

PROTHROMBINASE INHIBITION BY A COVALENT COMPLEX OF
ANTITHROMBIN & HEPARIN

INHIBITION OF THE PROTHROMBINASE COMPLEX ON
PHOSPHOLIPID VESICLES, ACTIVATED PLATELETS,
AND RED BLOOD CELLS BY A COVALENTLY-LINKED
ANTITHROMBIN-HEPARIN COMPLEX

By

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Abstract

Prothrombinase is composed of a proteinase, factor Xa (Xa), its cofactor Va (Va), Ca^{2+} and a zymogen, prothrombin (II), assembled on a phospholipid surface. During coagulation, prothrombinase accelerates II to thrombin conversion; but during anticoagulation, it protects the proteinase from inhibition by antithrombin (AT) $\pm$ unfractionated heparin (UFH). Although the degree of Xa protection by prothrombinase varies according to the reports in literature, moderate to significant protective effects have been consistently reported by most investigators. To overcome the limitations of UFH, our laboratory has developed a covalent complex of AT and UFH (ATH) with superior anticoagulant responses. To further understand the mechanisms of enhanced anticoagulant activity of ATH, we proceeded to study inhibition of the prothrombinase complex on synthetic vesicles, activated platelets and red blood cells (RBCs).

Using discontinuous inhibition assays, we determined the rate of inhibition of prothrombinase-complexed Xa compared to control Xa. With synthetic vesicles, Xa was protected from inhibition by AT+UFH when in prothrombinase, while only a mild protective effect was observed with ATH. Omission of various components of the prothrombinase led to a reduction in Xa protection for AT+UFH. However, an increased Xa protection against ATH was observed when II was omitted from the prothrombinase. In comparison to the synthetic vesicle system, activated platelets showed a similar trend for protection of Xa in reactions involving prothrombinase $\pm$ components, while no protection of Xa was observed for ATH reactions. Alternatively, RBCs showed differences relative to vesicles in that increased protection of Xa occurred with omission

of II and Va for AT+UFH, whereas omission of Va increased protection against ATH inhibition. In addition, ATH had improved inhibition of thrombin generation, fibrin formation and plasma coagulation compared to AT+UFH. Studies of fluorescently labelled Xa and inhibitors detailed binding interactions with prothrombinase subunits. Overall, the results suggest that a covalent linkage between AT and heparin improves inactivation of prothrombinase complexed-Xa leading to down-regulation of prothrombinase function.

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My PhD is dedicated to my father, my dearest mentor in my life and the inspirer for me to study medical research.

It was at age of 16 when I truly felt the passion for science, and when my life-long goal to pursue medical sciences had become evident to me. It was then that my father had undergone elective surgery for correction of a dissected aortic aneurysm. During the surgery, my father had succumbed to severe complications that forever changed his life and the lives of our family members. It was a bitter-sweet feeling. Sweet, because the surgery ultimately prolonged my father's life, but bitter, because he became disabled (paraplegic) and was left to deal with life-long complications. Ever since this unfortunate circumstance, my lifelong path had been modeled by asking three simple questions. Firstly, why has this happened to my father? Secondly, what was the causative reason for him to develop the aortic aneurysm/dissection in the first place? Lastly, how can I prevent this from happening to another family in the future? Therefore, it was the unfortunate circumstance that has shaped the path in my life. It has provided me with the driving force to succeed in life, to help as many people as I possibly can along this path, and to hopefully one day answer my three questions.

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motivated me to think outside the box and ask even more questions, which further fuels my hunger to find more answers.

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Abbreviations:

ADP – Adenosine diphosphate

A₂M – Alpha-2-macroglobulin

ANOVA – Analysis of variance

APC – Activated protein C

AT – Antithrombin

ATH – Covalent antithrombin-heparin complex

AT+UFH – Antithrombin + heparin non covalent conjugate

AT(H) – Antithrombin-heparin inhibitor

ATP – Adenosine triphosphate

Ca²⁺ – Calcium

CaCl₂ – Calcium chloride

CAT – Catalytic domain

CPB – Cardiopulmonary bypass

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal-like growth factor

EPCR – Endothelial protein C receptor

FITC - Fluorescein isothiocyanate

FPPAck – Fluoresceinyl-Phe-Pro-Arg chloromethyl ketone

FPPAck-Xa – Fluoresceinyl-Phe-Pro-Arg chloromethyl ketone – Xa

FSAP - Factor VII-activated protease

F-ATH – Fluorescein-ATH

F-UFH – Fluorescein-unfractionated heparin

F-Xa – Fluorescein-Xa

GAG – Glycosaminoglycan

Gla – γ -carboxyglutamic acid

GP – Glycoprotein

h – Hours

HCI – Heparin cofactor 2

HMWK – High molecular weight kininogen

IL-10 – Interleukin-10

II – Prothrombin

IIa – Thrombin

k_1 – First-order rate constant of inhibition

k_2 – Second-order rate constant of inhibition

k_{cat} – Rate of substrate turn-over

K_D – Dissociation constant

kDa – kiloDalton

LMWH – Low molecular weight heparin

MgCl₂ – Magnesium chloride

min – Minutes

PCPS – Phosphatidylcholine and phosphatidylserine

PDI – Protein disulfide isomerase

PK – Prekallikrein

PKC- α – Protein kinase C- α

PS – Phosphatidylserine

RCL – Reactive center loop

RBCs – Red blood cells

rTAP – recombinant tick anticoagulant peptide

s – Seconds

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide electrophoresis

SEM – Standard error of the mean

Serpin - Serine protease inhibitor

TAFI – Thrombin-activatable fibrinolysis inhibitor

TF – Tissue factor

TFPI – Tissue factor pathway inhibitor

TRIS – Tris-(hydroxymethyl)-aminomethane

TSP – 20 mM Tris-HCl, 140 mM NaCl, 0.6% polyethylene glycol, pH 7.4

UFH – Unfractionated heparin

vWF – von Willebrand factor

Chapter 1: General Introduction

1. INTRODUCTION:

Hemostasis is the process that maintains the integrity of the high-pressured cardiovascular system after vascular damage (1). Vessel wall injury and endothelial cell damage initiates rapid clotting events which are responsible for sealing the breach (1). *In vivo*, thrombus generation or clot formation is dependent on a number of factors, including plasma concentrations of pro- and anticoagulant proteins, cell surface stimulants, and interaction of molecules in the subendothelium with the intravascular environment (1,2). Ultimately, regulation of the clotting factors within the coagulation cascade affects the cleavage of prothrombin (II) to thrombin, which in turn cleaves fibrinogen to fibrin monomers, so that fibrin monomers can self-polymerize and become cross-linked (via factor XIIIa) into a solid clot. Figure 1.1 depicts the most current model of the plasma coagulation cascade.

Injury to the vessel wall arising from chemical, mechanical or electrical stimulation results in the activation of either the extrinsic or intrinsic pathways of coagulation, or both (1,3). Depending on the injury or disease state, one pathway may predominate over the other. However, both lead to the initiation of the coagulation cascade by activating clotting factors and generating thrombin (3). Evidence suggests that the extrinsic pathway is the main initiator of thrombin generation *in-vivo*, (1,3-6).

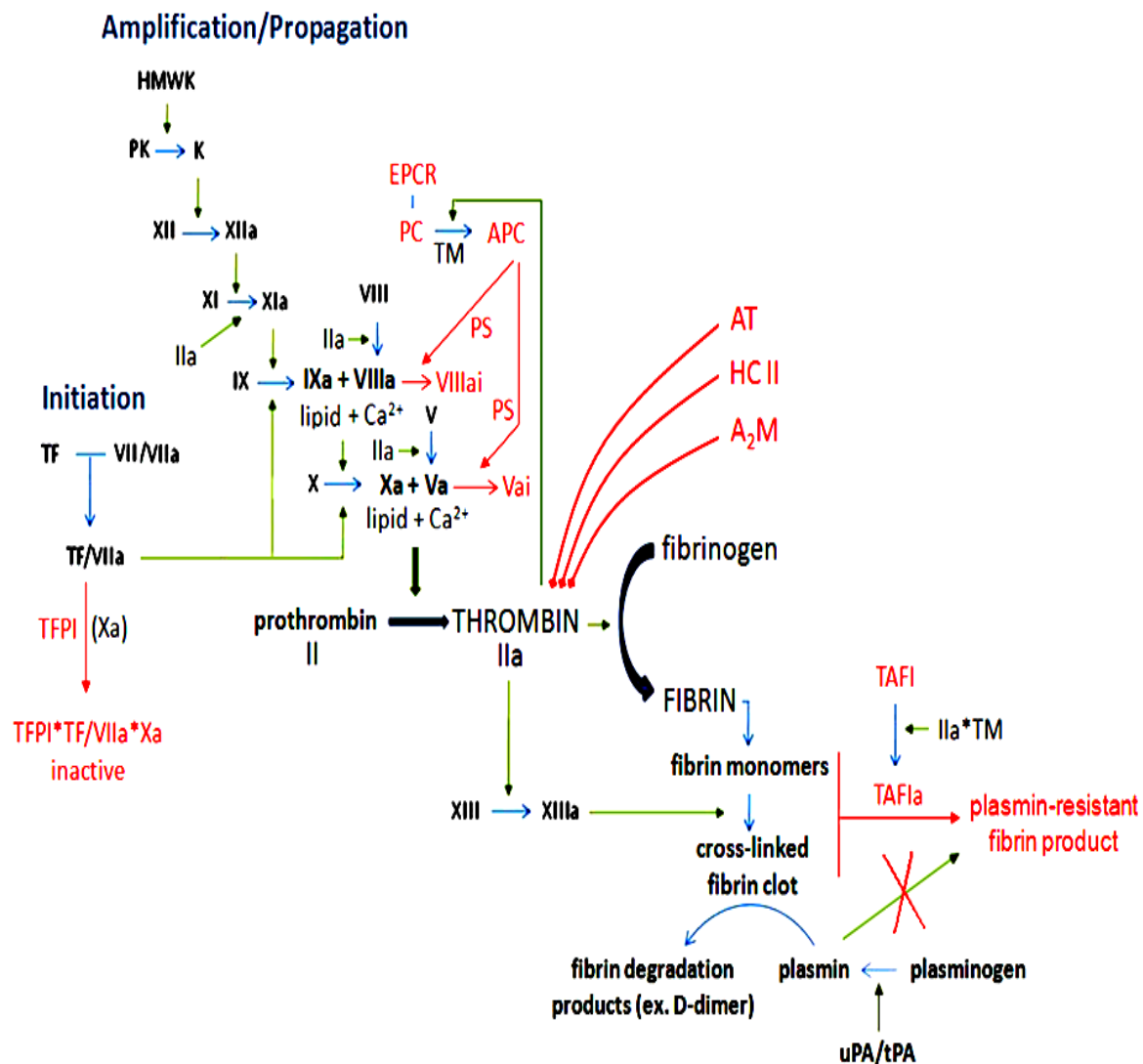


Figure 1.1: Plasma coagulation cascade. This figure illustrates the extrinsic and intrinsic pathways responsible for activating the coagulation cascade, and the pro- and anticoagulant factors responsible for regulating the cascade. Blue arrows are proceeding reactions, green arrows indicate activation of the reaction and red arrows are inhibitors of indicated reaction.

1.1 Initiation:

Injury to the blood vessel wall results in initiation of coagulation through tissue factor (TF) and factor VIIa (VIIa) interaction. TF has high affinity for VII/VIIa, and upon vessel wall injury, TF complexes with VII/VIIa and activates IX to IXa and X to Xa (1,3-5,7). The Xa then cleaves II to thrombin. Only a trace amount of thrombin is produced by the initiating pathway while larger amounts of thrombin will be generated during the amplification and propagation phases (3,4,8). A second initiation system called the contact pathway also exists to generate trace amounts of thrombin; the contact pathway will be discussed in more detail below.

1.1.1 Tissue factor

Tissue factor (TF) is a 43 kDa transmembrane glycoprotein that shares structural homology with the cytokine receptor super-family, particularly interleukin-10 (IL-10) and interferon- $\alpha/\beta/\gamma$ (9). It contains a transmembrane domain that is approximately 23 amino acids long and a short, 21 amino acids long intracytoplasmic domain with three serine residues that may act as potential sites for phosphorylation (9). Evidence exists to suggest that TF exists in two conformations, the active (de-encrypted) and encrypted forms (10). The current theory is that de-encryption of TF occurs when protein disulfide isomerase (PDI) forms a critical disulfide bond between Cys¹⁸⁶ – Cys²⁰⁹, which results in maturation of the VII/VIIa binding pocket of TF (11).

TF is capable of interacting with VII, the zymogen form, or the active protease VIIa. As the initiator of coagulation, TF acts as a cofactor that helps to localize factor

VII/VIIa to the phospholipid membrane *in-vivo*. Formation of the TF/VIIa complex accelerates the conversion of factor IX to IXa and X to Xa in excess of 1000-fold (12). Under normal physiological conditions, TF is expressed only in extravascular and perivascular sites of blood vessels. Thus, when blood vessels are breached, the subendothelial TF contacts the circulating factor VII/VIIa to initiate coagulation (13). In some circumstances, such as inflammation or cancer, TF can be stimulated on monocytes/macrophages (14), endothelial cells (15), or platelets (16). The latter is a matter of debate, as expression of TF on platelets either from *de-novo* synthesis, α -granule release, or fusion from TF-bearing monocytes microparticles has been much discussed. A recent study suggests there is no evidence for tissue factor expression on platelets based on advanced and highly sensitive TF antigen and TF activity assays (17). The battle to confirm, or refute, TF activity/expression on platelets had sparked further experiments that ascertained that some agonists (such as ADP, epinephrine and calcium ionophore A23187) may cause early expression of TF, and some agonists (such as TRAP-6 and PAR-1) may cause late expression of TF on the platelet surface (16), which may be responsible for the confounding reports.

Previous experimental studies have demonstrated that TF is important for survival. In a study utilizing TF-knockout mice, all of the pups died in utero between days 8.5-10.5, and all showed obvious phenotypic differences compared to the TF-bearing mice (such as pale skin, edema, and growth retardation), thus highlighting the importance of this protein for fetal development (18).

1.1.2 VII/VIIa

Factor VII, originally called proconvertin, is a single-chain, Gla-containing glycoprotein of approximately 50 kDa in size that is produced in the liver (19). The plasma concentration of VII is approximately 10 nM. Activation of VII is required for this protein to function as a serine protease, and binding to its cofactor, TF, significantly accelerates the conversion of factors IX to IXa and X to Xa.

Approximately 1% of the total plasma level of VII circulates in the active form, however the activity of VIIa based on those levels is negligible unless it interacts with TF (20). Limited understanding exists to explain the mechanism by which resident plasma VIIa is produced and circulates. It has been shown that Xa in the presence of Ca^{2+} and phospholipids, as well as thrombin and XIIa without cofactors, are capable of cleaving VII to VIIa (19). However, under these circumstances, coagulation has already been initiated and the activation of VIIa would be a feedback mechanism. Other investigators have shown that other factors such as RNA are also capable of activating VII (21), or that VII undergoes autoactivation by VIIa itself (22). More recent evidence suggests that factor VII-activated protease (FSAP), a potent activator of VII, is a leading candidate for the maintenance of plasma VIIa levels (20). However, the importance of FSAP in coagulation is still under investigation. The cleavage of a peptide bond through limited proteolysis at $\text{Arg}^{152}\text{-Ile}^{153}$ is required for the generation of the two-chained active VIIa (23).

1.2 Contact Pathway

Coagulation can also be activated through factor XIIa on charged surfaces, which is termed ‘contact activation’ (7). For example, subendothelial collagen or negatively charged surfaces can instigate the binding of XII to the exposed subendothelium in the presence of prekallikrein and cofactor high molecular weight kininogen (HMWK), which results in the activation of XII to XIIa (7,24). Subsequently, XIIa activates XI to XIa, which in turn, activates IX to IXa (2,7). The IXa (within the tenase complex) subsequently cleaves X to Xa, which then generates thrombin from II (via the prothrombinase complex).

Initially, the contact system was thought to be relevant only *ex-vivo*, in cases where blood came into contact with charged surfaces such as those found on glass, plastic or medical devices. In the absence of *in-vivo* factors that can activate XIIa, charged surfaces from synthetic products may also play a role in the autoactivation of XII to XIIa (25). Thus, XIIa can amplify its own generation through self feedback activation mechanisms. More recently, there is evidence to suggest that RNA (26), misfolded and aggregated proteins (27), and polyphosphates (28) may assist activation of XII to XIIa and initiate coagulation. Extracellular genetic material is often released under conditions of severe sepsis or inflammation, and thus may be a contributor to the procoagulant state witnessed in these conditions (29). Furthermore, it has been shown that platelet α -granules contain polyphosphates. Thus, activation of platelets and degranulation of the α -granules may result in activation of XII to XIIa through the released polyphosphates (28). The importance of the factors comprising the contact pathway has been a matter of

debate, because deficiencies of XII (30), prekallikrein (31), or HMWK (32) have not been associated with significant bleeding diatheses *in-vivo*.

