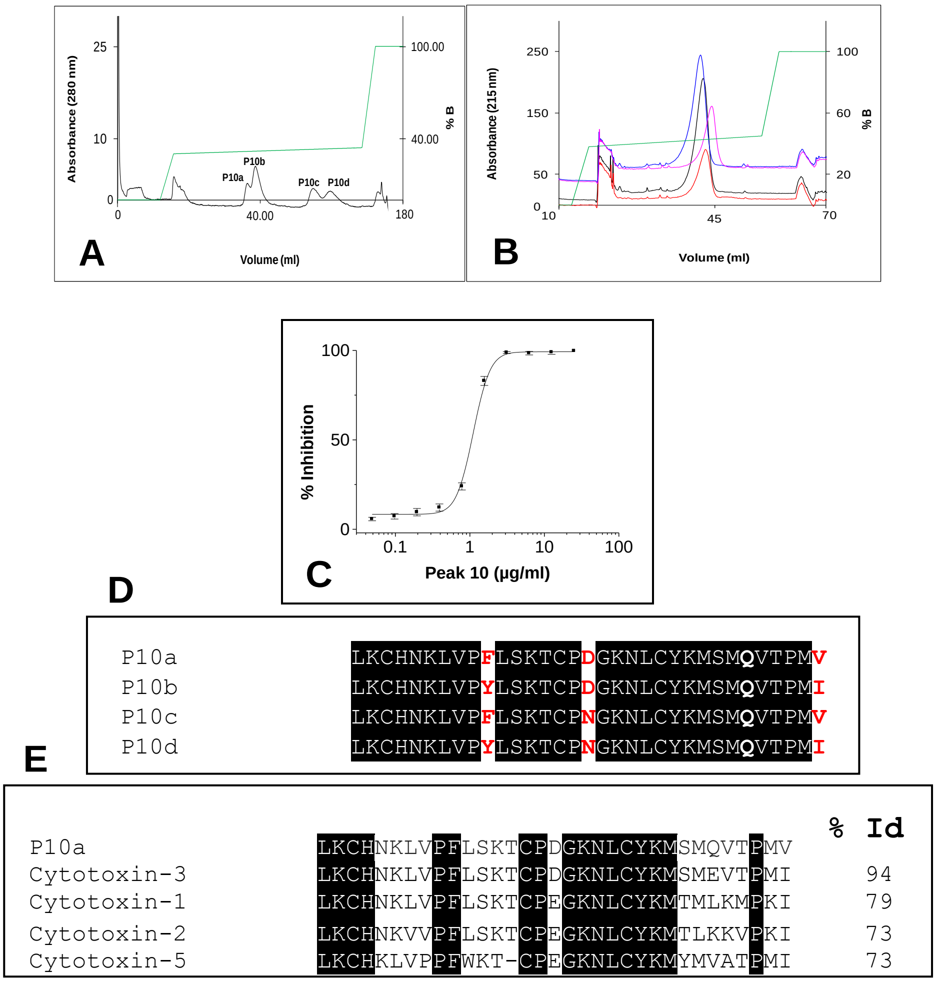


Figure 2.20: Characterization of anticoagulant proteins from peak 10. A) Peak 10 was separated into four proteins denoted as P10a, P10b, P10c and P10d on cation-exchange chromatography. The proteins were eluted using 1M NaCl in 25 mM citric acid buffer, pH 5. **B**) Proteins in each peak from cation-exchange chromatography co-eluted together in RP-HPLC, suggesting them to be isoforms. **C**) The effect of peak 10 having the four isoforms as evaluated on FX activation by the extrinsic tenase complex. An IC_{50} value of 1.11 ± 0.27 µg/ml was obtained. **D**) Represents the partial sequence of peak 10 isoforms. P10a, P10c and P10b, P10d differ at 17th position (D to N) explaining the 1 Da difference among these isoforms. However P10a, P10b and P10c, P10d differ by 30 Da that can be explained by the difference in 10th and 33rd position. **E**) Partial sequence of P10a was compared with other cytotoxins. Comparisons were made with cytotoxin-3, cytotoxin-1 and cytotoxin-2 from *H. haemachatus* and cytotoxin-5 from *N. annulifera*.

2.4. Discussion

Cardiovascular and cerebrovascular diseases resulting from unwanted clots are the major cause of death in developed countries (Mann *et al*., 2003; Gross and Weitz, 2008). Anticoagulants prevent the formation of unwanted clots and hence death. Due to their non-specific mode of action, currently used anticoagulants like warfarins and heparins have limited therapeutic window (Nutescu, 2006; Fareed *et al*., 2008). Thus there is a need for novel anticoagulants that can target specific steps in the coagulation pathway. The extrinsic activation complex is crucial for the initiation of the blood coagulation cascade in response to a vascular injury (Hedner and Ezban, 2008; Ott, 2011) and hence been identified as an important target for anticoagulation therapies. Over the years, several specific inhibitors to this complex have been characterized. These inhibitors are from two key sources- saliva/salivary gland extracts of hematophagous animals and snake venoms (Stark and James, 1996; Koh and Kini, 2008). Though various snake venom proteins targeting the extrinsic activating complex have been characterized (Kini, 2006; Yamazaki and Morita, 2007), very little information is known about anticoagulant 3FTxs. Recently an anticoagulant 3FTx, hemextin from the venom of *H. haemachatus* was characterized. Hemextin targets the extrinsic activating complex, specifically FVIIa non-competitively with nanomolar affinity (Banerjee *et al*., 2005; Banerjee *et al*., 2007). In search of novel anticoagulants targeting the extrinsic activation complex, the crude venom of *H. haemachatus* was screened. Preliminary studies showed proteins in both peak 2 and 3 of SEC prolonging the plasma clotting time (**Fig. 2.2**), indicating their anticoagulant properties. Peaks 2 and 3 contained proteins that mostly belong to phospholipase A_2 and 3FTx families, respectively (Joubert, 1975; Yang and King, 1980). Phospholipase A_2 mediates their anticoagulant activity through enzymatic mechanisms. Since major focus was given in characterizing the non-enzymatic anticoagulants, 3FTxs were purified from peak 3. Peak 3 on RP-HPLC revealed the presence of a number of anticoagulants targeting the extrinsic activation complex (**Fig. 2.3**). However, exactin was purified and characterized (*peak marked with black arrow*) because of the following reasons. Initial plasma clotting studies with all the peaks from RP-HPLC of peak 3 revealed that exactin peak (*peak marked with black arrow*) and peak 10 (*peak marked with green arrow*) prolonged the prothrombin time (>150s; n = 2) more significantly when compared to others. Moreover, exactin exhibited high inhibitiory potency towards FX activation by the extrinsic tenase complex when compared to peak 10 (**Fig. 2.3**).

The complete amino acid sequence of exactin was determined by Edman degradation. The sequence alignment has revealed its high identity towards short neurotoxins (>58 %). However, it shared low identity (<35 %) towards the well characterized short-chain α-neurotoxins (like erabutoxin A, toxin-α, haditoxin, α-neurotoxin) and anticoagulant 3FTxs (like hemextin, siamextin and naniproin). Anticoagulant 3FTxs characterized to date are highly identical to cardiotoxins/cytotoxins. Thus exactin shared a unique sequence when compared to other 3FTxs.