1.2.1 XII/XIIa

Previously called the Hageman factor (named after the first identified factor XII deficient patient), factor XII is a glycoprotein with an approximate molecular weight of 80 kDa. It is present in the plasma at concentration between 180 nM to 580 nM (33). Upon contact with negatively charged surfaces, XII undergoes limited proteolysis by kallikrein to yield two active forms of the enzyme, α -XIIa and β -XIIa (25,33). The α -XIIa is composed of a heavy chain and a light chain linked together by a disulfide bond. The heavy chain has an approximate molecular weight of 52 kDa and contains the surface binding site that is 353 amino acids long (33). The light chain, which is 28 kDa fragment and made up of 243 amino acids, contains the active site capable of cleaving factor XI to XIa. The β -form also contains a light and a heavy chain that have molecular weights of 2 and 28 kDa, respectively. The heavy chain of β -XIIa also contains the active site of the enzyme (33).

Negatively charged surfaces are believed to be responsible for binding and bringing the protein factors together for efficient interactions with one another. Thus, when bound to the negatively charged surfaces, XII and prekallikrein interact in a so-called reciprocal activation mechanism (34). For example, upon activation of XIIa on negatively charged surfaces by autoactivation, XIIa can then activate prekallikrein to kallikrein, which in turn activates additional XII to XIIa (25,32).

1.2.2 XI/XIa

Factor XI is a zymogen that is produced in the liver and circulates in the plasma as a dimer, with a total molecular weight of 160 kDa. The plasma concentration of XI is approximately 90 nM. The dimer contains identical subunits; each comprised of 607 amino acids and approximately 80 kDa, which are linked together at the A2 domains by a disulfide bond (35). The N-terminus of each subunit contains four 90-91 amino acid repeats called apple domains (A1-A4), and the C-terminus contains the trypsin-like catalytic triad (36-40). The other coagulation protein that contains the apple domains is prekallikrein. XI circulates in the blood complexed to HMWK. Unlike many other zymogens in the coagulation cascade, XI does not contain the Gla-domain that is required for the calcium-dependent interaction with anionic phospholipid membrane surfaces (35). A1-A2 domains have been implicated in binding to the HWMK, and A2-A3 domains have been shown to interact with the XIa substrate factor IX. The A3 domain also contains the binding site for platelets and for initial interaction with IX (41), whereas the A4 domain has been shown to mediate dimer formation. Even though XI does not contain the Gla-domains, it has been previously estimated that one activated platelet contains 1,500 binding sites for XI (42). Subsequent work determined that residues Ser²⁴⁸ and Arg²⁵⁰ on the A3 domain were responsible for interacting with glycoprotein 1b (GP1b) on platelets (42).

Given that the patients with known deficiencies in XII are still viable, this suggests that alternative pathways may exist for activating XI. Therefore, apart from XIIa, other known activators of XI include thrombin, the II activation intermediate

meizothrombin, and XIa (through autoactivation) (43). All of the aforementioned proteases cleave a peptide bond between Arg³⁶⁹-Ile³⁷⁰, which results in activation of the XIa subunit (35). Both subunits need to be cleaved in order to have a fully functional XIa. Available data for cleavage of individual XI subunits by thrombin requires the interaction of thrombin with residues Glu⁶⁶, Lys⁸³, and Gln⁸⁴ on the A1 domain, which are suitably positioned close to the Arg³⁶⁹-Ile³⁷⁰ peptide bond for further processing (35,44). In the zymogen complex containing the dual subunits, the mechanism of activation by thrombin is slightly more complicated. For example, thrombin will bind to A1 of one subunit and cleave the Arg³⁶⁹-Ile³⁷⁰ bond of the other subunit, thus acting in a *trans* mechanism of activation (35,40). During the *trans* mechanism of activation, an intermediate called ½-XIa is formed, where one subunit is in the active state while the other is not (figure 1.2) (45).

The exact residues responsible for XIIa interaction with XI has not been elucidated yet. There is evidence to suggest that the A4 domain may be a plausible candidate, however, experiments utilizing monoclonal antibodies that recognize the A2 domain prevent XI activation by XIIa. This suggests a more complex mechanism is required for activation of XI by XIIa (33,46).

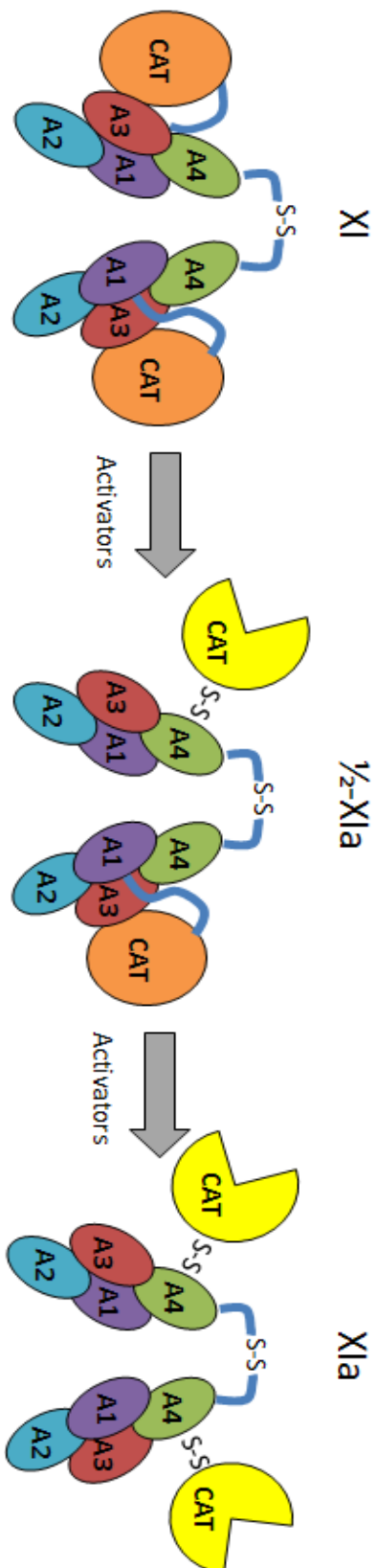


Figure 1.2: Sequential activation of factor XI dimer. XI exists in the circulation as a dimer of two identical XI subunits. Initially, XIIa or other activators (thrombin, meizothrombin and XIa (autoactivation)), bind to the A1 domain and subsequently cleave the Arg³⁶⁹-Ile³⁷⁰ peptide bond located on the adjacent subunit. This causes the production of a partially activated intermediate called ½-XIa. Then subsequent cleavage of the Arg³⁶⁹-Ile³⁷⁰ peptide bond on the second subunit results in the production of a fully activated XIa. Cleavage of the Arg³⁶⁹-Ile³⁷⁰ peptide bonds on both subunits causes the apple domain disks to move relative to the catalytic domain (CAT), which exposes a surface on the A3 domain that is capable of interacting with factor IX.

1.3 Amplification/propagation of coagulation

The trace amounts of thrombin generated during initiation provide further activation of V, VIII, and XI. IXa generated by XIa, complexes with VIIIa in the presence of phospholipids and Ca^{2+} ions to generate additional Xa (see section 1.3.1; tenase complex) (4). In turn, the Xa complexes with Va on the phospholipid membrane and in the presence of Ca^{2+} ions converts II to thrombin. This feedback mechanism produces the burst of thrombin generation required to convert fibrinogen to fibrin (see section 1.3.4; prothrombinase complex) (3,4). Thus, the added activation of V, VIII, and XI by thrombin ensures a positive feedback loop exists to sustain elevated levels of thrombin generation (1,4). Thrombin also activates XIII to XIIIa, a transglutaminase enzyme responsible for cross-linking fibrin and stabilizing clots (2,4,5). The reactions leading to thrombin generation and fibrin polymerization are complex, and each clotting factor must be positioned in proximity to its activating protease on the surfaces of activated platelets (4,8).

1.3.1 Tenase complex

The tenase complex is composed of the serine protease, IXa, its cofactor VIIIa, Ca^{2+} , anionic phospholipids and the precursor X. *In-vivo*, the anionic phospholipids are made available on the surface of activated platelets. Thus, assembly of the IXa within the tenase complex increases the conversion of X to Xa by up to 100,000-fold (47). Circulating vWF-complexed VIII binds to the GP Ib/IX receptor and, upon binding, the complex is cleaved by thrombin resulting in the activation of VIII to VIIIa and the

disassembling of the vWF from the VIII/VIIIa. The VIIIa remains bound to the platelet membrane (8) and helps to recruit IXa to the surface of activated platelets and, in the presence of Ca^{2+} , X becomes converted to the active enzyme, Xa. Factors IXa and VIIIa interact on the phospholipid surface in a 1:1 molar stoichiometric ratio (48). The activated Xa then diffuses adjacently on the platelet surface and complexes with cofactor Va to form the prothrombinase complex (discussed in more detail below) (8).

1.3.2 VIII/VIIIa

Factor VIII is synthesized in the liver and has a molecular weight of 300 kDa (49). It is a single chain polypeptide comprised of 2332 amino acids and circulates in plasma at a concentration of 300 pM (50). It contains three A domains (A1-A3), one B domain, and two C domains (C2-C3) (figure 1.3) (51). A1-A2 domains form the heavy chain, while A3, C1 and C2 form the light chain; the heavy and light chains are separated by a B domain (52). Thrombin, and to some degree Xa, cleave VIII at two sites located on the heavy chain. First, the Arg^{372} is cleaved between the A1-A2 domains, followed by Arg^{740} which separates the A2 domain from the B domain (53). This results in the formation of the 50 kDa A1 and 40 kDa A2 subunits of VIIIa. The B domain becomes variably degraded and is not part of VIIIa function. Exposure of the IXa binding site within the A2 subunit occurs when the Arg^{372} is cleaved, which produces a viable area on the A2 domain responsible for proper cofactor activity (53). Cleavage of Arg^{1689} in the A3 subunit releases a peptide of approximately 40 residues enriched in acidic amino acids (53).

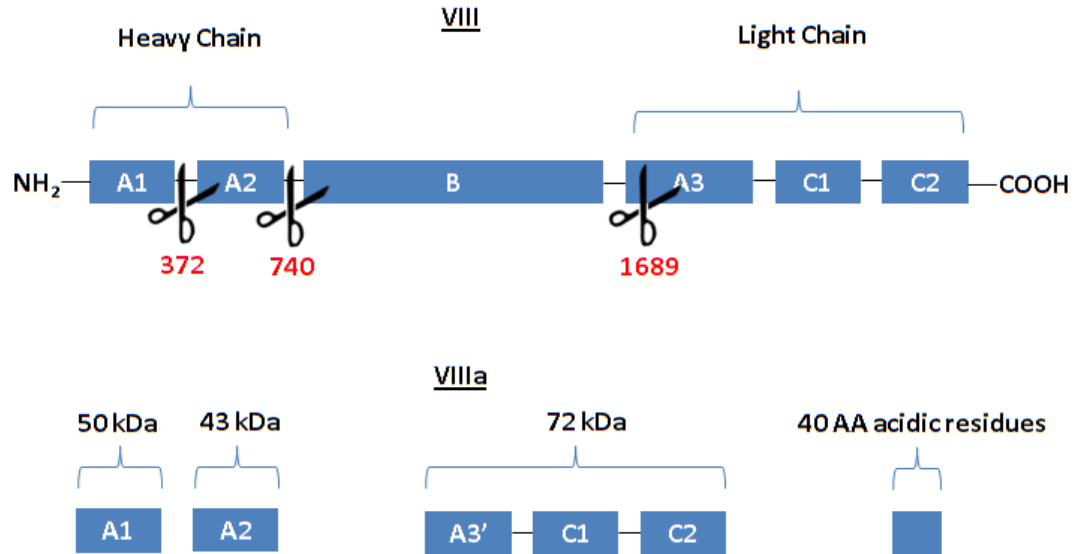


Figure 1.3: Cleavage process for conversion of factor VIII to VIIIa. VIII gets cleaved at Arg⁷⁴⁰ first, which facilitates subsequent cleavages at Arg³⁷² and Arg¹⁶⁸⁹. Furthermore, cleavage of the A3 domain at Arg¹⁶⁸⁹ can also facilitate the cleavage of Arg³⁷² between the A1-A2 domains. The cofactor activity of VIII is not fully developed until the final step at which cleavage of Arg³⁷² occurs, which exposes the binding site on the A2 domain responsible for IXa interaction.

Moreover, proteolytic cleavage of VIII at Arg³⁷² and Arg¹⁶⁸⁹ is important for its cofactor activity, although the latter releases VIII from the von Willebrand factor (vWF) (54). Even though VIII is synthesized as a single chain molecule in the liver, it circulates in tight association with vWF as a heterodimer. Heterodimer formation is assisted by specific intracellular proteases through the cleavage of a peptide bond at Arg¹⁶⁴⁸, which is located between the B and A3 domains (54). vWF primarily acts to stabilize VIII in the circulation. Factor Xa has been shown to cleave VIII at the same site as thrombin, even though the binding sites for the enzymes are different on the C2 domain (55). The order of magnitude higher plasma concentration of II compared to X suggests that thrombin is the more physiologically relevant activator of VIII. The main function of VIIIa in coagulation is to act as a cofactor for IXa in the tenase complex.

1.3.3 IX/IXa

Also known as the Christmas factor, IX is a 55 kDa single chain protein that is synthesized in the liver and circulates in the plasma at a concentration of approximately 90 nM. The molecule is comprised of a non-catalytic light chain located at the N-terminus and a heavy chain containing a trypsin-like active site at the C-terminus, both of which are separated by an ~11 kDa activation peptide region (figure 1.4) (56,57). IX also contains the Gla-domains on the N-terminus, which in the presence of Ca²⁺ help to bind the protein to membrane surfaces. The primary function of IXa in coagulation is to activate X to Xa via the tenase complex. Only trace amounts of Xa are produced when free IXa cleaves X to Xa, thus highlighting the importance of the tenase complex.

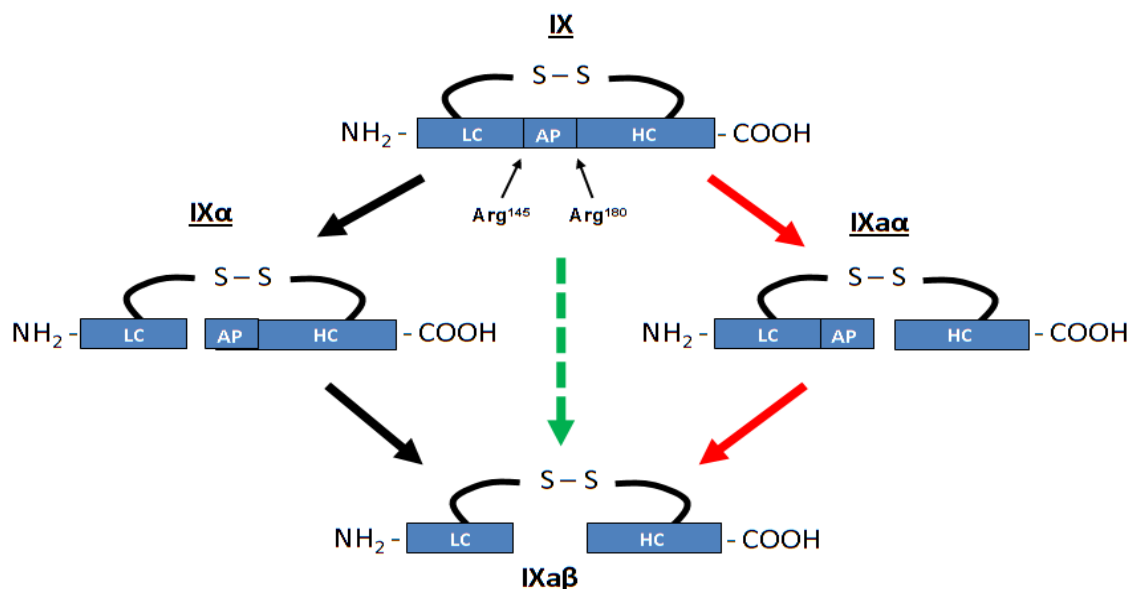


Figure 1.4: Activation of factor IX to IXa through three different pathways. Factor IX is comprised of the heavy chain (HC) and light chain (LC) linked together by a disulfide bond, and separated by the activation peptide (AP). Activators of IX (TF/VIIa and XIa) activate IX through the pathway denoted by large black arrows. First, the proteinases cleave at Arg¹⁴⁵ to produce the intermediate IXα, which is inactive, followed by cleavage at Arg¹⁸⁰ to yield IXaβ. A second pathway has been described (red arrows), but has been observed mostly with Russell's viper venom, which cleaves at Arg¹⁸⁰ first to yield a partially active intermediate called IXαα. An additional cleavage at Arg¹⁴⁵ then yields the active IXaβ. Lastly, since XIa is a dimer comprised of two identical active subunits, it is believed that XIa can cleave IX at both Arg¹⁴⁵ and Arg¹⁸⁰ simultaneously to yield the active IXaβ + activation peptide, without detection of intermediates.