Inorder to delineate the anticoagulant site of exactin in the coagulation cascade, we used the ‘Dissection approach’ (Kini and Banerjee, 2005). Previously, this approach was used to understand the anticoagulation site of hemextin (Banerjee et al., 2005) and naniproin (*Unpublished data*). Hemextin, a FVIIa inhibitor prolonged the prothrombin time without affecting the Stypven time and thrombin time suggesting its target as the extrinsic activation complex while naniproin from *N. nigricollis* prolonged Stypven time more significantly when compared to APTT and prothrombin time. But thrombin time was not affected, suggesting its target as the prothrombinase complex. Exactin like hemextin also prolonged the prothrombin time more significantly than APTT and Stypven time without affecting thrombin time, suggesting its target as the extrinsic activation complex. Inorder to validate the results with plasma clotting times, the effect of exactin towards the reconstituted extrinsic tenase complex, intrinsic tenase complex, prothrombinase complex and thrombin amidolytic activity was evaluated. Exactin exhibited high inhibitory potency towards FX activation by the extrinsic tenase complex (IC_{50}=116.49 ± 3.28 nM) compared to FX activation by intrinsic tenase complex (>30-folds), PT activation by prothrombinase complex (>100-folds) and thrombin amidolytic activity (>1000-folds). The results thus confirm the anticoagulation site of exactin to be the extrinsic activation complex.

Kinetic studies showed that exactin exhibits a mixed-type inhibition towards FX activation by extrinsic tenase complex, $FVIIa_{PL}$ and FVIIa/sTF. The affinity of the inhibitor towards the enzyme-substrate complex ($FVIIa/TF_{PL}$/FX), Ki' 30.62 ± 7.73 nM) was 5-fold higher compared to the enzyme complex ($FVIIa/TF_{PL}$), Ki 153.75 ± 17.96 nM) suggesting its preference to [ES] complex. Though exactin inhibited FX activation by extrinsic tenase complex and $FVIIa_{PL}$ with equi-potency (116.49 ± 3.28 nM versus 102.7 ± 11.71 nM), in the absence of TF Ki' dropped 3-fold to 103 ± 13.49 nM with a slight decrease in Ki of 184.25 ± 6.13 nM. Thus, exactin appears to bind to the complete complex better than FVIIa/FX complex. The 3-folds difference in affinity could be explained by the fact FX interacts with both TF as well as FVIIa on the membrane surface to form a perfect extrinsic activation complex for its efficient catalysis. These exosite interactions determine the affinity of FX towards the extrinsic tenase complex (Baugh *et al*., 2000). Even the macromolecular binding exosite on FVIIa is modulated by allosteric binding of TF (Ruf *et al*., 1992; Ruf *et al*., 1992;

Huang *et al*., 1996; Kirchhofer *et al*., 2000; Eigenbrot, 2002). However, the unaltered affinity to enzyme suggests that exactin can interact well with FVIIa even in the absence of its cofactor. The affinity of exactin was drastically reduced by >1000-folds for FX activation in the absence of phospholipids (Ki', 295 ± 7.07 µM; Ki, 1250 ± 56.6 µM), suggesting its preference towards membrane-bound complex. The importance of phospholipids in FX catalysis by extrinsic tenase complex is well studied (Bom and Bertina, 1990). It has been hypothesized that conformational alterations of factor **X** upon phospholipid binding change susceptibility of the Arg-Ile bond to proteolytic activation, presumably by influencing recognition and peptide bond hydrolysis by the tenase complex (Ruf *et al*., 1991). These conformational changes on FX upon phospholipid binding could be compared with that of prothrombin binding to the membrane surface via its Gla domain. FXa in the absence of phospholipids initially cleave at Arg^{273}-Thr^{274} to form prethrombin 2 as an intermediate. However, in the presence of cofactor FVa and phospholipids, FXa preferentially cleaves at Arg^{322}-Ile^{323} of prothrombin to form meizothrombin as intermediate. This reaction required Gla domain as Gla domainless prothrombin upon cleavage by FXa produced prethrombin 2 (Malhotra *et al*., 1985). The drastic drop in Ki' and Ki for exactin in the absence of phospholipids could be explained by the fact that substrate-membrane interaction is important for the efficient catalysis of human FX by the extrinsic tenase complex. Proper anchorage of coagulation factors FVIIa and FX on to the membrane surface via Gla domain enable in the efficient formation of the extrinsic activation complex (Nelsestuen et al., 1978; Krishnaswamy *et al*., 1992). Also FRET analysis has shown that once bound to the membrane surface, the active site of both FVIIa and FXa are oriented perpendicular above the membrane surface (Husten *et al*., 1987; Morrissey, 2001). Exactin however, demonstrated poor inhibition towards the amidolytic activities of FVIIa and FXa (**Fig. 2.9B**), suggesting that it does not target the active site of the coagulation factors. Moreover, the results support the fact that exactin preferably inhibits the proteolytic activation of the macromolecular substrate FX. We have also determined the kinetic parameters (K_M and K_{cat}) for the inhibition of FX activation in the presence of exactin. The K_M and K_{cat} for FX activation by extrinsic tenase complex, $FVIIa_{PL}$ and FVIIa/sTF in the absence of exactin were obtained as 14.57 ± 0.87 nM; 1.7 ± 0.017s^{-1}, 36.56 ± 2.76 nM; 0.0558 ± 0.0046 s^{-1}, 1.03 ± 0.089 µM; 0.0017± 6.13 E-05 s^{-1}, respectively. These values were significant when compared to those determined previously (Bom and Bertina, 1990; Soejima *et al*., 2002; Chen *et al*., 2004; Waters *et al*., 2006). However, the K_M value calculated (14 nM) for FX activation by extrinsic tenase complex was 4-5 folds lower compared to that reported earlier. Probable reason for this difference could be attribuited to the tissue factor used. In all the experiments with extrinsic tenase complex, human recombinant tissue factor Innovin was used. As per the suppliers manual, Innovin contained PE in undetermined ratios apart from PC and PS. The effect of PE in enhancing FVIIa/TF proteolytic activity has been studied (Neuenschwander *et al*., 1995; Tavoosi *et al*., 2011). The kinetic analysis has shown a decrease in K_M and K_{cat} with increase in inhibitor concentration (**Table 2.1, 2.2, 2.3**), characteristic of mixed-type inhibition. This has also been observed with nitrophorin 2, a tick inhibitor that specifically inhibits intrinsic activation complex (Zhang *et al*., 1998). It could be thus inferred that binding of exactin to the extrinsic activation complex, increases the affinity of FX towards the tenase complex, however with a reduced catalysis. The kinetic analysis has thus given a better understanding of the mechanism of inhibition of the extrinsic activation complex by exactin.

FX activation involves a proteolytic cleavage of its Arg^{152}-Ile^{153} bond resulting in the release of a 52 residue activation peptide. This activation is catalyzed by the extrinsic tenase complex, intrinsic tenase complex and the snake veom FX activator RVV-X (Fujikawa et al., 1974; Bom and Bertina, 1990; Duffy and Lollar, 1992; Takeya *et al*., 1992; Siigur and Siigur, 2006). Exactin exhibited high specificity towards extrinsic tenase (IC_{50} - 116 nM) when compared to intrinsic tenase complex (IC_{50} - 4.05 ± 0.32 µM) and RVV-X (IC_{50} - 6.1 ± 2.9 µM) in inhibiting FX activation. Kinetic analysis of FX activation by intrinsic tenase and RVV-X in the presence of exactin was also studied. Exactin non-competitively inhibited the proteolytic activation of FX by intrinsic tenase complex and RVV-X (Ki of 1.67 ± 0.35 µM and 2.79 ± 0.29 µM, respectively). The kinetic parameters, K_M and K_{cat} were determined and were significant when compared to that reported previously (Van Dieijen et al., 1981; Baugh and Krishnaswamy, 1996; Gilbert and Arena, 1996; Wilkinson *et al*., 2002, **Table 2.4, Table 2.5**). This implies that exactin interferes with the proteolysis of FX as both intrinsic tenase and RVV-X, like extrinsic tenase activates FX by a similar activation mechanism. Also FVIIa and FIXa share a serine protease domain while RVV-X has got a metalloprotease domain (Fujikawa et al., 1974; Bom and Bertina, 1990; Duffy and Lollar, 1992; Takeya *et al*., 1992; Siigur and Siigur, 2010). Thus exactin exhibits high specificity in inhibiting FX activation specifically by the extrinsic tenase complex.