Activation of IX by XIa occurs through two proteolytic cleavages between Arg¹⁴⁵-Ala¹⁴⁶ and Arg¹⁸⁰-Val¹⁸¹, which results in the release of the activation peptide (57,58). Three possible pathways may exist for activation of IX to IXa, and the activated IX is often referred to as IXa β (59). The first pathway (denoted by the large black arrows) produces an intermediate called IXa α by a cleavage at Arg¹⁴⁵ first (60), followed by a cleavage at the second site (Arg¹⁸⁰) which produces the active IXa β (59). Conversion of IX to IXa by this pathway has been described for XIa, TF/VIIa and thrombin, although the latter may not have significant physiological relevance. Cleavage of IXa at Arg¹⁸⁰ first has only been described for Russell's viper venom, which yields the partially activated intermediate IXa α (denoted by the red arrows) (61). In addition, some investigations have revealed that TF/VIIa and XIa dimer can directly convert IX to IXa β without detection of the intermediates (green dotted arrow) (56). At least for XIa, it is thought that the two active subunits on the dimer may cleave Arg¹⁴⁵ and Arg¹⁸⁰ simultaneously to produce the final products IXa β and the activation peptide (59).

1.3.4 Prothrombinase Complex:

The prothrombinase complex is comprised of Xa, its cofactor Va, anionic phospholipids, Ca²⁺ and the zymogen, II. Its sole purpose is to convert II to the active proteinase, α -thrombin (figure 1.5) (62,63). Thrombin is considered to be the final effector proteinase of the coagulation cascade, which is capable of causing feedback within the clotting cascade to regulate coagulation factors, inhibitory proteins and fibrinolytic enzymes.

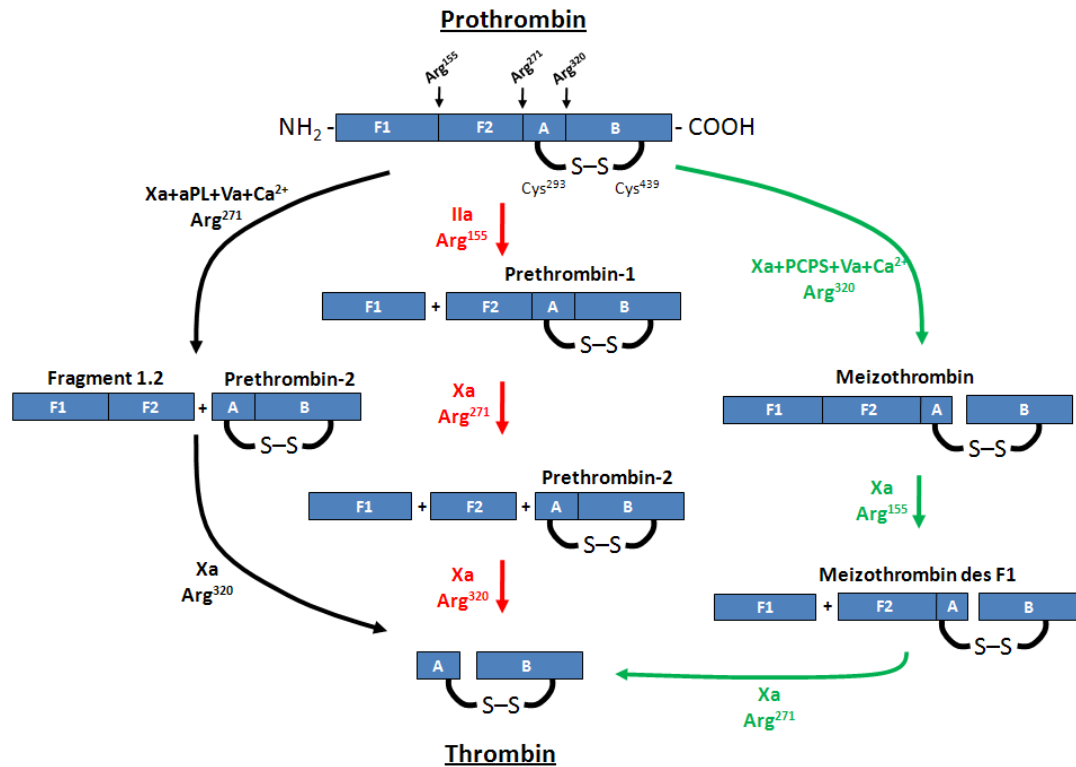


Figure 1.5: Prothrombin activation by thrombin and Xa. Both thrombin and Xa are capable of cleaving II at different sites to generate intermediates during activation. Thrombin can cleave II at Arg¹⁵⁵ to generate an intermediate prethrombin-1, which can be further processed by Xa to form the active thrombin (red arrows). In the presence of activated platelets (aPL) as the surface for prothrombinase, Xa cleaves II at Arg²⁷¹ to generate the intermediate prethrombin-2 and fragment1.2. Prethrombin-2 is further processed by Xa by cleavage of Arg³²⁰ to generate the active thrombin (black arrows). Whereas, when prothrombinase forms on phosphatidylcholine/phosphatidylserine (PCPS) phospholipids, Xa cleaves II at Arg³²⁰ to yield a partially activated intermediate called meizothrombin. Meizothrombin is then cleaved at Arg¹⁵⁵ to form meizothrombin des F1, which is further processed by Xa to generate the active thrombin (green arrows).

Prothrombinase is the cornerstone of the coagulation cascade. It is positioned in such a manner that both the intrinsic and extrinsic pathways, in addition to the feedback from generated thrombin, aid with the production and function of the prothrombinase complex. For example, TF/VIIa (extrinsic pathway) activates IX to IXa and X to Xa, so that Xa can activate II to thrombin. As discussed above, thrombin then feeds back to the cascade to activate other factors (V to Va, VIII to VIIIa, XI to XIa, and resting platelets to activated platelets), which will aid with further turnover of the tenase and prothrombinase complexes. Previous investigations of prothrombinase function have shown that incorporation of Xa within the prothrombinase complex enhances the conversion of II to thrombin up to 300,000-fold faster relative to non-complexed Xa (62).

Assembly of the proteinase in the complex greatly enhances the conversion of zymogen to its active component (62). Consequently, this produces a double edged sword. In hemostasis, it speeds up the rate of conversion of zymogen to active enzyme. However, during anticoagulation, it protects the proteinase from inhibition by anticoagulants such as heparin. Variable protection of Xa has been reported in literature, with values being anywhere from full protection to essentially no protection at all by unfractionated heparin (UFH) (64-69). The reason for the variability in reports arises from the complexity of the assay systems, as the inhibitor (AT) can either react with the Xa in the prothrombinase or the product of the complex, thrombin. The studies that determined the most protection utilized mutant forms of proteins to establish inhibition rates. For example, Rezaie in his work utilized a mutant AT that reacted with Xa but was unable to react with the product thrombin (68). Bruffato *et al.* utilized a mutant II that

produced a product that was incapable of reacting with AT, so that all of the AT was directed towards Xa (69). Nonetheless, the studies which utilized modified proteins in their inhibition assays attained the greatest inhibition rates (68-69). In comparison, studies which used native and unmodified proteins in their experiments showed a lesser protective effect (64-67).

1.3.5 Prothrombin/Thrombin

Prothrombin (II) is a glycoprotein consisting of a single polypeptide chain with a molecular weight of approximately 72 kDa. It circulates in the plasma at a concentration of 1.4 μ M. II contains four distinct structural domains: fragment 1 (F1) and 2 (F2), which are linked by a covalent bond between Arg¹⁵⁵-Ser¹⁵⁶, followed by A and B domains. F2 is linked to the A domain at Arg²⁷¹-Thr²⁷², and the A domain is linked to the B domain at Arg³²⁰-Ile³²¹ (70). The Gla-domains are located on F1, and F2 contains the triple-disulfide kringle domain, while A and B domains comprise the protease component. A and B domains are stabilized by a disulfide bond at Cys²⁹³-Cys⁴³⁹ (70,71).

Cleavage of II yields a serine protease, α -thrombin (referred to as thrombin), with an approximate molar mass of 37 kDa (72). When II is cleaved, partially activated intermediates are formed (figure 1.5). Only one half of the proenzyme is converted to the active form while the other half is released as a by-product during activation (73-75). The by-product fragments comprise the non-thrombin forming region of II which also includes the Gla-domain. Therefore, unlike other serine proteases in the cascade formed

from Gla-containing proenzymes, the active form of II (thrombin) does not have a Gla-domain.

II can be activated by Xa alone or with assistance from thrombin, all of which occur through different pathways (figure 1.5) (76). Thrombin cleaves II specifically at Arg¹⁵⁵ between F1 and F2, thus producing an intermediate called prethrombin-1 + F1 (red arrows, figure 1.5) (76). Xa then cleaves prethrombin-1 at Arg²⁷¹ to generate prethrombin-2 + F1 + F2 (77,78). In the final step, Xa catalyzes prethrombin-2 cleavage at Arg³²⁰ to yield the active serine protease, thrombin plus the degradation fragments F1 + F2 (77).

Wood *et al.* have shown that in the presence of activated platelets as the source of anionic phospholipids for prothrombinase, the prethrombin-2 + fragment1.2 (black arrows, figure 1.5) pathway predominates (79). Thus, in the presence of activated platelets the protease cleaves II at Arg²⁷¹ to yield prethrombin-2 + fragment1.2. Xa then further cleaves prethrombin-2 at Arg³²⁰ to produce the active thrombin (79). In the presence of strictly PCPS phospholipids, such as those found on synthetic vesicles or procoagulant red blood cells, the meizothrombin pathway predominates (80). Therefore, in the presence of PCPS, Xa exhibits a preference to cleave Arg³²⁰ of II to generate the intermediate meizothrombin (80). At this point, the only product, meizothrombin, is one intact covalent molecule with a disulfide bond keeping the A domain linked to the B domain (70). Xa subsequently cleaves meizothrombin at Arg¹⁵⁵ to generate the meizothrombin des F1 intermediate. In the last step, Xa cleaves Arg²⁷¹ to generate Xa and the by-products F1 + F2 (80).

The phenomenon of channelling has also been described for II activation. Channelling is the ability of the prothrombinase to generate the active thrombin from II without producing the intermediates (81,82). This method of II activation may account as much as 50% of all the thrombin generated *in-vivo* (82).

Meizothrombin, meizothrombin des F1 and thrombin contain catalytic activities (83,84). It has been shown that meizothrombin and meizothrombin des F1 have similar activities as thrombin for the cleavage of S-2238 (85). However, they both contain less than 10% fibrinogen-clotting activity of thrombin (85). Moreover, meizothrombin contains higher catalytic activity compared to thrombin or meizothrombin des F1 for thrombomodulin-dependent protein C activation, thus suggesting their participation in the anticoagulant response (85,86). There is evidence to suggest that meizothrombin can also activate factors V and VIII. Since meizothrombin contains the Gla-domains, this suggests that meizothrombin may interact with membrane surfaces to increase its catalytic efficiency. However, the exact role of the intermediates in clotting *in-vivo* is not well understood.

1.3.6 X/Xa

Factor X has a molecular weight of 59 kDa and circulates in the plasma as a two-chained glycoprotein with a concentration of approximately 130 nM. The precursor to X is synthesized in the liver as a single chain, but upon release in the plasma it becomes cleaved to a two-chained molecule (87). The heavy chain is composed of 206 amino acids and is linked to a 139 amino acids long light chain through a disulfide bond (88).

The N-terminal Gla-domain (Ala¹-Gla³⁹) contains 11 Gla-residues, followed by a short hydrophobic stack (Phe⁴⁰-Lys⁴⁵) and two epidermal growth factor (EGF)-like domains (EGF1; Asp⁴⁶-Phe⁸⁴ and EGF2; Thr⁸⁵-Gly¹²⁸) (89,90). The Gla-domain enables X to interact with membrane surfaces and the EGF domains are believed to participate in cellular signalling and/or interaction of X with VIIa. X also contains an activation peptide (Ser¹⁴³-Arg¹⁹⁴), however, prior to being released in the circulation a set of cleavages removes residues Arg¹⁴⁰-Arg¹⁴², thus partially cleaving the activation peptide (87). The second cleavage occurs at the peptide bond between Arg¹⁹⁴-Ile¹⁹⁵ by the proteinases during the activation process. The heavy chain contains the serine protease domain (Ile¹⁹⁵-Lys⁴⁴⁸), with the catalytic triad located at His²³⁶, Asp²⁸² and Ser³⁷⁹ (87).

Activators of X include IXa (in the tenase) and TF/VIIa complex, both of which cleave X at Arg¹⁹⁴, resulting in the release of the activation peptide. As discussed above, only one cleavage is required to release the full activation peptide from the heavy chain of X, because X already had a set of cleavages that removed the peptide bond which attaches the activation peptide to the light chain before being released into the circulation (87). Since the heavy and light chains are tethered together with a disulfide bond, fully activated Xa still contains the Gla-domains (89,90). Thus, the Gla-domain increases the ability of Xa to interact with membrane surfaces in the presence of Ca²⁺ and its cofactor Va (prothrombinase complex), to increase the catalytic efficiency of converting II to thrombin (91).

1.3.7 V/Va

Factor V is a 330 kDa glycoprotein that is synthesized in the liver and circulates in the plasma at a concentration of approximately 30 nM. Being a cofactor, V shares a similar structural homology to VIII (92). It contains 3 A domains, 2 C domains and one B domain (A1-A2-B-A3-C1-C2) (93,94). Similar to VIII, V does not contain a Gla-domain. Even though evidence exists to show that V binds to membranes, the mechanism is not completely understood, but it is believed that membrane binding occurs through the hydrophobic residues localized on the C2 domains (95,96). V is activated through a series of protease cleavages involving residues Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵, which generates a 168 kDa molecule with enhanced cofactor activity for Xa (97). When bound to the membrane, Va contains a unique structure, whereby the C1 and C2 domains are attached to the membrane and are adjacent to each other, and the A1, A2 and A3 domains interact with the C1 and C2 domains in a triangular configuration (figure 1.6) (98,99).

In-situ production of V has been controversial in the literature, as platelets do not contain a nucleus necessary for production of factor V mRNA (106). It has been reported that megakaryocytes, precursor cells that produce platelets by fragmentation (see section 1.4.2), engulf V from plasma via endocytosis and store it in the α -granules (100). Moreover, it has been shown that megakaryocytes can also express factor V mRNA, which could be passed on to platelets during the fragmentation process (101). A recent study on mouse and human platelet transcriptomes has shown that platelets in fact contain stable mRNA with active protein synthesis (102). However, unlike mouse platelets, the study was unable to detect V transcripts in human platelets, which further supports the plasma-derived storage of V in human platelets (102).

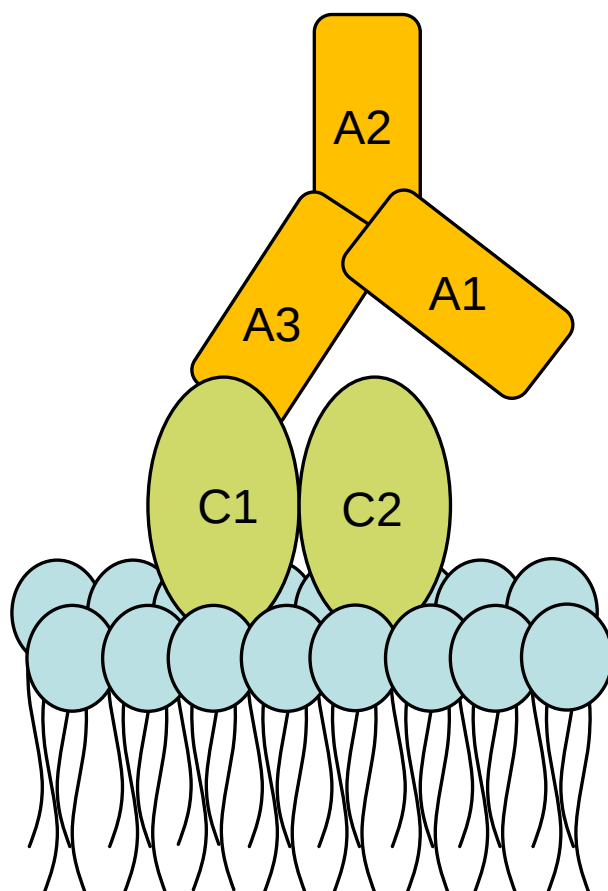


Figure 1.6: Structural association of Va with the phospholipid membrane. The C1 and C2 domains are adjacent to each other. They interact with the phospholipid membrane (blue circles) through their hydrophobic regions (C1; Leu¹⁹⁵⁶ and Tyr¹⁹⁵⁷ and C2; Trp²⁰⁶³ and Trp²⁰⁶⁴). Domains A1, A2, and A3 form a triangular shape, with the A3 domain interacting with the C1 domain.