The extrinsic tenase complex can proteolytically activate both the macromolecular substrates FX and FIX (Fujikawa *et al*., 1974; Zögg and Brandstetter, 2011). Though the mechanisms of activation are different, FX represents a better macromolecular substrate when compared to FIX as the former has a higher affinity to phospholipid membranes (K_d for FX, 0.25 µM and K_d for FIX, 2 µM). Also the rate of activation of

FX is faster when compared to FIX (Fujikawa *et al*., 1974; Nelsestuen *et al*., 1978; Zur and Nemerson, 1980; Warn-Cramer and Bajaj, 1986; Komiyama *et al*., 1990; Zögg and Brandstetter, 2011). Exactin exhibited >200-folds inhibition to FX activation (IC_{50}, 116.49 ± 3.28 nM) when compared to FIX activation (IC_{50}, 29.66 ± 5.27 µM). Exactin exhibited a mixed-type inhibition (Ki', 38.66 ± 10.27 µM**;** Ki, 128.6 ± 12.54 µM) towards FIX activation as observed from a decrease in K_M and K_{cat} with increase in inhibitor concentration (**Table 2.6**). The kinetic parameters K_M and K_{cat} determined for FIX activation by extrinsic tenase complex were significant when compared to that reported previously (Zur and Nemerson, 1980; Warn-Cramer and Bajaj, 1986). Thus exactin specifically inhibits activation of FX compared to FIX, though both FX and FIX serves as the macromolecular substrate for the extrinsic tenase complex.

A mechanism was proposed to explain the inhibition of FX activation by exactin (**Fig. 2.21**). The kinetic analysis suggests that exactin specifically inhibits FX activation by the extrinsic tenase, preferably binding to FX (based on the non-competitive mode of inhibition of FX activation by intrinsic tenase complex and RVV-X), hence preventing its proteolytic cleavage. The affinity of exactin towards enzyme remains unaltered in the absence of TF, suggesting that exactin can interact well with FVIIa but not the active site (as per the results from FVIIa amidolytic activity). The removal of PL has drastically reduced the inhibition which can be attributed to the fact that PL helps in organizing the extrinsic activation complex for proper proteolysis (Tavoosi *et al*., 2011; Morrissey *et al*., 2011). Thus exactin prefers the entire enzyme-substrate complex (FVIIa/TF_{PL}/FX) to exhibit its inhibition. To date, the only functionally characterized classical molecules targeting the extrinsic activation complex (FVIIa/TF_{PL}/FX) are tissue factor pathway inhibitor TFPI (Girard *et al*., 1990;

Hamamoto *et al*., 1993), ixolaris (Francischetti *et al*., 2002; Monteiro *et al*., 2008), NAPc2 (Bergum *et al*., 2001; Vlasuk and Rote, 2002) and hemextin (Bannerjee *et al*., 2005). TFPI, ixolaris and NAPc2 inhibits the extrinsic activation complex with picomolar affinity (for details see Introduction) by binding to both FX(a) and FVIIa, thus affecting their amidolytic activities. Moreover, ixolaris and NAPc2 bind to the heparin binding exosite on FX(a). Hemextin on the other hand inhibits FVIIa alone without binding to FX (for details see Introduction). Exactin differs from these classical inhibitors in that it does not affect the amidolytic activities of either FVIIa or FXa. However, exactin binds to both FX and FVIIa (as exactin exhibits mixed-type inhibition with two different affinities to [ES] complex and E) and is highly specific in inhibiting FX activation by the extrinsic tenase complex. Currently, we do not have any information regarding the binding site of exactin on FX and FVIIa. Future experiments will thus focus on elucidating the binding site of exactin on the extrinsic activation complex.

Apart from exactin, we have also partially characterized other anticoagulants from the same venom. The anticoagulant protein with mass 7438 Da had a partial sequence homologous with neurotoxin like proteins and muscarinic toxins. It would be interesting to understand their anticoagulant mechanism as both exactin and 7438 were less identical. Similarly, peak 10 isoforms can also be an interesting target for anticoagulation studies, as our preliminary results suggest that there could be a synergism among the isoforms for their anticoagulant activity. The individual isoforms were not active at the highest possible concentration used (1 µM); however the peak 10 having the four isoforms exhibited potent inhibition towards FX activation. To conclude, *Hemachatus* crude venom has shown to be a repertoire of anticoagulants.

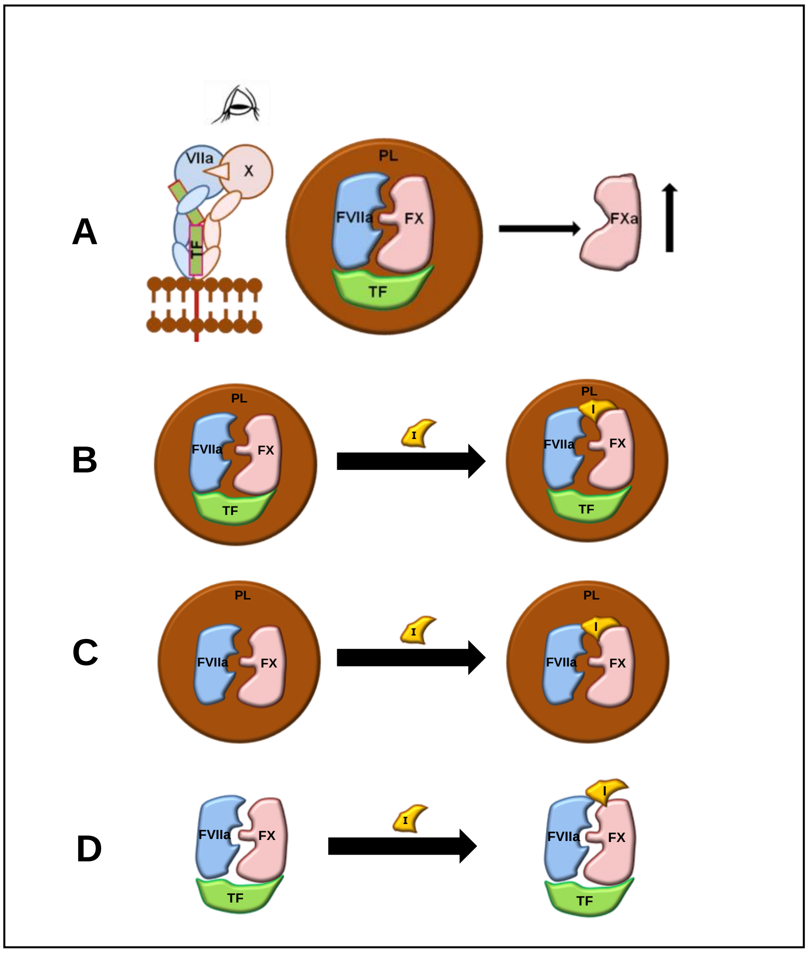


Figure: 2.21: Proposed inhibitory mechanism of exactin. **A**) Proteolytic activation of the macromolecular substrate FX by extrinsic tenase complex via the cleavage of Arg^{152}-Ile^{153}. Schematic representation of the extrinsic activation complex along with the top view is shown **B**) Exactin (represented as I) binds to the full extrinsic activation complex there by significantly reducing FX proteolysis. **C)** The removal of TF from the extrinsic activation complex however, does not alter the binding affinity of exactin and the inhibitor is able to inhibit FX proteolysis significantly. **D)** The removal of phospholipids from the extrinsic activation complex drastically reduced (>1000-folds) the inhibition of FX proteolysis by exactin, explaining the importance of phospholipids in exactin's inhibitory mechanism.