Factor V is able to undergo post-translational modification involving the addition of an N-glycan on Asn²¹⁸¹ in the C2 domain, which results in the production of two variants: V₁ and V₂. They exist in the plasma and α -granules in a approximate molar ratio of 1:3 (V₁:V₂) (103,104). Thus when activated, the N-glycosylated form, Va₁, binds with a much lower affinity to the phospholipid membrane compared to the non-glycosylated Va₂ (105). However, it is Va₁ that is considered more thrombogenic, not because of its enhanced cofactor activity, but because of its relative resistance to inactivation by activated protein C (105).

Thus, the primary function of V/Va in coagulation is to act as a cofactor for Xa for enhanced prothrombinase complex assembly and function. In order for Xa to bind effectively to the membrane it requires the binding of Va first. In the prothrombinase complex, the molar ratio of Va:Xa is reported to be 1:1, and addition of excess Va did not change the catalytic efficiency of prothrombinase (62).

1.4 Role of cell membranes

All cells within the body are surrounded by a lipid bilayer comprised of various constituents, including but not limited to various types of phospholipids, embedded proteins, cholesterol, and carbohydrates. For example, to maintain fluidity of blood in vessels, blood cells ensure that anionic phospholipids, such as PS, are sequestered on the inner leaflet of their respected membranes (106). Otherwise, PS exposure on the cell surface, irrespective of the blood cell type, may result in a procoagulant state. The speeds at which many of the enzymatic reactions take place in the coagulation cascade are

significantly governed by the presence of an appropriate membrane surface (107). It is reported that the enzymatic reactions are enhanced by membranes, in part, by close localization and proper alignment of the participating proteins (108).

1.4.1 Synthetic phosphatidylcholine/phosphatidylserine vesicles

Synthetic phospholipid vesicles prepared from a 3:1 mixture of PC:PS have been the foundation on which cell-based models of coagulation have been developed (109). PCPS vesicles readily reproduce inhibition results similar to those observed with platelets, are easier to maintain and are simple to prepare, thus they are a very useful platform for studying coagulation (110). However, since membranes from PCPS vesicles lack many of the constituents that are present on cell membranes *in-vivo*, making conclusions from results attained from synthetic PCPS vesicles requires caution (111,112).

With respect to prothrombinase function, it has been established that II activation on synthetic vesicles comprised of 75% PC to 25% PS proceeds through the meizothrombin pathway (described in figure 1.5 green arrows). Upon prothrombinase formation on PCPS vesicles, Xa cleaves II at Arg³²⁰ to yield a partially activated intermediate called meizothrombin (80). Meizothrombin is then cleaved at Arg¹⁵⁵ to form meizothrombin des F1, which is further cleaved at Arg²⁷¹ by Xa to generate the active thrombin (80).

1.4.2 Platelets

The normal platelet count range in human blood is $150-400 \times 10^9/L$. They are formed by vesiculation of megakaryocytes as anucleated cells of diameters ranging from 2-4 μm . One megakaryocyte can produce over 1,000 platelets (113). Platelets are discoidal in shape when at rest, and upon activation produce pseudopodia-like projections protruding from the platelet cell surface (114). They can further vesiculate and shed microparticles, which contain similar membrane material as activated platelets (114), but are obviously much smaller, ranging from 40 to 500 nm in size (115). Platelet membranes also contain numerous proteins imbedded in their membrane, such as glycoproteins (GP) that are involved in the clotting process (e.g. GPIa, GPIb, and GPIIb/IIIa). Inside the cell, platelets contain microfilament and microtubular systems required for shape change and mobility of intracytoplasmic contents (114). Furthermore, platelets contain two kinds of granules called α - and dense granules. Some of the contents contained within α -granules include V, polyphosphates, vWF, fibrinogen, platelet factor-4, insulin-like growth factor, transforming growth factor β , and platelet-derived growth factor (116). Dense granules contain the adenosine nucleotides (ATP and ADP) and serotonin (116). The factors in the dense granule are often procoagulant and are released in the circulation upon platelet activation to cause a positive feedback for additional platelet activation/aggregation (114). It is believed that α - and dense granules have been produced during the megakaryocytic differentiation by engulfing plasma contents via endocytosis and storing the granules in the cytoplasm, although some contents of the granules may be produced *in-situ* (114).

With respect to coagulation, activated platelets contain approximately 3,000-5,000 binding sites for Xa and Va, thus suggesting a significant capability for enzyme complex formation on the platelet membrane (63). It has recently been shown that II activation by a prothrombinase on activated platelets proceeds through the prethrombin-2 fragment1.2 pathway (as described in figure 1.5 large black arrows). In this pathway, Xa cleaves II at Arg²⁷¹ to generate the intermediate prethrombin-2 and fragment1.2, followed by cleavage of prethrombin-2 at Arg³²⁰ to generate the active thrombin and by-product fragment 1.2 (117). Thus, it is believed that *in-vivo* the prethrombin-2 pathway serves to optimize the procoagulant activity expressed by activated platelets, by limiting the anticoagulant effects of the active intermediate, meizothrombin (117). Platelet-derived microparticle membranes contain 40- to 100-fold greater procoagulant activity compared to activated platelets (118).

1.4.3 Red and white blood cells

Red blood cells (RBCs) are the most abundant cells in human blood. RBCs from patients with various hematological conditions, such as sickle cell anaemia (119), beta-thalassemia (120), or preeclampsia (121), have been to having increased procoagulant activity. Evidence suggests that overt exposure of PS on the outer surface of the plasma membrane of RBCs or production of RBC-derived microvesicles rich in PS may be a potential mechanism to explain participation of RBCs in hemostasis and thrombosis. PS exposure on the outer surface of the membrane may provide docking sites for formation

of procoagulant enzyme complexes (tenase or prothrombinase) that help with amplification/propagation of the coagulation cascade.

Previous investigations have shown that intracellular signalling through increased Ca^{2+} flux, enhanced Ca^{2+} -dependent activation of phosphokinase C- α (PKC- α), and depletion of ATP can mediate PS exposure on the outer membrane surface of RBCs (122-123). Other signalling factors have been implicated, including arachadonic acid (124), prostaglandins (125), and platelet-activating factor (126). However, the extent of PS exposure through these means is minuscule, ranging from 3-5% of the total lipids comprising the RBC membrane (127). Thus, another signalling factor must exist to explain the rapid shift in membrane asymmetry. As demonstrated by a recent study, RBCs treated with phosphatidic acid (PA) responded quickly by decreasing intracellular ATP levels, increasing Ca^{2+} and activating PKC- α , which resulted in activation of the scramblase and attenuation of the flippase enzymes (127). Cumulatively, this caused rapid exposure of PS on the outer surface of RBCs and accelerated thrombin generation through enhanced prothrombinase activity (127).

A recent study by Mann *et al.* has demonstrated that RBCs and platelets work in synergy to cause enhanced thrombin generation in plasma (128). Furthermore, the authors have shown that non-platelet membranes, such as those from RBCs, can contribute approximately 35% to II activation. Moreover, the pathway for II activation on the surface of RBCs is mediated through the meizothrombin pathway (figure 1.5, green arrows) (128).

In addition to RBCs, it has been shown that certain white blood cells, such as monocytes, lymphocytes and neutrophils, are capable of forming the prothrombinase complex on the surface of their membranes (63). Moreover, all of the cell surfaces contained a significant capability to bind Xa and Va. For example, the number of bindings sites for Xa and Va on monocytes, lymphocytes and neutrophils have been estimated to be 16000, 45000, and 8000, respectively (63). Moreover, the catalytic efficiency of prothrombinase on monocytes and lymphocytes is comparable to platelets. The prothrombinase efficiency on neutrophils has been shown to contain 25% of the efficiency of platelet-prothrombinase (63).

1.5 Inhibitors

If coagulation was allowed to proceed without down-regulation, it would lead to thrombosis, which is defined as abnormal and excess clotting. Thus, inhibitor systems have been developed by the body to tackle each of the coagulation proteinases discussed above. Since the majority of active enzymes in the coagulation cascade are serine proteases, a set of proteins called serine protease inhibitors (serpins) have evolved with the task of down-regulating coagulation in order to prevent abnormal thrombosis. Serpins have a unique method of inhibiting the serine proteases, often referred to as the ‘suicide’ mechanism of inhibition. Each serpin type contains a loop, the reactive centre loop (RCL), which possesses a specific peptide bond for which a specific serine protease has high affinity (129-131). Upon recognition and cleavage of the peptide bond, a covalent bond is formed between the serpin and serine protease, followed by structural changes of

the serpin molecule which causes the serine protease-attached loop to become trapped within the body of the serpin (129). The resultant serpin-protease complex is quickly cleared from the circulation. The exact mechanism of inhibition will be discussed in more detail below.

1.5.1 Antithrombin

Antithrombin (AT), also called AT-III, is a single-chain plasma glycoprotein whose structure and function resembles that of other members of the serpin family. It is produced in the liver and has a molecular weight of 58 kDa. AT circulates in plasma at a concentration of approximately 3 μ M and has a half-life of 3 days (132). AT is composed of 31% α -helix, 16% β -sheet, 9% β -turn and 44% random coil, which make up 3 β -sheets (A to C), 9 α -helices (A to I) and a Reactive Center Loop (RCL), typical for all other serpins (129,131,133). There is a stretch of positively charged arginyl and lysyl residues comprising the D α -helix that represents the heparin-binding region (129). It has been demonstrated by both structural and biochemical analyses that the three amino acid residues Lys¹¹⁴, Lys¹²⁵, and Arg¹²⁹ interact with the high affinity pentasaccharide sequence of heparin. Moreover, residues at Arg¹³², Lys¹³³, and Lys¹³⁶ have been shown to contribute to the binding of longer heparin chains (129).

AT exists in two forms; 90% α -AT and 10% β -AT. The α -AT contains four glycan chains linked via N-amido glycosidic bonds to the R-group of Asn residues at positions 96, 135, 155 and 190 (2,129). However, β -AT is missing a glycan at Asn¹³⁵

(129) This is important, as glycosylation of Asn¹³⁵ obscures the heparin-binding region of AT, thus decreasing its catalytic capability (2,129,133).

AT and other serpins inhibit the serine proteases by a stress-release mechanism (129,133). Initially, the serine protease interacts non-covalently with AT until it cleaves a specific peptide bond in the RCL of AT. The target peptide bond in the RCL normally exists between Arg³⁹³ and Ser³⁹⁴, and is designated as P₁-P_{1'} (129,133). The serine protease, by means of a deprotonated serine hydroxyl residue situated at the active site, acts as a nucleophile which reacts with the target peptide bond in the RCL of AT. This forms an ester bond between the hydroxyl group of serine from protease with the Arg³⁹³ carboxyl-carbonyl from AT, thus covalently linking the serine protease to AT (2). In this reaction, the serine protease is treating AT as its substrate until the covalent ester bond is formed between the two molecules. This stage of the reaction is referred to as the acyl-enzyme intermediate stage of proteolysis (129,133,134). Prior to cleavage, the RCL of active AT exists in a stressed (S) configuration, which makes it readily accessible to nucleophilic attack by the serine protease. When the high-tension P₁-P_{1'} bond on the RCL becomes cleaved by the serine protease, the AT undergoes conformational change from the S-configuration to the relaxed (R) configuration (135). During this transitional change, the RCL-protease complex springs backwards into the β -sheet A pocket of AT and irreversibly traps the serine protease (129,133,135).

Serine proteases that are capable of being inhibited by AT include VIIa, IXa, Xa, XIa, XIIa and thrombin (129). AT's inhibition rate for the serine proteases can be

accelerated approximately 1,000-fold in the presence of heparin (136), which will be discussed in more detail below.

1.5.2 Heparin Cofactor II

Heparin cofactor II (HCII) is a 66 kDa protein which is synthesized in the liver and which circulates in plasma at a concentration of 1.4 μ M. Although it shares structural homology to AT, HCII is a highly specialized serpin in that thrombin is its only known target enzyme, and the specificity of HCII for thrombin is much higher than AT (137,138). HCII has adopted similar mechanisms as AT for inhibiting thrombin (139). HCII also contains a heparin-binding domain, and binding of heparin to HCII causes a change of conformation of the RCL in a similar manner described for AT (140,141). At its N-terminus, HCII also possesses an acidic tail that unfolds when glycosaminoglycans (such as heparin or dermatan sulfate) bind to it (142). It has been shown that deletion of the acidic tail results in 100-fold decrease in the rate of thrombin inhibition by HCII (142). Furthermore, the acidic tail of HCII was added to α_1 -antitrypsin which resulted in a >20-fold increase in the rate of thrombin inhibition (143,144). Thus, these results suggest that the acidic tail of HCII interacts with thrombin to cause the enhanced inhibition, and the exosite-1 of thrombin is believed to be the primary site candidate for this interaction. *In-vivo* studies in mice have shown that total absence of HCII from the circulation may not be lethal, and thus HCII has been referred to as a redundant serpin for inhibition of its target serine proteases (145).

1.5.3 Tissue factor Pathway Inhibitor

Although several anticoagulant proteins exist to down-regulate the various reactions in the coagulation cascade (figure. 1.1 above), tissue factor pathway inhibitor (TFPI) is the only one known to inhibit the TF/VIIa complex (146). TFPI is synthesized primarily by endothelial cells and functions to regulate the extrinsic pathway of coagulation. Thrombin stimulates the release of TFPI from endothelial cells to act at sites with excess TF/VIIa activity. However, TFPI can also remain locally bound to endothelial cells because it has the capability of interacting with glycolipid microdomains or glycosaminoglycans found on the surface of endothelial cells (147,148).

TFPI is composed of an acidic amino terminus, followed by three tandem Kunitz-type proteinase inhibitory domains, and a basic carboxy-terminus (146,149). Kunitz domain-1 and -2 inhibit the active sites of VIIa and factor Xa, respectively, when TF/VIIa cleaves X to Xa. While the inhibitory role of Kunitz domain-3 is yet to be determined, it does show capability of interacting with cellular surfaces (146,150). In order to act as an inhibitor of the TF/VIIa complex, the TFPI protein must form a quaternary complex with VIIa, TF and Xa. The process is as follows: TFPI binds reversibly to Xa, and then the TFPI/Xa complex inhibits the VIIa which is bound to TF, thereby forming a quaternary complex. Thus, TFPI mediates inhibition of the initiation complex after the TF/VIIa complex has had a chance to activate some X to Xa (146,151). No documented cases of TFPI deficiency has been described to date, suggesting a deficiency in TFPI may lead to embryonic lethality. However, a murine model involving a homozygous Kunitz domain-1 mutation has been described to cause embryonic death between days 9.5 and birth (146).

1.5.4 Protein C/Activated protein C

Protein C is synthesized in the liver, has a molecular weight of 63 kDa, and circulates in the plasma at a concentration of 65 nM. Protein C becomes activated to active protein C (APC) by thrombin. The reaction becomes greatly enhanced if protein C is bound to the endothelial protein C receptor (EPCR) and thrombin to thrombomodulin (152). Thus, thrombin on thrombomodulin enhances the conversion of protein C on EPCR to APC by 1,000-fold relative to conditions without cofactors (153,154). Thrombin cleaves protein C at Arg¹⁶² to release an activation peptide and generate APC (155). The primary role of APC is to inactivate Va and VIIIa in order to down-regulate the prothrombinase and tenase functions, respectively. Inhibition of Va and VIIIa by APC is significantly accelerated by binding of APC to its cofactor, Protein S (156). The half-life of APC in the circulation is significantly extended when bound to protein S (157). Inactivation of Va by APC occurs through limited proteolysis of the heavy chain. APC cleaves Va at Arg⁵⁰⁶, followed by Arg³⁰⁶ and Arg⁶⁷⁹ (158). Cleavage at Arg⁵⁰⁶ causes a reduction in Va cofactor activity by 30%, and the subsequent cleavage at Arg³⁰⁶ completely inactivates Va (158). Although it is not completely understood, cleavage of Va at Arg⁶⁷⁹ seems to play a lesser role for its inactivation.

Down-regulation of VIIIa can occur enzymatically by APC or by non-enzymatic means. For enzymatic inhibition, APC cleaves VIIIa at Arg³³⁶ of the A1 domain and at Arg⁵⁶² of the A2 domain (159,160). The first cleavage reduces the binding affinity of A1 for A2, and the second cleavage disrupts the interaction of VIIIa with IXa (161,162). However, cleavage of VIIIa by APC at the A1 domain seems to play a greater role for

VIIIa inactivation than the latter (162). For non-enzymatic inactivation, the A2 domain of VIIIa is capable of spontaneously dissociating from the A1/A3-C1-C2 dimer (51). Interestingly, APC is only capable of inactivating membrane-bound Va and VIIIa, whereas the zymogen forms are poor substrates for APC (161).

1.5.5 Alpha-2-Macroglobulin

Alpha-2-macroglobulin (A₂M) is not a member of the serpin family of inhibitors, but is one of the key players for inhibiting the serine proteases *in-vivo*. A₂M has a molecular weight of 725 kDa and circulates in plasma as a dimer at a concentration of 3 µM (163). The 4 subunits are arranged in such a way that they form a cage, in which A₂M irreversibly entraps the enzyme upon inhibition without blocking the functional active site of the proteinase (164). Thus, bound proteinases often retain activity towards small molecule substrates, whereas activity for large substrates is generally eliminated (165,166). For example, chromogenic substrate cleavage by serine proteases entrapped by A₂M can still be detected, thus suggesting activity of the serine proteases persist even when inhibited by A₂M (166).