2.5. Conclusions

To summarize the results of this chapter, we have isolated, purified and determined the mechanism of inhibition of exactin, a novel 3FTx from the venom of *H. haemachatus*. Though the initial screening revealed the presence of a large number of inhibitors targeting the extrinsic activation complex, we purified and characterized exactin due to its high potency. Sequence alignment studies showed its identity to short neurotoxins. The 'Dissection approach' has assigned its target as 'extrinsic activation complex' (FVIIa/TF_{PL}/FX). Further detailed studies, involving the kinetics of inhibition as well as effect on macromolecular complexes (both enzyme and substrate), has shown that exactin specifically inhibits FX activation by extrinsic tenase complex (FVIIa/TF_{PL}). Based on the results, a mechanism was proposed to explain the inhibitory mechanism of exactin. Exactin inhibits FX activation, by preferably binding to the macromolecular substrate FX and preventing its catalysis by the extrinsic tenase complex. Even the removal of TF does not alter the inhibition significantly. However, in the absence of PL, the inhibition of FX activation dropped drastically, suggesting that exactin prefers the entire enzyme-substrate complex (FVIIa/TF_{PL}/FX) to exhibit its inhibition. The binding site of exactin on FVIIa and FX has yet to be elucidated. We have also partially characterized other anticoagulants. Currently, we are looking into their structure-function studies. Thus to conclude, we have successfully purified and characterized a novel 3FTx with a unique sequence and a unique mechanism of action when compared to the classical exogenous inhibitors of the extrinsic activation complex.

Chapter 3

Neurotoxic properties of exactin

3.1. Introduction

Snake venom toxins are known to interfere with cholinergic transmission (Chang and Lee, 1963; Karlsson *et al*., 1966; Karlsson, 1979) in the central and peripheral nervous systems and can be subdivided into three major classes - muscarinic toxins, fasciculins and neurotoxins. Since this thesis deals with the neurotoxic effects of exactin, a brief account of neurotoxic 3FTx has been given.

3.1.1. Snake venom neurotoxins

Snakes use their venom neurotoxins to paralyze the prey so that they can feed the prey more effectively. When bitten by the snake, these neurotoxins cause rapid paralysis of the voluntary muscles resulting in lack of movement and respiratory paralysis and finally death of the prey. Snake venom neurotoxins can be classified as either postsynaptic or presynaptic, depending on their site of action. Most of the postsynaptic neurotoxins fall into the group of 3FTxs also called as 'curaremimetic toxins' due to their similarity in action to the arrow poison curare, a competitive nicotinic acetylcholine receptor antagonist (Endo & Tamiya, 1991). Based on the target receptors as well as primary structures, postsynaptic neurotoxins can be classified as - curaremimetic or α-neurotoxins, κ-neurotoxins and non-conventional neurotoxins. The α-neurotoxins can be subdivided into short-chain α-neurotoxins (60-62 amino acid residues with four conserved disulfide bridges) and long-chain α-neurotoxins (66-74 amino acid residues with four conserved disulfide bonds with an additional disulfide bond in the tip of loop II) (Endo and Tamiya, 1991; Servent and Menez 2001). Both short-chain and long-chain α-neurotoxins target muscle (αβγδ or α1subtype) nAChRs (Nirthanan and Gwee, 2004; Tsetlin, 1999; Changeux, 1990). Apart from the structural differences, both short and long-chain neurotoxins differs functionally, mainly in the kinetics of association and dissociation with skeletal muscle nicotinic acetylcholine receptors (Chicheportiche *et al*., 1975; Tsetlin, 1999). Also long-chain neurotoxins but not short-chain neurotoxins targets the α7- nAChRs associated with neurotransmission in the brain (Servent *et al.*, 1997; Servent *et al*., 2000).

κ-neurotoxins are homodimeric complexes of 3FTxs (Dewan *et al.*, 1994). They are structurally similar to long chain α-neurotoxins with five disulfide bonds (the additional one is in the tip of loop II) but have a shorter C-terminal tail like the short chain α-neurotoxins, and intermediate number of (66) amino acid residues (Grant and Chiappinelli 1985; Fiordalisi *et al.*, 1994). The κ-bungarotoxin (*Bungarus multicinctus*), a member of this group show specificity for other neuronal subtypes, α3β2- and α4β2-nAChRs (Grant and Chiappinelli, 1985; Wolf *et al.*, 1988).

Non-conventional 3FTxs are characterized by the presence of 62-68 amino acid residues and a fifth disulfide bond in loop I compared to long chain α-neurotoxins where the fifth disulfide linkage is present in the second loop. These toxins are also referred as 'weak toxins' based on their low *in vivo* toxicity (LD_{50} ~ 5–80 mg/kg) when compared to the highly lethal α-neurotoxins (LD_{50} ~0.04–0.3 mg/kg) (Utkin *et al*., 2001). However, candoxin isolated from the venom of *Bungarus candidus* (Nirthanan *et al*., 2002) are exceptional to this convention in that candoxin produced a potent reversible neuromuscular blockade and also interacted with α7-nAChRs with nano molar affinity (Nirthanan *et al.*, 2002).

To understand the usefulness of exactin as an anticoagulant lead, we studied its toxicity on animals. This chapter thus covers the *in vivo* and *ex vivo* neurotoxicity studies of exactin. [*Note: the experiments done for this chapter were with the help of Ms. Sheena Foo, Mr. Bhaskar Barnwal and Mr. Bidhan Nayak*].

3.2. Material and Methods

3.2.1. Materials

Lyophilized *H. haemachatus* crude venom was purchased from South African Venom Suppliers (Louis Trichardt, South Africa) and exactin was purified as described in chapter 2. The drugs used for pharmacological studies - acetylcholine chloride and carbamylcholine chloride (carbachol) were purchased from Sigma-Aldrich (Missouri, USA). α-bungarotoxin from *B. multicinctus* was also purchased from Sigma-Aldrich. Erabutoxin b was purchased from Latoxan (Rue Léon Blum, France). Potassium chloride (KCl) was from Merck KGa (Darmstadt, Germany). All other chemicals and reagents used were of the highest purity. The stock solutions of the drugs and exactin were prepared fresh by dissolving in deionized water.

3.2.2. Animals

Animals (Swiss albino mice) acquired from the National University of Singapore Laboratory Animal Center were acclimatized to the Department Animal Holding Unit for at least 3 days before the experiments. Food and water were provided *ad libitum* in a light controlled room (12-h light/dark cycle, light on at 7:00 a.m.) at 23 °C and 60% relative humidity. Domestic chicks were purchased from Chew's Agricultural Farm, Singapore, and delivered on the day of experimentation. Animals were sacrificed by exposure to 100% carbon dioxide. All experiments were conducted according to the Protocol (021/07a) approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

3.2.3. *In vivo* toxicity studies

Exactin in 0.9% saline was injected intraperitoneally (i.p) using a 27-gauge 0.5-inch needle (BD Biosciences) into male Swiss albino mice (32 ± 2 g) at doses of 10 and 100 mg/kg (n=2). The symptoms were observed and the time it took to kill the mice was also noted. The control group was injected with 0.9% saline. Once the death was confirmed, postmortem examinations were conducted on all animals.

3.2.4. *Ex vivo* organ bath studies

The effect of exactin on chick biventer cervicis muscle (CBCM) preparations were carried out on a conventional organ bath (6 ml) containing Krebs solution [(in mM): 118 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, 2.4 $MgSO_4$, and 11 D-(+) glucose], pH 7.4 at 37 ºC (Nirthanan *et al*., 2002; Pawlak *et al*., 2009). The solution was continuously aerated with carbogen (5% CO_2 in oxygen). The resting tension of the tissues maintained at 0.5-1.5 g were allowed to equilibrate for 30–45 min. Electrical field stimulation was provided through platinum ring electrodes using a Grass stimulator S88 (Grass Instruments, West Warwick, RI). The magnitude of the contractile response was measured in gram tension. The CBCM nerve-skeletal muscle preparation (Ginsborg and Warriner, 1960) isolated from 6-day old chicks were mounted in the organ bath chamber under similar experimental conditions as described previously (Ginsborg and Warriner, 1960; Nirthanan *et al*., 2002). The effect of exactin (0.1 µM to 30 µM) on nerve-evoked twitch responses of the CBCM were examined. A dose-response curve for exactin's effect on neuromuscular blockade after 15 min of exposure was derived by continuously recording the data on a PowerLab/Chart 5 data acquisition system via a force displacement transducer (Model MLT0201) (AD Instruments, Bella Vista NSW, Australia). In separate experiments, the recovery from complete neuromuscular blockade was assessed by washing out the protein with Krebs solution at regular cycles of 30 s wash followed by 1 min interval over a period of 30 min.

3.3. Results

3.3.1. Neurotoxicity of exactin

3.3.1.1. *In vivo* toxicity studies

Exactin (10 and 100 mg/kg) were injected *i.p.* to study the *in vivo* toxicity. At 10 mg/kg, after 10 min the mice were stretching their hind limbs and were immobile. However, it regained its normal activity after 30 min. At 100 mg/kg, immediately after IP injection, the mice showed typical symptoms of peripheral neurotoxicity such as hind limb paralysis, immobilization and labored breathing (Laothong and Sitprija, 2001). The mice died within 35 min, presumably due to respiratory paralysis. Postmortem examination showed no significant changes, particularly hemorrhage or internal bleeding, were observed in their internal organs.

3.3.1.2. *Ex vivo* organ bath studies

To evaluate the observed peripheral neurotoxic symptoms, we studied the effect of exactin (0.1 to 30 μM) on neuromuscular junctions in CBCM preparations. Exactin (10 μM) produced a time-dependent neuromuscular blockage **(Fig. 3.1A)**. It also inhibited contractile responses of the exogenously applied nicotinic agonists (ACh and CCh), whereas responses to exogenous KCl and twitches evoked by direct muscle stimulation were not inhibited. These results indicated that exactin induces a postsynaptic neuromuscular blockage but lacks direct myotoxicity. Intermittent washing resulted 80% recovery of the twitch height in 8.5 min **(Fig. 3.1B)** denoting that neuromuscular block by exactin is reversible. Exactin induces a dose-dependent block with an EC_{50} value of 4.4 μM. When compared to erabutoxin b and α-bungarotoxin, exactin was 47- and 176-fold less potent (**Fig. 3.2**).

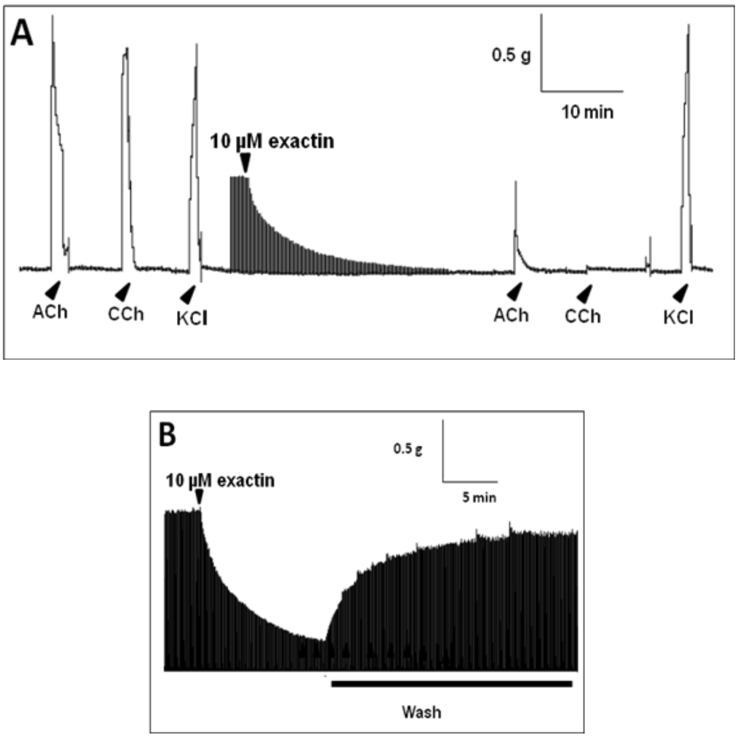


Figure 3.1: Effect of exactin on CBCM preparations. **A)** A segment of tracing showing the effect of 10 µM exactin on the twitch response of CBCM elicited by nerve stimulation and exogenously supplied acetylcholine (ACh), carbachol (CCh) and potassium chloride (KCl). The vertical bar represents the magnitude of twitch response in gram tension and the horizontal bar depicts the time in minute. **B)** Reversibility of the nerve evoked twitch response blockade produced by exactin in CBCM. The segment of tracing shows complete blockade of nerve evoked twitch response by 10µM exactin. Once complete blockade is achieved, exactin is removed from the bath chamber by extensive washing (at regular cycles of 30 s wash followed by 1 min interval over a period of 30 min) with Krebs solution. The vertical bar represents the magnitude of twitch response in gram tension and the horizontal bar depicts the time in minute. All the experiments were conducted in n=2.