1.6 Natural and synthetic anticoagulants

Natural regulators of activation, amplification/propagation and down-regulation of coagulation have all been discussed above. However, a failure in any one part may lead to severe consequences such as severe bleeding or thromboembolic complications.

Thrombosis, where the natural systems cannot down-regulate coagulation, readily requires the use of exogenous, commercially prepared, anticoagulants.

For the past seven or so decades, heparin has been used worldwide as the gold standard anticoagulant for the treatment of thromboembolic diseases. However, heparin has numerous clinical limitations (discussed below in more detail) which have led to the development of better anticoagulants, including various fractions of heparin (low molecular weight heparin or pentasacharride sequence) and direct inhibitors of Xa and thrombin. Direct inhibitors of Xa (e.g. Apixaban or Rivaroxaban) and thrombin (such as Dabigatran Etaxilate) are not within the scope of this study and will not be discussed further. This report will focus on the heparinoid anticoagulants where clinical data is abundant.

1.6.1 Unfractionated Heparin

Unfractionated heparin (UFH) is a member of the glycosaminoglycan (GAG) family of molecules that are exclusively synthesized by mast cells. GAGs are straight polysaccharide chains composed of repeating uronic acid-hexosamine disaccharide units (2). GAGs are biosynthesized by O-linkage to protein cores, and one protein core may have as many as 10 heparin chains ranging from 30 – 100 kDa in size, linked via O-glycosidic bonds to serine residues in a glycine-serine sequence repeat (2,167,168). GAGs provide several functions *in vivo*. In the proteoglycan form, GAGs act as structural components of the extracellular matrix and can act as tissue-chemoattractant molecules by binding growth factors, interleukins and other cell messengers (169,170). In addition,

via the pentasaccharide sequence, UFH can function as an anticoagulant by catalyzing the reaction of AT with thrombin and other serine proteases (2,167).

UFH has an average molecular weight of 15 kDa, with a range between 5-50kDa. The anticoagulant capability of UFH occurs through a high affinity pentasaccharide sequence (figure 1.7) which is specific for recognizing the heparin binding region of AT. Initially, residues D, E and F from the pentasaccharide bind to AT through charge interaction and hydrogen bonding (129). Although the bonds are relatively weak, they do induce a conformational change in AT. The 2-O-sulfated iduronic acid (monosaccharide G) converts from a chair to a skewed-boat configuration, thus allowing its residues to interact with AT's Arg⁴⁷ to a greater degree (2). In addition, the skewed boat configuration of G and the conformational change of AT, enables the 3-O-sulfate of the central monosaccharide (F) and a cluster of functional groups on G and H to bind to AT with high affinity, thus securing the GAG and serpin together (2,130,134,171). The 3-O-sulfated glucosamine residue on the F-monosaccharide of the high affinity pentasaccharide sequence has been determined to be critical for binding to AT (172,173).

In the native AT molecule that has not reacted with UFH, the N-terminal region of the RCL (including the hinge region) is joined to strand 4 of β -sheet A, which restricts and partially hides the RCL and the P₁-P_{1'} side chains from being readily recognized by the serine proteases (134,174). Binding of UFH to the D α -helix of AT induces a conformational change in AT, which results in the release of the hinge region from β -sheet A. The conformational change of the P₁-P_{1'} side chain becomes reoriented, which makes it more accessible for recognition by the serine proteases (134,174). Interestingly,

re-chromatography of pure AT that was once previously reacted with heparin yielded a population of purified AT molecules that did not interact with the UFH (135).

The reaction of AT with serine proteases can be accelerated with UFH by two methods (129,130). In the first method, a conformational activation process can be induced where binding of UFH to AT causes an allosteric change in AT that results in a reconfiguration of the RCL and making it accessible to the serine proteases (130,134). The second method is through a template mediated effect (10), whereby UFH simultaneously binds AT and the serine protease, thus bridging the two in close proximity to each other and increasing the rate of protease inhibition by AT (130,175). Once the protease-AT complex has formed, the affinity of UFH for AT decreases and the UFH subsequently dissociates from the ternary complex (2). Inhibition of thrombin by AT+UFH occurs through the template mediated mechanism, whereas inhibition of Xa occurs through the conformational activation mechanism (129,130). Moreover, UFH chains with molecular weights less than 5.4 kDa cannot inhibit thrombin (130).

UFH is prepared by extraction from tissues obtained from animal slaughter houses (i.e. porcine intestine, bovine lungs, etc.). There are five basic steps utilized for preparation of commercial heparin: (1) preparation of tissue; (2) extraction of heparin from the tissue; (3) recovery of raw heparin; (4) purification of heparin and (5) recovery of purified heparin (176). However, the methodologies associated with each of the steps are complex and intricate, and are outside the scope of this report.

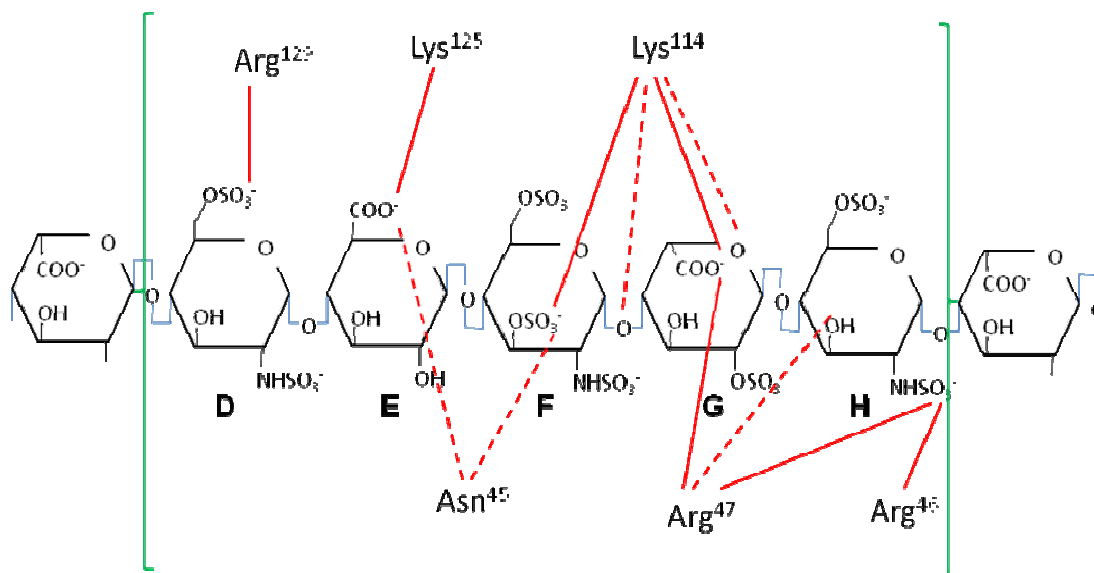


Figure 1.7: High affinity pentasaccharide sequence. The high affinity pentasaccharide sequence is listed D-H between the green lines. Direct side chain interactions of AT with the pentasaccharide sequence are illustrated schematically, with solid red lines depicting salt bridges and dashed red lines representing hydrogen bonding.

1.6.2 Low Molecular Weight Heparin

Low molecular weight heparins (LMWHs) are defined as having an average molecular weight of less than 8 kDa (176). LMWH must also have a potency of 70 units/mg of anti-Xa activity and a ratio of anti-Xa activity to anti-thrombin activity of ≥ 1.5 (176). The rationale for development of LMWH was mostly to address the limitations of the pharmacokinetic and pharmacodynamic properties of heparin. But also it was based on the belief that anti-Xa activity supported efficacy whereas anti-thrombin activity was responsible for bleeding effects (177). However, activity of LMWHs is much more complex than their anti-Xa and anti-thrombin effects.

LMWH is derived from standard UFH through partial depolymerisation by chemical or enzymatic means (176). An early way of preparing LMWH was through fractionation, however nowadays partial depolymerisation is the most common way of producing LMWH. The most common method used is deaminative cleavage of heparin with nitrous acid or an organic nitrite (amyl nitrite), which breaks the heparin chains at the N-sulfated glucosamine residues. This method usually leaves an anhydromannose reducing-end residue which can be reduced to anhydromannitol (178). The second method utilizes enzymatic (limited heparinase) or chemical beta-elimination (benzoyl ester formation, followed by base elimination), which leaves an unsaturated uronic acid derivative at the non reducing terminus. Lastly, oxidative depolymerisation (H_2O_2 and Cu^{2+}) is the third method employed to produce LMWH from UFH (178).

LMWH is considered to be the standard of care for clinical management of thromboembolic complications, particularly venous thromboembolism (177). Various clinical investigations have been performed in the past to show potential advantages of LMWH over UFH. Some of the advantages include: decreased non-specific protein interaction, increased bioavailability, more predictable dose response, increased half-life, and decreased side effects such as severe bleeding or heparin-induced thrombocytopenia (179).

Fondaparinux

Fondaparinux is a pentasaccharide sequence derived strictly from chemical synthesis and not from animal origins as in the case of UFH or LMWH. There are over 50 different steps involved in synthesizing this agent, however the final product is a pure chemical compound, without the potential for contamination from animal sources (180). Fondaparinux has a molecular weight of 1.8 kDa, thus all of the activity AT+fondaparinux is against Xa. Fondaparinux consists of five monosaccharide units and is prepared as the α -methyl glycoside of the pentasaccharide which represents the unique sequence in UFH that binds AT with high affinity (181).

Fondaparinux becomes 100% bioavailable after subcutaneous injection, and reaches peak plasma concentrations at 1 to 3 h (180). The half-life of fondaparinux is 13-15 h and the primary route of elimination is through the kidneys. Fondaparinux is not metabolized by the liver (180). The major complication associated with fondaparinux is severe bleeding, which seems to be a dose dependent effect. One of the most beneficial

aspects of fondaparinux is that no cases of heparin-induced thrombocytopenia have been reported in any of the available studies (180).

1.6.4 Limitation of the Heparinoids

Although heparin is clinically useful, it still has a number of limitations. Firstly, UFH has a short, dose-dependent intravenous half-life ranging from 0.3 to 3 hours (182). Clearance of UFH and LMWH occurs through renal filtration or hepatocyte metabolism (183). Also, UFH binds non-specifically to plasma proteins, platelets, and endothelial cells, which results in decreased concentrations of free UFH in the plasma (184,185). In addition, the binding of UFH to plasma proteins decreases the formation of thrombin-AT complexes, resulting in a reduced anticoagulation effect (184-186). Protamine is used to counter-act the effect of UFH (2). However, due to their small size, LMWHs and fondaparinux are unable to interact with protamine (2), which increases the bleeding risk (187). Also, UFH and LMWH readily pass through physiological compartments and can exchange between the circulation and extravascular space, thus adding to the unpredictability in their plasma half-lives (2,188). Furthermore, only 1/3 of commercial UFH contains the high affinity pentasaccharide sequence necessary for catalytic activation of AT (2,189). Lastly, UFH is unable to catalyze AT inhibition of fibrin-bound thrombin, due to its ability to form a ternary complex between fibrin and thrombin (2,190).

Numerous side effects have been observed with the use of UFH. Approximately 3% of adult patients receiving UFH tend to develop heparin-induced thrombocytopenia, a

condition in which an immune response is elicited against UFH bound to platelets (2,187). LMWH is not recommended for patients with heparin-induced thrombocytopenia, as LMWH cross-reacts with 80% of the antibodies produced against the original UFH treatment (2,187). However, use of LMWH prior to heparin-induced thrombocytopenia shows a lower bleeding risk profile and decreased immune response (2). In addition, prolonged use of UFH is associated with osteoporosis in approximately 17-36% of patients (2).

1.7 Covalently-Linked Antithrombin-Heparin (ATH) Complex:

To address some of the limitations of the heparinoids discussed above, researchers have studied methods of stabilizing the interaction of AT with UFH in order to enhance the anticoagulant effectiveness of the serpin. It was proposed that covalent conjugation of AT with UFH (ATH) would improve the safety and efficacy of UFH (79,191).

The rationale for the use of these covalent conjugates is several-fold. For example, AT should remain in a permanently activated conformation when heparin is irreversibly bound to it (191). The rate limiting step in anticoagulation is the rate at which heparin complexes with AT, thus the act of covalently linking heparin to AT eliminates this step and results in an increased inhibition rate (192). Also, in covalently linked ATH, most of the high-affinity pentasaccharide would be interacting with the heparin-binding region of AT. This would decrease the ability of heparin to interact with other molecules and thus decrease the non-specific binding of ATH to other plasma proteins. Cumulatively, this may result in a more predictable antithrombotic response, an

increased half-life, and decreased risk of hemorrhagic side effects compared to heparin (2,191,193).

1.7.1 Formation of ATH:

Currently, there are only three approaches to synthesizing ATH by permanently linking AT with heparin (2). However, this report will focus on the synthesis of ATH by the method of Chan *et al.* (191), which from now on will be referred to as ATH. ATH was synthesized via Schiff base formation and a spontaneous Amadori rearrangement of heparin together with AT (2). The production methodology of ATH stems from *in vivo* observations of haemoglobin in red blood cells becoming glycated during hyperglycemia in diabetes.

Briefly, AT and a vast excess of UFH (approximately 200-fold higher than AT) from various sources, were mixed together in phosphate-buffered saline (PBS; 0.016 M Na_2HPO_4 , 0.004 M NaH_2PO_4 , 0.15 M NaCl (pH 7.3)), and was heated for 16 days at 40°C (191). Under these conditions, a Schiff base forms between an amino group of AT and a terminal aldehyde of heparin, followed by a spontaneous Amadori rearrangement, to yield the covalent linkage between AT and heparin (191).

Some of the Schiff base intermediates may not have been stabilized by Amadori rearrangements, so the reaction mixtures were further incubated with a reducing agent, NaBH_3CN . The isolation of ATH required a two-step procedure involving hydrophobic chromatography on butyl-sepharose, followed by anion exchange chromatography on diethylaminoethyl Sepharose (DEAE-Sepharose). Butyl-sepharose chromatography was

used to remove any excess unbound heparin, whereas DEAE Sepharose chromatography was used to remove any unbound AT, resulting in the isolation of pure ATH (2,191,193).

Employing the above procedure, the percent recovery of AT to ATH was approximately 50%, the highest recovery reported to date involving the covalent linkage of AT to heparin. Analysis of ATH preparations revealed that AT and heparin exist in a 1:1 molar ratio, and that 20-40 % of the ATH molecules contain 2 or more high-affinity pentasaccharide sequences (191).

1.7.2 Characterization of the ATH Complex:

In vitro investigation involving inhibition experiments revealed that ATH inhibited Xa and thrombin more effectively than non-covalently linked AT and heparin (AT+UFH) (191). Kinetic rate experiments of thrombin inhibition showed bimolecular and second-order rate constants that were one order of magnitude faster for ATH relative to AT+UFH (191,193). Interestingly, the second-order rate for ATH represents one of the highest values ever reported at $3.1 \times 10^9 \pm 0.4 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$ (191,194,195).

Structural analysis of the ATH molecule revealed that 45% of ATH conjugates contained the β -AT isoform (196). In addition, inhibition experiments showed that the β -AT isoform was able to inhibit thrombin 2-fold faster than α -AT (196-199). As discussed above, only 1/3 of commercial UFH molecules contain the high affinity pentasaccharide sequence, compared to all of the ATH molecules (189,194). Thus, ATH formation selects for UFH molecules possessing the high-affinity pentasaccharide sequence (191,194). Inhibition experiments involving the addition of exogenous AT to the system revealed a

15-fold increase in Xa and thrombin inhibition activity, thus suggesting ATH molecules were able to interact with the exogenous AT (191).

Numerous *in-vitro* and *in-vivo* investigations have been performed to evaluate the potential use of ATH as an alternative anticoagulant to UFH for the prophylaxis and treatment of thromboembolic conditions (200). The first study looked at the effect of ATH on fibrin clot accretion on existing clots in a rabbit model (201). ATH was compared to AT+UFH, UFH, AT and saline, and it was found that ATH decreased clot mass by approximately 17 %, whereas the other treatments caused an increase in clot mass by an astonishing 24%, 60%, 182% and 135%, respectively (201).

Also using a rabbit model, single-exponential half lives were calculated for UFH, AT+UFH and ATH, which were 0.32, 0.41 and 2.6 h, respectively (191). From this study, it was shown that ATH has a 6-8-fold longer half-life than free UFH (191). In addition, plasma appearance of subcutaneous injections of ATH was evaluated and yielded important pharmacokinetic properties of ATH (191). The appearance of UFH in plasma occurred at 1 h post-subcutaneous injection, while ATH reached peak plasma concentration at 24-30 h, and was still present up to 96 h post-injection (191). The findings from this experiment suggest that ATH was poorly absorbed into the bloodstream and remained localized to the site of injection (191).

To further verify sequestration of ATH at the site of injection, tracheal instillation experiments were performed in rabbits (202). Measurable concentrations of ATH were noted in lung lavage up to 48 h post-injection, whereas heparin readily escaped into the surrounding tissue and into the systemic circulation immediately after injection (202).

These findings for ATH may prove useful for the treatment of neonatal respiratory distress syndrome, where prolonged anticoagulation in the lung may reduce pulmonary fibrin deposition (2,193).

In a recent investigation involving a pig model of cardiopulmonary bypass (CPB), ATH significantly reduced the development of microemboli during CPB compared to UFH or AT+UFH. In addition, the catalytic effects of ATH can also be reversed with protamine sulfate. This suggests that ATH may be a good alternative anticoagulant to use for the prevention of microemboli while patients are on CPB during cardiac and cardiovascular related surgeries (181).

Lastly, an important area of application of ATH is in the coating of biomaterial surfaces. Numerous *in vitro* and *in vivo* (rabbit jugular vein model of thrombosis) experiments have been performed and the results may indicate that biomaterials (catheters and stents) coated with ATH were less thrombogenic compared to UFH or saline coated ones (203,204,205). Stents and catheters are often used to treat patients with acute coronary syndromes and cancer, respectively, and application of ATH in these conditions may prove to be more beneficial than UFH.

Overall, these studies suggest an obvious advantage of ATH over UFH ($\pm$ AT) for the possible treatment of thrombosis *in-vivo*. To further evaluate this comparison, we decided to pursue inhibition of the prothrombinase complex by AT+UFH versus ATH.

1.8 Specific aims, hypotheses and rationales

1.8.1 Specific aim 1:

To investigate the mechanism of inhibition of the prothrombinase complex by ATH versus AT+UFH when the prothrombinase complex is formed on synthetic phosphatidylcholine/serine phospholipid vesicles.

1.8.2 Hypothesis 1:

We hypothesize that ATH will inhibit Xa within the vesicle-prothrombinase complex to a greater degree compared to AT+UFH.

1.8.3 Rationale 1:

We have previously established that ATH inhibits coagulation factors in isolation much faster compared to AT+UFH. Unlike unfractionated heparin, we have demonstrated that ATH is capable of inhibiting thrombin-bound thrombin.

1.8.4 Specific aim 2:

To investigate inhibition of Xa within the prothrombinase complex by ATH versus AT+UFH when the prothrombinase complex is formed on a more physiologically relevant surface such as activated platelets.

1.8.5 Hypothesis 2:

We hypothesize that ATH will inhibit Xa within the platelet-prothrombinase complex to a greater degree compared to AT+UFH.

1.8.6 Rationale 2:

Variable protection of Xa with the prothrombinase has been reported in the literature when prothrombinase forms on platelet surfaces. Given that the heparin moiety in covalent ATH cannot dissociate from the AT, as demonstrated by our previous studies, we expect that platelet-prothrombinase will be inhibited more avidly by the ATH.

1.8.7 Specific aim 3:

To investigate inhibition of Xa within the prothrombinase complex by ATH versus AT+UFH when the prothrombinase complex is formed on the surface of procoagulant red blood cells.

1.8.8 Hypothesis 3:

We hypothesize that ATH will inhibit Xa within the red blood cell-prothrombinase complex to a greater degree compared to AT+UFH.

1.8.9 Rationale 3:

It has been determined that red blood cells can become procoagulant by expressing anionic phospholipids, such as phosphatidylserine, on the outer surface of their membrane. Moreover, previous investigations have shown that the function of

prothrombinase complex is greatly enhanced by the procoagulant red blood cell surface. Since surfaces containing anionic phospholipids are the foundation for prothrombinase formation in vesicles and platelets, we similarly expect ATH to inhibit this complex in the red blood cell system.

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Chapter 2: Manuscript One

***Mechanism of inhibition of the prothrombinase complex by
covalent antithrombin-heparin complex***

Foreword

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Ivan Stevic performed the research and drafted the manuscript. Leslie Berry provided scientific discussion and revised the manuscript. Anthony Chan oversaw the work.

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**MECHANISM OF INHIBITION OF THE PROTHROMBINASE COMPLEX BY
A COVALENT ANTITHROMBIN-HEPARIN COMPLEX**

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Running Title: Prothrombinase inhibition by covalent antithrombin-heparin

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Abbreviations: AT, antithrombin; UFH, unfractionated heparin; ATH, covalent antithrombin-heparin complex; PCPS, phosphatidylcholine/serine phospholipids; II, prothrombin

SUMMARY:

Factor-Xa assembly into the prothrombinase complex decreases its availability for inhibition by antithrombin+heparin (AT+UFH). We have developed a novel covalent antithrombin-heparin complex (ATH), with enhanced anticoagulant actions compared to AT+UFH. The present study was performed to extend understanding of the anticoagulant mechanisms of ATH by determining its inhibition of Xa within the critical prothrombinase. Discontinuous inhibition assays were performed to determine final k_2 values for inhibition of Xa. Fluorescent microscopy was conducted to evaluate inhibitor-prothrombinase interactions. The k_2 for inhibition of prothrombinase versus free Xa by AT+UFH was lower, whereas for ATH were much higher. Relative to intact prothrombinase, rates for Xa inhibition by AT+UFH in complexes devoid of prothrombin/vesicles/factor-Va were higher. For ATH, exclusion of prothrombin decreased k_2 , removal of vesicles increased k_2 , and exclusion of factor-Va gave no effect. While UFH may displace Xa from prothrombinase, Xa is detained within prothrombinase during ATH reactions. We confirm prothrombinase hinders inhibitory action of AT+UFH, whereas ATH is less affected, with prothrombin being a key component in the complex responsible for the opposing effects. Overall the results suggest that covalent linkage between AT-heparin assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function compared to conventional non-conjugated heparin.

INTRODUCTION:

The coagulation cascade is a series of enzymatic reactions involving conversion of proenzymes to activated coagulation factors. Some of these factors combine to form macromolecular complexes responsible for generating the key enzymes of the cascade. The prothrombinase complex, which is composed of factor Xa (Xa), its cofactor Va (Va), phospholipid surface (PCPS), calcium ions and prothrombin (II), is the enzymatic complex responsible for converting II to thrombin (1-3). The catalytically-active component of the prothrombinase is Xa, which requires Va in a minimum ratio of 1:1 for enhanced II to thrombin conversion (1;4;5). Formation of the full prothrombinase complex may enhance the conversion of II to thrombin by 3.0×10^5 fold (6;7). Several studies have looked at omitting components of the prothrombinase complex on thrombin generation, and it has been observed that absence of any components from the prothrombinase significantly reduces its capability to generate thrombin (8-10).

The cascade is a fine balance between pro- and anticoagulant activity and a net shift in this activity may lead to development of thrombotic complications or severe hemorrhaging (11-15). Inhibitors of the coagulation cascade are important and are required to adequately regulate the cascade's activity. Antithrombin (AT) is the major *in vivo* serine protease inhibitor (serpin) that is variously capable of inhibiting thrombin (16), factors VIIa (17), IXa (18), Xa (19), XIa (20), and XIIa (21). Inhibition of the serine proteases by AT is significantly accelerated by the presence of unfractionated heparin (UFH) (22). UFH is part of the glycosaminoglycan family, and UFH interacts with AT

through a high affinity pentasaccharide sequence (23-25). Reportedly, UFH catalytically increases the rate of AT reaction with thrombin or Xa in excess of 1000-fold (26).

Clinical use of heparin has yielded many of its limitations, including a short dose-dependent intravenous half life and unpredictable anticoagulant response (27-29). Complications from UFH may arise, such as heparin-induced thrombocytopenia or severe bleeding (30). Also, only one-third of UFH molecules contain the high affinity pentasaccharide sequence (31;32). In addition, several studies have shown that assembly of Xa in the prothrombinase complex results in protection from inhibition by AT+UFH (33-35). The extent of this protection is a matter of debate. Reports range from complete protection (10;36) to less than 5- to 10-fold differences (33;37-39).

To overcome some of these limitations, we have employed a covalent complex of AT and UFH (ATH) to study its effect on blood coagulation. Over the past decade, we have determined that the covalent conjugate results in a higher intravenous half-life (40), increased inhibition of fibrin-bound thrombin (41), reduced interaction of its heparin chains with endothelial surfaces (42), more potent antithrombotic activity (43), predictable anticoagulant response (44), and higher rates for inhibition of coagulation factors in isolation (45). However, interaction of ATH with surface-based enzyme complexes has not been studied before. Therefore, we investigated inhibition of the prothrombinase complex by ATH versus AT+UFH, and determined potential mechanisms involved in the enhanced anticoagulant actions of ATH.

MATERIALS and METHODS:

Chemicals: All chemical reagents were of analytical grade. Sodium chloride and Tris (tris (hydroxymethyl) amino-methane) were purchased from Bioshop (Burlington, ON). Polyethylene glycol 8000, ethylene-diamine tetraacetic acid-disodium salt (Na₂EDTA) and calcium chloride dihydrate (CaCl₂) were from BDH Inc. (Toronto, ON). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI) and sucrose was purchased from ACP Chemicals Inc. (Montreal, QC). Human factor Xa, II and thrombin were purchased from Enzyme Research Laboratories Ltd. (South Bend, IN). Human factor Va and fluorescein FPR-chloromethylketone (FPPack) was obtained from Haematologic Technologies Inc. (Essex Junction, VT). Substrates for Xa (S-2222; N-benzoyl-isoleucyl-glutamyl-glycylarginyl-p-nitroanilide-hydrochloride and its methyl ester) and thrombin (S-2238; H-D-phenylalanylpipecolyl-arginyl-p-nitroanilide-dihydrochloride) were purchased from DiaPharma Group Inc. (Westchester, OH). AT was purchased from Affinity Biologicals (Ancaster, ON, Canada). Unfractionated sodium heparin (UFH) was obtained from Sigma (St. Louis, MO). Thrombin-specific inhibitor, Pefabloc-TH (N α -(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine piperidide acetate (NAPAP)) was purchased from Pentapharm Ltd. (Engelgasse, Switzerland). L- α -phosphatidylcholine type XVI-E (PC) and L- α -phosphatidyl-L-serine (PS) phospholipids and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON). FPR-chloromethylketone dihydrochloride (PPack) was obtained from Calbiochem (La Jolla, CA).

Preparation of ATH: ATH was synthesized as described by Chan *et al.* (40). Briefly, 1052 mg of human AT and 64 g of UFH was separately dialyzed against 2 M NaCl followed by Phosphate Buffered Saline (PBS) and mixed to a volume of 900 ml. The reagents were incubated at 40°C for 16 d. Following incubation, 0.5 M NaBH₃CN reducing agent was added to the mixture and incubated for another 5 h at 37°C. ATH was purified using a two-method extraction process involving butyl-sepharose hydrophobic chromatography (Amersham, Uppsala, Sweden) and DEAE-sepharose anion exchange chromatography (Amersham, Uppsala, Sweden) for the removal of any unbound heparin or AT, respectively. ATH was analyzed for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and was found to be > 95 % pure (40). We have previously shown that the AT content (amino acid analysis) and heparin content (by 3 different mass analysis methods) of ATH preparations are in a mole ratio of 1:1(46).

Preparation of phosphatidylcholine/serine (PCPS) vesicles: PCPS vesicles in a 3:1 (PC:PS) concentration ratio were made by taking 60 µl of 250 mg/ml of PC and mixing with 100 µl of a 50 mg/ml solution of PS, followed by dissolution in 2 parts (200 µL) chloroform to 1 part (100 µL) methanol in a KIMAX borosilicate glass round bottomed tube (Daigger, Vernon Hills, IL, USA). The mixture was then dried under a light stream of argon gas. To remove any remaining organic solvents, the product was lyophilized for approximately 1 h. After the lipids were completely dried, they were re-suspended in 5 ml of 20 mM Tris, 0.15 M NaCl, pH of 7.4 buffer and sealed under an argon gas atmosphere. Tubes were then sonicated at 4°C for approximately 2 h or until the cloudy

solution turned clear. The sonicated vesicles were transferred to Beckman Clear Ultracentrifuge tubes (Beckman Coulter, Brea, CA, USA), and centrifuged at 40,000 rpm in a SW55 rotor (Beckman Centrifuge) for 3 h. Four ml of the resultant vesicle solution was then mixed with 0.4 g of sucrose and aliquots were stored at -80°C . One ml of vesicle solution was used for quantification by phosphate analysis.

The PCPS vesicles were analyzed for concentration by phosphate analysis. Thirty μl of magnesium nitrate solution (1 g of $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ per 10 ml of 95% ethanol) was added to 5 μl of sample. The solution was heated over a Bunsen burner flame in a fume hood until brown fumes were produced by the solution. After cooling, 0.3 ml of 0.5 M HCl was added to hydrolyze any pyrophosphate that may have been formed. The tubes were then capped with a marble and put in a boiling water bath for 15 min. After cooling, 700 μl of 1 part 10% ascorbic acid to 6 parts 0.42% ammonium molybdate $\cdot 4\text{H}_2\text{O}$ in 0.5 M H_2SO_4 was added and incubated for 20 min at 45°C . After cooling for 15 min, 200 μl of solution was transferred to a 96-well round bottom well plate. Absorbance readings at 820 nm were taken using a SpectraMax Plate Reader for the samples and reference phosphate standards. A standard curve was produced from readings of 0, 5, 10, 15, 25, 50 and 70 μl of 1 mM phosphate standards. The PCPS vesicle concentration was determined by comparing the 820 nm absorbance readings from the samples against the standard curve.

Determination of second-order rate constants (k_2 values): k_2 values for inhibition of $\text{Xa} \pm$ prothrombinase complex by AT+UFH or ATH were determined by performing

discontinuous second-order rate constant assays under pseudo first-order conditions, as described previously (45). Briefly, rate experiments were performed at physiological temperature (37°C) and a minimum inhibitor:enzyme ratio of 10:1 was maintained for all reactions. To determine the concentration of UFH that yields maximal inhibition of Xa by AT, a range of UFH concentrations were tested (figure 2.1). It was determined that maximal inhibition of Xa was achieved with a 10 fold mole ratio of UFH to AT, or 300 nM UFH. For rate experiments involving the inhibition of free-Xa by AT+UFH or ATH, the reaction protocol was as follows: 10 µl aliquots of 6 nM Xa (3 nM final) containing 8 mM CaCl₂ (4 mM final) in TSP buffer (0.02 M Tris HCl + 0.15 M NaCl + 0.6 % polyethylene glycol, pH = 7.4) were incubated in 7 separate wells of a 96-well flat bottom microtiter plate (Fisher, Nepean, ON, Canada) for 5 min. To well number 7, 10 µl of TSP buffer was added to represent the control reaction of the experiment. At time intervals ranging from 2–5 s, a 10 µl volume of inhibitor (30 nM AT+300 nM UFH or 30 nM ATH final) was added to the remaining wells (1 through 6). At 2-5 s following addition of inhibitor to well 6, the reactions were neutralized by the addition of 80 µl of developing solution containing 1.25 mg/ml of polybrene + 0.53 mM Xa-specific substrate S-2222 + 1.14 mM of Na₂EDTA in TSP buffer. After terminating the inhibition reactions, residual Xa activity for each well was determined by measuring the absorbance at 405 nm (SpectraMax Plus 384 plate reader) for a specific period of time (10-30 min). For prothrombinase reactions, 5 µl of solution (containing 9.6 nM Va, 48 µM PCPS, 1.2 µM II, 4 mM CaCl₂ and 40 µM Pefabloc-TH, final) were first incubated in 7 wells for 2 min, followed by simultaneous addition of 5 µl of enzyme (3 nM Xa final) to all wells.

Inhibitors were then added as described above. To calculate the pseudo first-order constant k_1 , plots of $\ln V_t/V_o$ (V_t represents the enzyme activity at time t , and V_o is the initial enzyme activity in well number 7) versus time were generated in order to determine the slope. The negative value of the slope gave k_1 ($k_1 = -\text{slope}$). The k_2 value was then calculated by dividing k_1 by the concentration of inhibitor (AT(H)) used ($k_2 = k_1/[AT(H)]$). Averages from at least 5 experiments were used to generate the final k_2 value.

To investigate the roles of individual components of the prothrombinase complex on the anticoagulant effects of ATH versus AT+UFH, additional experiments were performed where components of the complex (II, PCPS or Va) were excluded prior to reaction with the inhibitors. To further understand the mechanisms, additional rate experiments were performed to evaluate the effect of mixing 300 nM UFH with 30 nM ATH on inhibition of prothrombinase-based Xa, with additional experiments repeated in the absence of II.