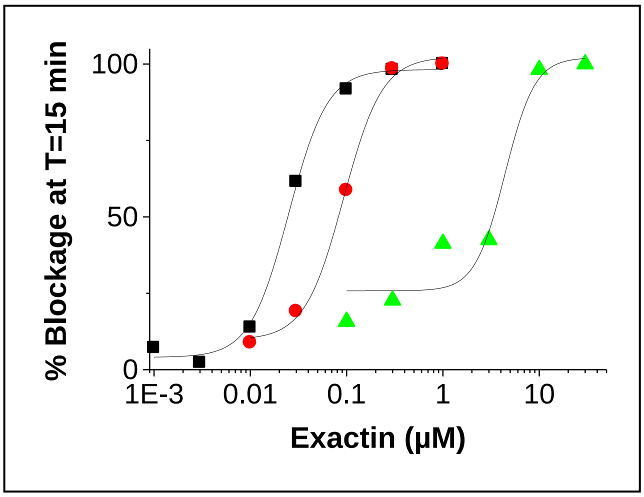


Figure 3.2: Dose-response effect of exactin on CBCM. The EC_{50} value for exactin (▲) on CBCM at T=15 min was determined to be as 4.4 μM. It was compared to erabutoxin b (●) (EC_{50} = 80 nM) a short-chain α-neurotoxin and α-bungarotoxin (■) (EC_{50} = 25 nM), a long-chain neurotoxin. All the experiments were conducted in n=2

```
Exactin       LECYQK-----SKVVTCQPEQKFCYSDTTMFFPNHPVYLSGCTFSCTEEGN----RRCCTTDKCNR
Toxin-α       LECHNQQSSQPPTTKTC-PGETNCYKKVWRDH-RGTIIERGCG--CPTVKP-GIKLNCCTTDKCNN  33
Haditoxin     TKCYNHQSTTPETTEICPDSGYFCYKSSWIDG-REGRIERGCTFTCPELTPNGKYVYCCRRDKCNQ  33
α-neurotoxin RICYNHQSTTRATTKSC--EENSCYKKYWRDH-RGTIIERGCG--CPKVKP-GVGIHCCQSDKCNY  26
Erabutoxin A RICFNHQSSQPQTTKTCSPGESSCYNKQWSDF-RGTIIERGCG--CPTVKP-GIKLSCCESEVCNN  26
Erabutoxin B RICFNHQSSQPQTTKTCSPGESSCYHKQWSDF-RGTIIERGCG--CPTVKP-GIKLSCCESEVCNN  26
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Figure 3.3: Sequence comparison of exactin to the well characterized short-chain α-neurotoxins. The conserved eight cysteine residues are highlighted in black. The key functional invariant residues responsible for neurotoxicity in erabutoxin, based on mutational studies are highlighted in red. The comparison was made with the following sequences: Toxin-α from *N. nigricollis*; haditoxin from *O. hannah*, α-neurotoxin from *D. polylepis polylepis*; erabutoxin a and erabutoxin b from *L. semifasciata*.

3.4. Discussion

Anticoagulant 3FTxs characterized to date belongs to cardiotoxins/cytotoxin group (Banerjee *et al*., 2005; naniproin, siamextin [unpublished observations]). Interestingly, exactin showed only <35% identity to this group **(Fig. 2.6B)**. Exactin shared highest identity to toxin CM-1b, CM-2a and CM-3. However, CM-1b from *Hemachatus* (Joubert and Taljaard, 1979) and CM-2a and CM-3 from *Naja haje annulifera* (Joubert, 1977) venom were not functionally characterized. Exactin also exhibited sequence identity (>58%) to many short-neurotoxins from *O. hannah* **(Fig. 2.6A)**. Most of them were not functionally characterized (He *et al*., 2004; Li *et al*., 2006) except for the toxin OH9-1. OH9-1 was found to be a potent irreversible postsynaptic neurotoxin on CBCM preparations. It exhibited EC_{50} value of 88 nM, 4 folds lower to α-bungarotoxin (Chang *et al*., 2002).

To evaluate the biological effect and hence its usefulness as a lead molecule, we studied the *in vivo* toxicity of exactin in mice. Typical symptoms of peripheral neurotoxicity such as hind limb paralysis, immobilization and labored breathing (Laothong and Sitprija, 2001) were observed in mice administered with high dose of exactin (100mg/kg) suggesting its biological role in affecting the neurotransmission. These observations were also similar to that found with curare-mimetic neurotoxins (Chang, 1979). Postmortem analysis of the toxin administered mice revealed the absence of hemorrhage or bleeding of the internal organs, suggesting that exactin might not have any cytotoxic effects. This is also supported by the fact that exactin exhibited <35 % sequence identity to cytotoxins (**Fig. 2.6B**). We then evaluated the effect of exactin on neuromuscular junction using CBCM preparations. Exactin exhibited a reversible postsynaptic blockade of neuromuscular junction with an EC_{50} value of 4.4 μM. However compared to erabutoxin b, a short-chain neurotoxin and α-bungarotoxin, a long-chain neurotoxin, the potency of exactin was 47 and 176 folds lower (**Fig. 3.2**). Also compared to OH9-1, exactin was found to be 50 fold less potent and readily reversible on neuromuscular blockade as derived from CBCM experiments. (Chang *et al*., 2002). The site directed mutagenesis studies have revealed the presence of many functional invariant residues in short-chain neurotoxins and long chain-neurotoxins towards the muscle type receptor (αβγδ) (Pillet *et al*., 1993; Trémeau *et al*., 1995, Fruchart-Gaillard et al., 2002; Dellisanti et al., 2007). The crucial residues for α-neurotoxins to bind to muscle (αβγδ) nAChRs are Lys-27, Trp-29, Asp-31, Phe-32, Arg-33, and Lys-47. Apart from these, residues His-6, Gln-7, Ser-8, Ser-9, and Gln-10 in loop I; Tyr-25, Gly-34, Ile-36, and Glu-38 in loop II and Lys-47 in loop III of short-chain α-neurotoxins are also involved in the recognition of the receptors (Teixeira-Clerc *et al*., 2002). Arg-36 is a muscle subtype-specific determinant residue for long chain neurotoxins like α-bungarotoxin. However, exactin lacks all the functional invariant residues, explaining its low neurotoxicity. Morever, exactin also shared low identity towards the well characterized short-chain α-neurotoxins (<35 % identity) (**Fig. 3.3**). Inorder to determine the affinity of exactin towards the muscle (αβγδ) nAChRs, we have collaborated with Prof. Daniel Bertrand of HiQSCREEN Sàrl, Geneva, Switzerland. We are yet to study the binding affinity of exactin towards the muscle type receptor.

3.5. Conclusions

To summarize the results of this chapter, we have studied the neurotoxic properties of exactin. Exactin is a highly potent anticoagulant towards the extrinsic activation complex. However, we need to understand its toxicity in animals for future therapeutic purposes. Our toxicity studies (*in vivo* and *ex vivo*) have shown that exactin exhibited a weak neurotoxicity in both mice and chick preparations. Though exactin shared homology with neurotoxins, compared to the well characterized short-chain α-neurotoxins it was less potent. Thus we conclude that exactin is a novel three-finger toxin with dual function; a potent and specific anticoagulant effect on the FX activation by the complete extrinsic tenase complex and a weak, reversible, postsynaptic neurotoxicity.

Chapter 4

Crystallization of exactin

4.1. Introduction

In our previous studies (chapter 2), we have shown that exactin is a novel 3FTx having a unique amino acid sequence. It exhibited low sequence identity (<35%) towards anticoagulant 3FTxs (**Fig. 2.6B**) as well as the well characterized short-chain α-neurotoxins **(Fig. 3.3)**. The CD profile showed the presence of predominant β-sheet structure. Apart from this, exactin also exhibited weak neuromuscular blockade (EC_{50} of 4.4 µM compared to 80 nM of erabutoxin b) on the CBCM preparations. There was a 47 folds difference in the potency when compared with erabutoxin b though exactin lacks all the functionally invariant residues. Moreover, our kinetic studies have shown that exactin is a potent inhibitor of FX activation. Thus exactin is characterized by its dual role in anticoagulation and neurotoxicity. This makes it an important candidate for structure-function studies for which it is necessary to deduce the structure of exactin.

X-ray crystallography was employed to determine the three-dimensional structure of exactin. This is based on the fact that electrons in the atoms forming the crystal can diffract X-rays and hence produce a particular diffraction pattern. A three-dimensional electron density map of the molecule can thus be created from the diffraction pattern. Protein crystallization marks the most important part of protein crystallography studies. It is impossible to carry out any crystallographic structural studies without perfect protein crystals. The aim of protein crystallization is thus to produce well-ordered protein mono-crystals large enough to obtain a good quality diffraction data. Keeping this in mind, the crystallization screening of exactin was started to identify the condition which can generate crystals. The initial crystals obtained can be further optimized to yield diffraction quality crystals. In general, this chapter deals with the crystallization of exactin. We have optimized the crystallization condition for exactin, in which we got good quality diffracting crystals. [*Note: the crystallization of exactin was done in collaboration with Prof. Sivaramans's lab. Mr. Thangavelu and Dr. Jobichen have helped me with diffraction of the exactin crystals as well as initial refinement and structure determination*].