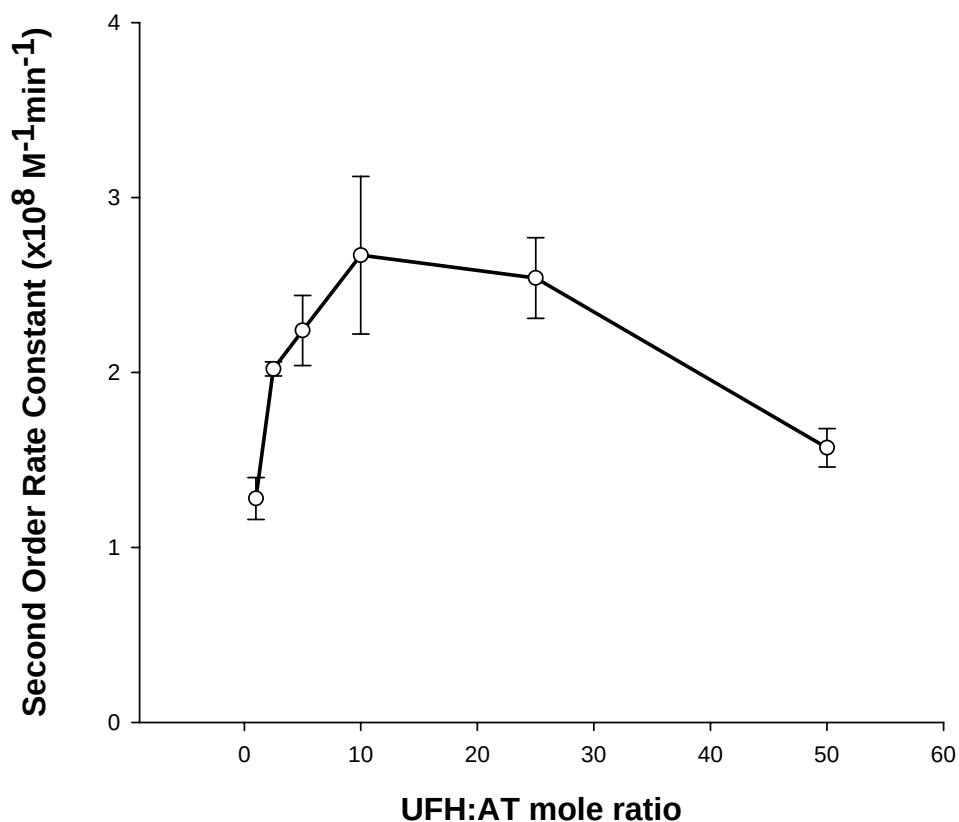


Figure 2.1: Rate of Xa inhibition by AT and varying levels of UFH. Three nM Xa was reacted with 30 nM AT + 30 – 3000 nM UFH and the k_2 values determined. Plots of maximal k_2 values versus relative concentration of UFH (with respect to 30 nM AT) was produced, and it was determined that a 10-fold mole ratio of UFH:AT (300 nM UFH) yielded the highest Xa inhibition by AT.

Thrombin generation: Thrombin-specific substrate S-2238 was used to assess the effect of inhibitors on thrombin generation by the prothrombinase complex. Varying levels of II (20-1,200 nM) were utilized to refine examination of inhibition by 30 nM AT + 300 nM UFH versus 30 nM ATH on prothrombinase inhibition. The concentration of the prothrombinase components were similar to those used above. Briefly, 10 μ l of the prothrombinase mixture was added to each of the seven wells of a 96-well flat bottom plate. In well one, 10 μ l of TSP buffer was added as a control, and in wells two-seven 10 μ l of the appropriate inhibitor was added at 5 s intervals. Five s after addition of inhibitor to the last well, 80 μ l of 0.53 mM solution containing thrombin-specific substrate S-2238 in TSP buffer was added simultaneously to all wells, and immediately read in the plate reader at 405 nm, for 10 min. Linear portions of the $V_{\max}$ curves were utilized and compared against a standard curve to determine the concentration of thrombin being generated. Plots of thrombin concentration versus time were produced to compare the effect of inhibitors on thrombin generation by the prothrombinase complex, and the area under the curve was used to determine thrombin potential for the system.

Fluorescent microscopy: Xa was labeled with Fluorescein FPR-chloromethylketone (FPPack-Xa) and experiments were performed to determine the binding interactions of active site-blocked Xa to the vesicle surface in combination with various components of the prothrombinase complex (PCPS, Va or II). Use of fluorescent, active-site blocked FPPack-Xa allowed us to observe direct interaction of inhibitors with stable/static prothrombinase complexes since II remained in the complex without being cleaved.

Concentrations of the prothrombinase components in the fluorescence experiments were as follows: FPPack-Xa was 8 nM, Va was 9.6 nM, PCPS was 48 μ M, II was 1,200 nM, and CaCl_2 was 4 mM. The concentrations of AT(H) were 30-300 nM and UFH were 300 nM – 2,000 nM. To avidly view vesicles containing prothrombinase under the microscope, only the large vesicles were studied in the reactions. All experiments were performed in the dark, under the same conditions and experimental protocols, with systematic addition of CaCl_2 , PCPS vesicles, Va and FPPack-Xa to TSP buffer, followed by addition of appropriate concentrations of inhibitors. To determine if the inhibitors play a role on FPPack-Xa assembly within or displacement from the prothrombinase complex, inhibitors were added in the reaction mixture either before or after FPPack-Xa. The reaction mix was transferred to a glass microscope slide, and viewed under the Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany) using white light, from 40-400X magnification field. The 40X magnification field was used to locate the general area containing vesicles, whereas higher magnifications were used to hone in on the individual vesicle. The 400X magnification field was used to observe all vesicles in this study, and all digital photographs were taken at 12X digital zoom using a Canon Power Shot: S31S digital camera attached to the #54150 assembly unit (Martin Microscope Company, South Carolina, USA). Once vesicles were located under the 400X magnification field, a picture was acquired for a reference micrograph under white light. Immediately, the filter was changed to blue/UV light (Leica EL6000 External UV Assembly, Leica Microsystems, Wetzlar, Germany)

and another photograph was taken capturing fluorescence of the vesicle harboring the prothrombinase complex, which was used for fluorescence analysis.

Additional experiments were performed to validate that fluorescence was indeed occurring as a result of a full prothrombinase complex being constructed on the vesicle surface. These results were confirmed functionally by comparing thrombin generation of a system in which the conversion of II to thrombin occurred in the presence of all of the components of the prothrombinase complex compared to the conversion of II to thrombin by Xa alone (data not shown). Fluorescence intensity was determined by converting all photographs from JPEG to TIFF followed by conversion of the micrographs from color to 16-bit grey scale. Photographs were uploaded onto a Molecular Imager[®] GelDoc XR System (BioRad Laboratories Inc, Hercules, California, USA) and vesicles appearing on the grey-scale micrograph were outlined by the manufacturer's image software. The software determined the intensity within the highlighted area and simultaneously converted the results to intensity per mm² (Int/mm²) in order to normalize for any discrepancies in vesicle size.

To further understand the mechanism of ATH inhibition, additional fluorescence was conducted utilizing a functional prothrombinase system, by labeling amino groups on the Xa with FITC (F-Xa). The concentration of F-Xa was 8 nM and concentrations of the prothrombinase complex components were the same as above. Fluorescence experiments for the functional prothrombinase system was performed as described above, with the use of F-Xa instead of FPPack-Xa in the presence of various concentrations of inhibitors (10-30 nM AT + 100-300 nM UFH or 10-30 nM ATH). Once vesicles harboring the

prothrombinase complex were located on the slide, digital micrographs were immediately taken at 15 s intervals for 75 s, and analyzed for fluorescence intensity utilizing the procedure described above. Plots of fluorescence intensity per unit area (Int/mm^2) versus time (s) were produced.

Experiments utilizing inhibitors labeled with FITC (F-UFH and F-ATH) were also carried out to examine interactions of the inhibitors with prothrombinase complex containing active site inhibited factor Xa (PPack-Xa). Additionally, various prothrombinase components were excluded from the active site blocked prothrombinase prior to interaction with fluorescent inhibitors and measurement of fluorescent intensity on the vesicles were determined.

Data analysis: The inhibition rate experiments were performed at $n=5$, since previous work showed this number of replicates is sufficient to show statistical significance between groups, and the number of replicates for the fluorescence experiments were conducted at a minimum of $n=3$. Statistical analysis for multiple groups was performed using ANOVA. In the case of comparison between groups, a student t-test was used. Values with a $p < 0.05$ were considered significant.

RESULTS:

Comparison of k_2 values for inhibition of Xa $\pm$ prothrombinase complex: Discontinuous second-order rate constant assays were performed to determine the effect of prothrombinase complexation on k_2 values for inhibition of Xa by AT+UFH versus ATH

and the underlying mechanisms involved. Results for inhibition of Xa alone or Xa within the prothrombinase complex are shown in table 2.1. Maximal k_2 values for non-conjugated AT+UFH occurred when reacted with Xa alone, whereas the k_2 value significantly decreased when Xa was incorporated into the prothrombinase complex ($p<0.001$). Inhibition of free Xa by ATH resulted in significantly higher k_2 values, compared to those of AT+UFH ($p<0.01$). In addition, the k_2 values for ATH inhibition of Xa within the prothrombinase was also significantly higher than either of the AT+UFH results ($p<0.001$). Protection of Xa by the components of the complex was less observed for ATH compared to AT+UFH ($p<0.0001$).

Table 2.1. Inhibition of free Xa and prothrombinase complex by AT+UFH versus ATH

Condition	AT+UFH (k_2 values $\pm$ SD)	ATH (k_2 values $\pm$ SD)
Free Xa	$1.47 \times 10^8 \pm 0.18 \times 10^8$	$2.83 \times 10^8 \pm 0.42 \times 10^8$
Prothrombinase complex	$0.67 \times 10^8 \pm 0.11 \times 10^8$	$2.11 \times 10^8 \pm 0.85 \times 10^8$
Prothrombinase devoid of II	$1.24 \times 10^8 \pm 0.08 \times 10^8$	$1.57 \times 10^8 \pm 0.08 \times 10^8$
Prothrombinase devoid of PCPS	$1.49 \times 10^8 \pm 0.18 \times 10^8$	$2.79 \times 10^8 \pm 0.32 \times 10^8$
Prothrombinase devoid of Va	$1.44 \times 10^8 \pm 0.18 \times 10^8$	$2.09 \times 10^8 \pm 0.19 \times 10^8$
Prothrombinase + UFH	-	$0.62 \times 10^8 \pm 0.07 \times 10^8$
Prothrombinase devoid of II + UFH	-	$1.8 \times 10^8 \pm 0.04 \times 10^8$

Inhibition of thrombin generation: Thrombin generation was performed to examine the effect of AT+UFH versus ATH on functionality of the intact prothrombinase complex with varying levels of II in the reaction mixture (table 2.2). When 20 nM and 300 nM of II was used to construct the prothrombinase, there was enhanced inhibition of thrombin generation by ATH compared to AT+UFH. Both inhibitors significantly decreased the thrombin potential ($p < 0.0001$), with ATH having a greater effect ($p < 0.001$).

Comparison of k_2 values for the inhibition of Xa by combining/excluding prothrombinase components: To examine the roles of prothrombinase components on mechanisms of Xa inhibition by AT+UFH versus ATH, discontinuous second-order rate constant assays were performed comparing the inhibition of the full prothrombinase to a prothrombinase where various components were omitted prior to reaction with inhibitors (table 2.1). When II was excluded from the complex, the k_2 values for inhibition of Xa by AT+UFH increased ($p < 0.001$), relative to intact prothrombinase. Similar results were observed for reactions devoid of PCPS or Va ($p < 0.001$). In contrast, exclusion of II from prothrombinase significantly decreased the k_2 for Xa inhibition by ATH ($p < 0.05$). Although removal of PCPS from the system resulted in a higher k_2 ($p < 0.01$), exclusion of Va resulted in no significant difference compared to the intact prothrombinase for ATH reactions ($p < 0.80$).

Differences in effects of II exclusion on Xa inhibition by AT+UFH versus ATH were studied more closely. We posited that mechanisms behind these opposing results might lie in the interactions of free UFH not permanently bound to AT. Thus,

experiments were carried out to examine the impact of excess UFH on inhibition of Xa by ATH, with or without II in the prothrombinase complex (table 2.1). The k_2 values for inhibition of Xa within an intact prothrombinase significantly decreased when excess UFH was added to reactions containing ATH ($p<0.001$). To test whether excess UFH may prevent non-specific interactions with the vesicle surface by the conjugate, we examined the rate of inhibition by ATH + UFH of Xa in prothrombinase devoid of II. For this experimental reaction, the k_2 values significantly increased compared to reactions without excess UFH ($p<0.001$), suggesting that free UFH may compete with ATH for sites at the vesicle surface which keep ATH from reacting with Xa.

Table 2.2. Inhibition of thrombin potential by AT+UFH versus ATH

Condition	control (nM•sec ± SD)	AT+UFH (nM•sec ± SD)	ATH (nM•sec ± SD)
20 nM	$1.15 \times 10^3 \pm 0.34 \times 10^3$	$0.40 \times 10^3 \pm 0.07 \times 10^3$	$0.25 \times 10^3 \pm 0.06 \times 10^3$
300 nM	$2.19 \times 10^4 \pm 0.58 \times 10^4$	$1.82 \times 10^4 \pm 0.21 \times 10^4$	$1.41 \times 10^4 \pm 0.16 \times 10^4$

Fluorescence microscopy and formation of the prothrombinase complex: Fluorescence microscopy was performed to determine the structural construction of the prothrombinase complex on the vesicle surface, and to examine the effects of the inhibitors on Xa stability within the prothrombinase. Table 2.3 shows numerical values for fluorescent intensity of a prothrombinase complex with or without various components. Systematic exclusion of prothrombinase components from the reaction mixture results in a significant loss of overall fluorescence from the system. Furthermore, it is evident that high intensity of fluorescence is achieved only when all of the components of the prothrombinase are present ($p < 0.05$). Additional functional experiments (chromogenic thrombin generation activity) were performed to validate these results (data not shown).

Effect of UFH, AT+UFH and ATH on fluorescence intensity of a static prothrombinase complex: Labeling Xa with FPPack at its active site inhibits the serine hydroxyl of the protease, which creates a static and non-reactive prothrombinase complex. Fluorescence intensity for reactions of inhibitor with the fluorescently-tagged prothrombinase is listed in table 2.4. Interacting AT+UFH or UFH alone with the prothrombinase complex resulted in diminished fluorescence intensity ($p < 0.01$), which was not evident with ATH. UFH reactions with the static prothrombinase complex reduced FPPack-Xa fluorescence intensity in a dose dependent manner, albeit statistically not significant. Fluorescence intensity was similar whether UFH was added before or after FPPack-Xa. Similar results were observed when AT was included with UFH during interactions with the prothrombinase; however the dose-dependent effect was not evident. In contrast, addition

of ATH to the prothrombinase minimally reduced fluorescence intensity and lack of impact by the conjugate was dose- or order-independent.

Effect of UFH, AT+UFH and ATH on fluorescence intensity of a functional prothrombinase complex: A functional prothrombinase complex was produced by developing an F-Xa containing prothrombinase complex. The lysl groups of Xa were reacted with FITC, thus leaving the serine hydroxyl group of Xa unaffected and capable of reacting with II or AT. Functional assessment of F-Xa revealed no compromise in Xa function (results not shown). Figure 2.2A depicts the fluorescence intensity of the functional prothrombinase complex over a period of 75 s, while reacting with either 300 nM UFH, 30 nM AT + 300 nM UFH or 30 nM ATH. The fluorescence of the functional prothrombinase without any inhibitors decreased somewhat linearly over the time course due to loss of prothrombinase complexes as II was consumed. However, adding UFH to the prothrombinase caused a relative drop in fluorescence, suggesting that UFH may be slightly disrupting Xa within the prothrombinase and not allowing it to concentrate on the vesicle surface. These results are similar to the ones observed in the static prothrombinase complex. When AT was mixed with UFH, fluorescence was knocked down to a background level over the time course. Similar results were observed for ATH reactions, although the background fluorescence appeared slightly higher compared to the AT+UFH.

We then reacted lower concentrations of AT+UFH (10 nM AT + 100 nM) and ATH (10 nM) with the functional prothrombinase to better differentiate effects of these 2

inhibitors on F-Xa fluorescence within the prothrombinase (figure 2.2B). Reacting the lower concentration of AT+UFH with the functional prothrombinase strongly increased the initial fluorescence by approximately $1.50 \times 10^3 \text{ Int/mm}^2$, but intensity declined over the experimental time period to the same degree as the higher levels of inhibitors. Similarly, when ATH was reacted with the prothrombinase, there was an apparent increase in initial fluorescence by $2.80 \times 10^3 \text{ Int/mm}^2$ with a steady decline over the period of time. Reacting the two concentrations of ATH with the functional prothrombinase seemed to have retained fluorescence intensity, relative to AT+UFH. These strong trends suggest that, although ATH more readily inhibits Xa within the prothrombinase, the Xa (bound to ATH inhibitor) may not be displaced from the prothrombinase to the same degree as AT+UFH, consistent with results from the static prothrombinase.