4.2. Materials and Methods

4.2.1. Materials

Exactin was purified from *Hemachatus* crude venom as described in chapter 2. All the crystallization screens (Hampton), crystallization trays, cover slips and grease were purchased from Hampton Research (CA, USA).

4.2.2. Crystallization of exactin

Crystallization conditions for exactin were screened with Hampton research screens (Crystal screen 1 HR2-110, Crystal screen 2 HR2-112 and SaltRx HR2-108) using a hanging-drop vapour-diffusion method. The lyophilized protein was dissolved in 10 mM Tris-HCl buffer pH8.5. Crystallization experiments were carried out with 5 mg/ml, 10 mg/ml, 20 mg/ml 50 mg/ml and 100 mg/ml concentrations of exactin. The drops were prepared by mixing equal volumes of (1 µl) of protein and crystallization solutions. In each well, 500 µl of reservoir solution (crystallization solution) was placed. The plates were set at 25 ºC, 16 ºC and 4ºC. The crystals once got were subjected for optimization to generate diffraction quality crystals. For X-ray diffraction data collection, the crystals were briefly soaked in reservoir solution supplemented with various cryoprotectants like 10% glycerol, 70% sucrose, 15% inositol, 2-propanol, PEG-400 and propylene glycol. Once data collected, the crystals were flash-freezed in liquid nitrogen for future synchrotron studies.

4.2.3. X-ray diffraction and data collection

The crystals were mounted on a nylon cryo-loop and frozen in liquid nitrogen stream. Diffraction up to 2.7 Å was obtained using a CCD detector mounted on a Bruker V8 rotating anode generator (Bruker AXS, WI, USA). The complete data set collected was processed and scaled using the program HKL2000 (Otwinowski and Minor, 1997).

4.3. Results

4.3.1. Crystallization of exactin

In all the Hampton screens used, crystals were produced at 4ºC only in a single condition of crystal screen HR2-112 (condition 17: 0.1 M sodium citrate tribasic dehydrate pH 5.6, 35% tert-butanol). We have tried crystallization with various exactin concentrations. However, crystals were obtained only at 100 mg/ml concentration. The crystals obtained in this condition were optimized for better diffraction quality by preparing grid screens of varying concentration of tert-butanol (26% to 32%). Through these optimizations, diffracting quality crystals were obtained in the condition, 0.1 M sodium citrate tribasic dehydrate pH 5.6, 29% tert-butanol (**Fig. 4.1A**). The crystals diffracted up to 2.7 Å (**Fig. 4.1B**). The diffraction data was indexed and scaled using HKL2000. The crystals belonged to the C222 space group (orthorhombic crystal system) (**Table 4.1**). However, ice rings were observed in the diffraction pattern, even with the various cryoprotectants used. The data obtained was used to solve the structure of exactin using molecular replacement method. For this muscarinic toxin 2 isolated from *D. angusticeps* was used as a search model (pdb code 1FF4; sequence identity – 33%). There were 4 molecules in the asymmetric unit with 52% solvent content.

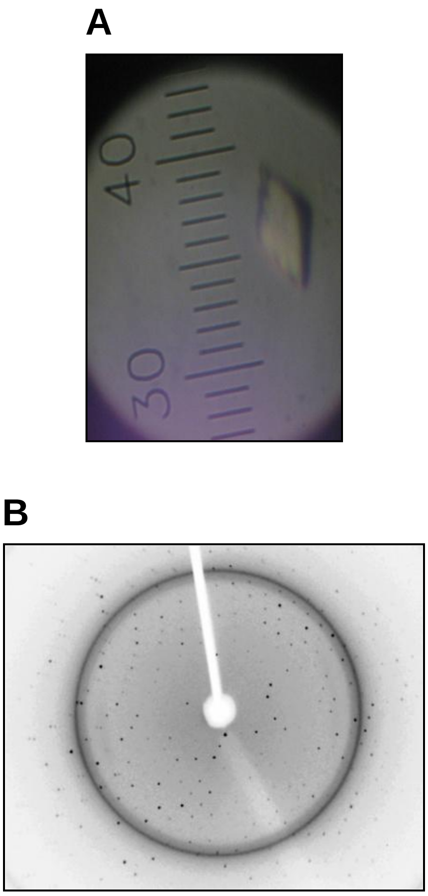

Figure 4.1: Crystallization of exactin. **A)** The diffraction quality crystals of exactin were obtained in 0.1 M sodium citrate tribasic dehydrate pH 5.6, 29% tert-butanol. **B)** The diffraction pattern of exactin collected on a Bruker V8 rotating anode generator (Bruker AXS, WI, USA).

Table 4.1: Crystallization parameters

Data set	
Cell parameters and space group	a=45.14, b=89.44, c=133.86 $\alpha=\beta=\gamma=90^{o}$ C222
Resolution range (Å)	50 to 2.7
Wavelength (Å)	1.5418
Observed reflections >1σ	94907
Unique reflections	7754
Completeness (%)	99.6 (100)
Overall (I/σI)	7.3
$R_{sym}{}^{a}$ (%)	9.7 (16.5)

4.4. Discussion

Even though very good diffracting quality crystals were, attempts to solve the structure using the molecular replacement was not successful. Various programs like Phaser and Molrep using the 33% identical homolog were used to obtain the phases. The data was subjected to twinning analysis and it showed the presence of twinning. This might be one reason for the failure of molecular replacement programs. Further crystallization trials have to be carried out to crystallize the protein in a different form so that an untwined data can be obtained. Another aspect might be the low sequence identity (33%) of the model used for molecular replacement with the protein of interest. Even with some small differences in the structure between the model and target protein, the molecular replacement programs will not be able to get a correct solution.

The classical way to overcome this issue is going for heavy atom phasing, like MIR, SAD or MAD. But in this case the protein is purified from a native source which makes it difficult to carry out the heavy atom labelling. Only option available is soaking of the protein crystal with heavy atom like, Au, Hg etc. For soaking experiments there is a need to generate many crystals that can withstand the soaking condition. In this case the crystals obtained are in volatile conditions. The crystals degraded very fast in room temperature, making it very difficult for soaking experiments. Currently, attempts are made to crystallize the protein in other screening conditions which will enable heavy metal soaking and finally the elucidation of the three-dimensional crystal structure of this protein.

4.5. Conclusions

To summarize this chapter, we attempted with crystallization of exactin. We got the crystals for exactin at high protein concentrations (100 mg/ml). Moreover, exactin crystallized in only one crystallization condition during the screening process. The crystals were optimized through grid screening. The diffraction quality crystals were taken to the in house x ray machine to collect data. However, we could not succeed in solving the structure of exactin through molecular replacement as it exhibited problems with twinning. Currently, we are optimizing more crystallization conditions for exactin in order to get better diffraction quality crystals.

Chapter 5
Conclusions and Future prospectives

5.1. Conclusions

The thesis deals with the mechanism of a novel anticoagulant protein from the venom of *Hemachatus haemachatus*. Since the crude venom contained proteins of various molecular masses, SEC was used as an initial step of purification. The crude venom was separated into five peaks; with peak 2 and 3 exhibiting anticoagulant activity based on the prolongation of prothrombin time. Since peak 3 was comprised of 3FTxs, it was separated on a RP-HPLC column and the effect of RP-HPLC fractions on FX activation by extrinsic tenase complex was evaluated. A large number of anticoagulants targeting the extrinsic activation complex were identified. However, exactin was purified and characterized further, as previously it was shown that exactin prolongs the prothrombin time much significantly when compared to others. Apart from exactin, a few other anticoagulants were also partially characterized. Currently their inhibitory mechanism is under investigation. The anticoagulant protein, exactin was purified to homogeneity and its mass determined as 6621 Da. The complete amino acid sequence of exactin was determined and was found to be unique because of its highest identity to short-neurotoxins. However, it exhibited least identity to the well characterized classical short-chain α-neurotoxins and anticoagulant cytotoxins. The 'Dissection approach' assigned the anticoagulant target of exactin to extrinsic activation complex, which was further validated through evaluating the effect of exactin to reconstituted blood coagulation complexes. Inorder to understand the mechanism of inhibition, the effect of exactin on the extrinsic activation complex was evaluated by sequentially removing each component of the complex. Removal of TF however, did not alter the IC_{50} value (102.70 ± 11.71 nM), but there was a >1000-folds decrease in the exactin inhibition, upon removal of PL. This was further studied with enzyme kinetics where exactin exhibited a mixed-type inhibition. However, exactin inhibited FX activation by the extrinsic tenase complex and $FVIIa_{PL}$ with nanomolar affinities compared to that in the absence of PL (in the absence of PL the affinity towards [ES] dropped to >1000-folds). Specificity of exactin to macromolecular complexes (activators as well as substrates) was also determined. Compared to intrinsic tenase complex and RVV-X (exactin non-competitively inhibited FX activation with an affinity of ~ 2 μM), exactin exhibited nanomolar affinity to extrinsic tenase complex. Exactin was also found to be highly specific in inhibiting FX activation (>1000-folds) when compared to FIX, though both FX and FIX served as macromolecular substrates for the extrinsic tenase complex. Thus exactin specifically inhibited FX activation by extrinsic tenase complex and hence the name Extrinsic activation inhibitor/Exactin. Based on the sequence alignment, we found that exactin is identical to short neurotoxins. Inorder to study the usefulness in anticoagulant therapy, the neurotoxic effects of exactin was evaluated in i*n vivo* and *ex vivo* experiments. Exactin was found to be a weak, reversible postsynaptic neurotoxin. Crystallization of exactin was also undertaken. Though diffracting quality crystals were obtained at high protein concentration (100 mg/ml), attempts to solve the structure were unsuccessful due to crystal twining. Currently efforts are being made to get good quality untwined crystals using various other crystallization conditions.

Overall, we have functionally characterized a novel anticoagulant 3Ftx targeting the extrinsic activation complex. This is the first report of a three-finger toxin exhibiting a dual function of anticoagulants and neurotoxicity.

5.2. Future prospectives

Though this thesis has addressed the purification and characterization of a novel anticoagulant protein exactin, further work needs to be carried out to better understand its binding site. This can not only help in structure-function studies but also improve our basic knowledge regarding exogenous factors targeting the coagulation cascade, specifically the extrinsic activation complex. Some of these answers can help in designing lead therapeutic molecules for future anticoagulation therapies.

5.2.1. Co-crystallization of exactin with FX

To understand the binding site of exactin on FX, co-crystallization studies will be carried out. FXa structure in complex with many exogenous factors is well studied (Murakami *et al*., 2007). For crystallization studies, the human FX will be subjected to a limited chymotryptic cleavage to remove the Gla domain as previous works revealed that presence of Gla domain might interfere with crystallization purpose (Morita *et al*., 1986). Though we crystallized exactin at very high concentrations (100 mg/ml), the crystals obtained showed twining. Thus co-crystallization might also help to get better diffracting crystals of the complex.

5.2.2. Biophysical studies with exactin

The thermodynamics of binding of exactin to individual chains of FVIIa can be studied using biophysical techniques like ITC (isothermal titration calorimetry). The FVIIa heavy and light chain can be prepared through partial reduction using β-mercaptoethanol and separation on an ion-exchange column (Kazama *et al*., 1993). The interaction studies will not only help to understand the binding site but will also determine the binding affinity of exactin to FVIIa.

5.2.3. Structure-function studies with exactin

Determining the binding site of exactin on the extrinsic activation complex can help in delineating the functional site in exactin. This can be achieved through chemically modifying exactin and then examining its anticoagulant activity. The loop I-III of exactin harbours various basic and hydrophobic residues that can be subsjected for chemical modifications (Kini and Evans, 1989; Menez *et al*., 1990). Once the functional site (residues responsible for anticoagulant activity) is determined, short anticoagulant peptides can then be designed. The novelty of exactin lies in its dual role of anticoagulation and neurotoxicity. We thus predict these dual functional sites to present on different regions of exactin (Kini, 2002). Hence determining the anticoagulant site on exactin is important for designing lead molecules for future anticoagulation therapy.

5.2.4. Characterization of other anticoagulants

Though we have partially characterized other anticoagulants (with mass 7279 Da, mass 7438 Da and peak 10), it is important to understand their mechanism of action since these anticoagulants like exactin also inhibit FX activation by extrinsic tenase complex. Partial sequencing of these anticoagulants has shown the difference in sequence when compared to exactin. Thus structure-function studies will not only explain how these distinct anticoagulants recognize the extrinsic activation complex but also will help in identifying novel exosites in the complex, binding to which can exhibit anticoagulant activity. The knowledge thus gained can be used for designing lead therapeutic molecules.

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Appendix

Publications

Vallerinteavide Mavelli Girish, Sundramurthy Kumar, Lissa Joseph, Chacko Jobichen, R Manjunatha Kini and J. Sivaraman. Identification and Structural Characterization of a New Three-Finger Toxin Hemachatoxin from *Hemachatus haemachatus* Venom (Submitted to Plos One).

Vallerinteavide Mavelli Girish, Foo Chun Shin, Bhaskar Barnwal and R Manjunatha Kini. Exactin – a specific inhibitor of factor X activation by extrinsic tenase complex isolated from *Hemachatus haemachatus* venom (Manuscript under preparation).

International presentations

A novel extrinsic tenase complex inhibitor from the venom of *Hemachatus haemachatus* (African Ringhals Cobra) [Poster presentation] **Vallerinteavide Mavelli G** and Kini R.M.; *Sixth INTERNATIONAL CONFERENCE ON STRUCTURAL BIOLOGY AND FUNCTIONAL GENOMICS*, Singapore, **6-8 December 2010.**

A novel extrinsic tenase complex inhibitor from the venom of *Hemachatus haemachatus* (African Ringhals Cobra) [Poster presentation] **Vallerinteavide Mavelli G** and Kini R.M.; *XXIII CONGRESS OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS,* Japan, **23-28 July, 2011**.