Table 2.3. Fluorescence intensity of the prothrombinase complex and its components

Condition	Fluorescence Intensity/mm²
Full Prothrombinase	$4.25 \times 10^3 \pm 0.98 \times 10^3$
Vesicles + FPPAck-Xa	$0.204 \times 10^3 \pm 0.068 \times 10^3$
Vesicles + Ca ²⁺ + FPPAck-Xa	$1.04 \times 10^3 \pm 0.15 \times 10^3$
Vesicles + Ca ²⁺ + Va + FPPAck-Xa	$1.66 \times 10^3 \pm 0.27 \times 10^3$
Vesicles + Ca ²⁺ + II + FPPAck-Xa	$1.13 \times 10^3 \pm 0.096 \times 10^3$

Table 2.4. Effect of UFH on FPPack-Xa binding within the prothrombinase complex

Condition	Fluorescence Intensity/mm ²
Full Prothrombinase	$4.25 \times 10^3 \pm 0.98 \times 10^3$
Prothrombinase plus 300 nM UFH*	$1.46 \times 10^3 \pm 0.35 \times 10^3$
Prothrombinase plus 2000 nM UFH*	$1.05 \times 10^3 \pm 0.13 \times 10^3$
Prothrombinase plus 300 nM UFH [#]	$1.41 \times 10^3 \pm 0.015 \times 10^3$
Prothrombinase plus 2000 nM UFH [#]	$1.30 \times 10^3 \pm 0.086 \times 10^3$
Prothrombinase plus 30 nM AT + 300 nM UFH*	$1.28 \times 10^3 \pm 0.25 \times 10^3$
Prothrombinase plus 30 nM AT + 2000 nM UFH*	$2.84 \times 10^3 \pm 0.68 \times 10^3$
Prothrombinase plus 30 nM AT + 300 nM UFH [#]	$1.16 \times 10^3 \pm 0.12 \times 10^3$
Prothrombinase plus 30 nM AT + 2000 nM UFH [#]	$1.23 \times 10^3 \pm 0.18 \times 10^3$
Prothrombinase plus 300 nM ATH*	$3.24 \times 10^3 \pm 0.33 \times 10^3$
Prothrombinase plus 2000 nM ATH*	$3.22 \times 10^3 \pm 0.80 \times 10^3$
Prothrombinase plus 300 nM ATH [#]	$3.99 \times 10^3 \pm 0.64 \times 10^3$
Prothrombinase plus 2000 nM ATH [#]	$3.388 \times 10^3 \pm 0.49 \times 10^3$

(AT) UFH or ATH added *before** and *after*[#] FPPack-Xa during prothrombinase assembly

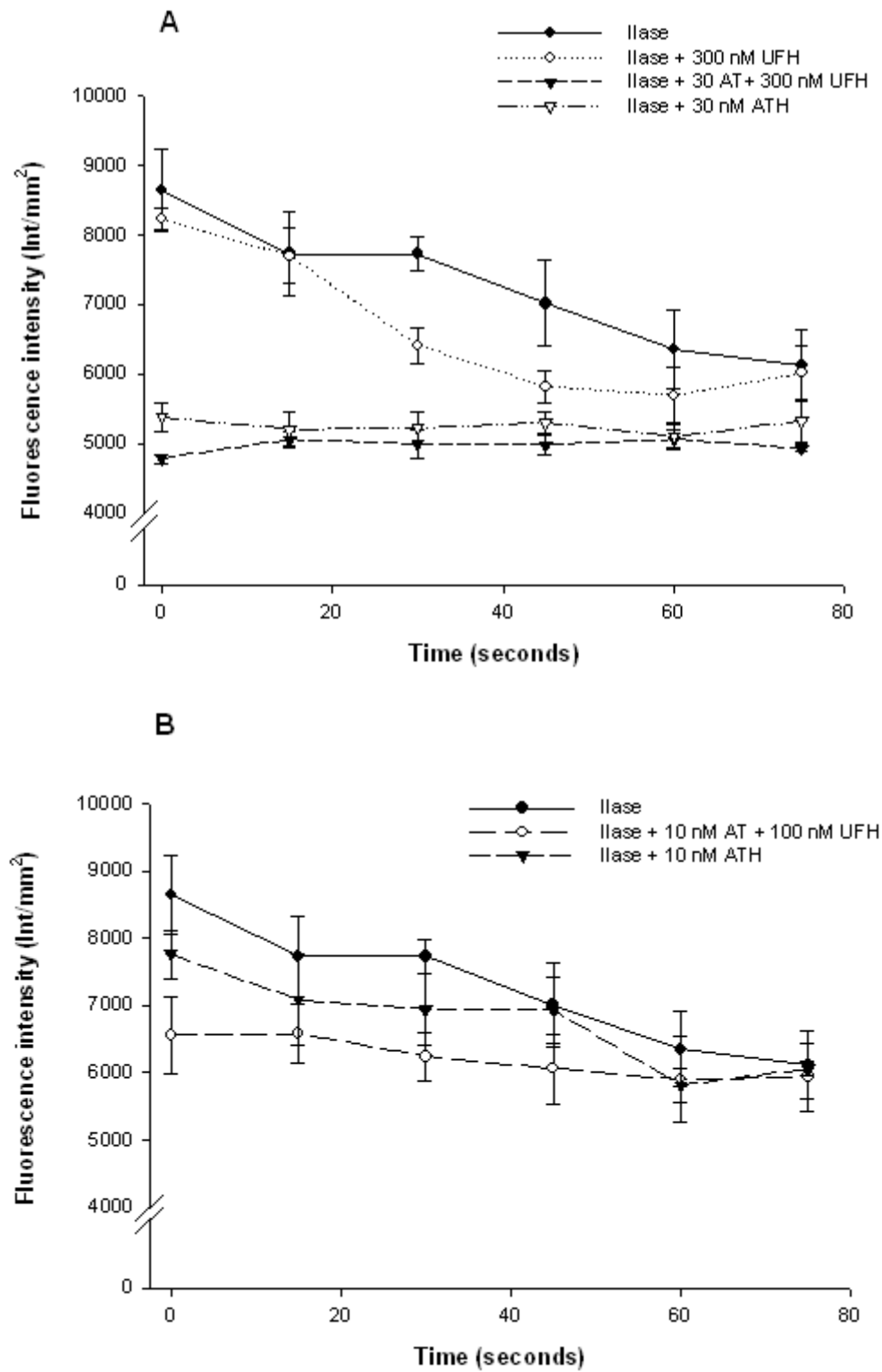


Figure 2.2: Fluorescent microscopy. Fluorescent microscopy illustrating the effect of UFH, AT+UFH and ATH on fluorescence intensity over time of a functional prothrombinase (IIase) complex. Using higher levels of inhibitors (**A**) the fluorescence intensity is quenched to a baseline reading over time. UFH alone decreases the fluorescence prematurely, suggesting fluorescent F-Xa displacement from the IIase complex in the presence of UFH. When lower levels of inhibitors were used (**B**) the fluorescence increased relative to the higher concentrations, however the intensity was still lower than the control.

Dependence of various prothrombinase components on F-UFH and F-ATH binding to prothrombinase: In order to study a stable (static) prothrombinase complex with F-UFH and F-ATH, PPack-Xa was used (table 2.5). The F-UFH bound to prothrombinase, prothrombinase without factor Va, and prothrombinase without II, suggesting that F-UFH may interact with variable combinations of components within the prothrombinase complex. Noteworthy, the F-UFH may also bind to PPack-Xa, but addition of other components greatly enhanced F-UFH binding. In contrast, F-ATH showed no significant binding capabilities except for cases where the full prothrombinase or prothrombinase without II was present.

Table 2.5. Fluorescence intensity of F-UFH and F-ATH interactions with prothrombinase $\pm$ components of the complex.

Condition	F-UFH (INT/mm²)	F-ATH (INT/mm²)
Full prothrombinase	$1.51 \times 10^4 \pm 0.16 \times 10^4$	$1.10 \times 10^4 \pm 0.22 \times 10^4$
Vesicles + Ca ²⁺	$3.41 \times 10^3 \pm 0.34 \times 10^3$	$4.34 \times 10^3 \pm 0.35 \times 10^3$
Vesicles + Ca ²⁺ + Va	$4.54 \times 10^3 \pm 0.15 \times 10^3$	$3.86 \times 10^3 \pm 0.45 \times 10^3$
Vesicles + Ca ²⁺ + II	$4.81 \times 10^3 \pm 0.67 \times 10^3$	$5.14 \times 10^3 \pm 2.04 \times 10^3$
Vesicles + Ca ²⁺ + PPack-Xa	$6.48 \times 10^3 \pm 1.21 \times 10^3$	$3.68 \times 10^3 \pm 0.78 \times 10^3$
Vesicles + Ca ²⁺ + Va + II	$3.79 \times 10^3 \pm 0.92 \times 10^3$	$4.56 \times 10^3 \pm 0.13 \times 10^3$
prothrombinase without II	$8.16 \times 10^3 \pm 1.99 \times 10^3$	$9.32 \times 10^3 \pm 2.91 \times 10^3$
prothrombinase without Va	$9.13 \times 10^3 \pm 0.75 \times 10^3$	$5.33 \times 10^3 \pm 0.22 \times 10^3$

DISCUSSION:

Anticoagulation by UFH has been clinically used for the treatment and prophylaxis of thrombotic complications (22). Mechanistically, it is well established that a specific pentasaccharide sequence on UFH is involved in conformational activation of AT which gives rise to a structure of AT that is more favorable for reacting with most serine proteases (23;47). A separate mechanism exists for thrombin inactivation called template-mediated inhibition, which additionally requires the UFH molecule to bridge AT and thrombin together (48;49). Inhibition of thrombin through the template-mediated reaction is chain length dependent, requiring the UFH molecule to contain 18 or more saccharides (33). AT interaction with UFH occurs through non-covalent means, which allows for UFH to dissociate from AT and may attenuate its catalysis of surface-bound enzyme inhibition (50;51). Other limitations have been discussed above, however it is well demonstrated that Xa is protected from AT+UFH inhibition when it is incorporated in the prothrombinase complex (33;34). The degree to which this protection occurs has been variably reported due to the difficulty of measuring Xa activity in the presence of II, as AT binds both Xa and the reaction product thrombin (34;52). Several attempts have been made to measure Xa activity using a mutant AT that only binds Xa and not thrombin (33), or using a mutant II that generates a thrombin which does not react with AT (34) (the latter potentially giving more accurate inhibition rates). However, these methods fail to use an assay containing a native prothrombinase complex, where none of the enzymes or components have been modified. In our study, we utilized an assay containing a native prothrombinase complex in the presence of a serine protease inhibitor

called pefabloc-TH. Pefabloc-TH is highly reactive with thrombin, and we have verified that pefabloc-TH minimally cross-reacts with Xa (data not shown). Therefore, by using vast excess of pefabloc-TH in our assay, this ensured that any thrombin generated by prothrombinase was quickly quenched by the pefabloc-TH, allowing us to study only AT+UFH or ATH reaction with the Xa.

Regulating the prothrombinase complex is very important in individuals with thrombotic complications. Since we have previously demonstrated that ATH significantly accelerates the inhibition of clot-bound thrombin (41), we therefore wanted to determine whether ATH is similarly effective for inhibition of the prothrombinase complex and propose a mechanism of action for the anticoagulation by ATH in relation to the non-conjugated inhibitor + catalyst system.

Based on our findings, we have shown that the prothrombinase complex hinders the inhibitory action of AT+UFH on Xa (consistent with previous findings), whereas ATH is less affected. In fact, ATH inhibition of the prothrombinase complex was significantly higher than the inhibition rate for either free or prothrombinase-bound Xa by AT+UFH. Similar to inhibition of fibrin-bound thrombin (41), UFH in the non-covalent AT+UFH dissociates and binds to components or products of the prothrombinase complex, which leaves AT alone in the non-activated state. Furthermore, UFH complexation with prothrombinase or its products may act to repel any incoming AT+UFH to cause protection of Xa from inhibition. However, formation of these complexes on the prothrombinase cannot occur when AT is covalently linked to UFH, as

in the case of ATH, thus allowing for enhanced inhibition of prothrombinase-bound Xa by ATH.

Previous studies have shown that in the presence of all the prothrombinase components, Xa was most protected from inhibition by pentasaccharide (fondaparinux), followed by LMWH (enoxaparin) and then regular heparin (34). In our study, we clearly showed that ATH overcame the protective effect and gave a modest 3.11-fold increase in the rate of Xa inhibition in the presence of all of the components of the complex, compared to heparin. Although a small protective effect by the prothrombinase was observed for Xa reaction with ATH, this difference was minor compared to the inhibition rates observed with AT+UFH. One possibility to explain this feature could be non-specific interaction of heparin chains from some ATH molecules with the prothrombinase complex. During the synthesis of ATH, the AT selects for relatively large UFH molecules that contain the high affinity pentasaccharide sequence, with up to 30% of the ATH molecules containing two or more pentasaccharide sequences (40). The longer heparin chains in this small subpopulation of ATH molecules may interact non-specifically with the prothrombinase, giving a small hindrance in Xa inhibition. Our work also suggests that the calcium-phospholipid surface associating with prothrombinase components may be an important player responsible for deterring ATH from inhibiting Xa, particularly in absence of II. Whereas for AT+UFH, excess UFH molecules may neutralize exposed calcium-phospholipid that normally bind II so that incoming AT+UFH inhibits Xa and is not attracted to these sites when II is absent. However, with no free heparin chains to interrupt such binding site associations, ATH will tend towards

these non-useful interactions. To confirm this clustering effect for ATH in the absence of II, we attempted to interrupt ATH interactions with any such binding sites by addition of excess UFH. This caused a moderate increase in Xa inhibition by the ATH. However, when experiments with added UFH were reproduced with a fully intact prothrombinase, the rate of Xa inhibition by ATH significantly decreased (suggesting bridging of free heparin with combinations of components in the full prothrombinase to repel binding of ATH through its heparin chain). Thus, the only aspects which might differentiate ATH's improved inhibition of Xa in prothrombinase are: covalent attachment of heparin to AT and absence of free non-pentasaccharide UFH within the reaction system.

Thrombin generation assays revealed strong trends to indicate that ATH is able to inhibit the function of the prothrombinase complex to a better degree than AT+UFH. When moderate to low levels of II were used (more indicative of local bursts of thrombin generation *in vivo*), ATH was capable of inhibiting thrombin generation better than AT+UFH. At the lower levels of II reaction, less thrombin was generated and not all of the inhibitors reacted with the thrombin, consequently the inhibitors could inhibit Xa to a better degree.

Our fluorescence results indicate that UFH alone may somewhat destabilize the prothrombinase, in part, by displacing the Xa from the complex. This is in sync with previously published work, which showed that heparin may displace Xa, and to a degree II, from the complex (53). In our work, we show that fluorescence intensity of the prothrombinase complex decreased in both the static and functional prothrombinase assays in the presence of UFH. We also found that the UFH in AT+UFH mixtures may

displace the Xa. Non-covalent interactions of heparin with AT in AT+UFH enables the UFH to dissociate from AT, which provides free UFH molecules to destabilize the prothrombinase complex. However, if UFH interacts with prothrombinase to displace Xa, AT dissociated from its UFH will poorly react with any free Xa. In the static prothrombinase, the displacement of Xa was not observed with ATH, and in the functional prothrombinase the effect was not observed to the same degree as with AT+UFH or UFH alone. Although ATH reacts avidly with prothrombinase-incorporated Xa compared to AT+UFH, slower loss of F-Xa fluorescence suggests that Xa-ATH complexes may have affinity to assist retention in the prothrombinase. Cumulatively, these results imply that ATH may not be mechanistically acting in the same manner as UFH for inhibiting the prothrombinase complex.

Binding studies using the fluorescent inhibitors similarly demonstrate that F-UFH is capable of binding to the prothrombinase and, with varying affinities, to its component combinations. Prothrombinase-complexed UFH may prevent additional AT+UFH complexes from inhibiting Xa. Removal of II decreases these non-productive free UFH associations and allows better access/approach of AT+UFH to enhance Xa inhibition. F-ATH showed moderate binding only to full prothrombinase and, more importantly, to prothrombinase devoid of II. We propose that ATH may be interacting with sites in close proximity to Xa normally occupied by II, thus causing inappropriate interaction with Xa. Moreover, these associations may be occurring through the long heparin chain of ATH, since addition of excess UFH provided recovery of Xa inhibition.

These findings reveal important mechanisms involved in the enhanced anticoagulant action of ATH against surface-bound coagulation enzymes, particularly the prothrombinase complex. It is clear that the prothrombinase components hinder the ability of AT + conventional heparins to inhibit Xa. However, covalent linkage between AT and UFH assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function. Enhanced inhibition of serine proteases on surfaces and within supramolecular complexes encourages further investigation of ATH for application in thrombosis.

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Chapter 3: Manuscript Two***Covalently linking heparin to antithrombin enhances inhibition
of the prothrombinase complex on activated platelets*****Foreword**

This manuscript has been submitted for publication. The authors are: Ivan Stevic, Howard H.W. Chan, Ankush Chander, Leslie R. Berry, and Anthony K.C. Chan. The corresponding author is Dr. Anthony Chan. The manuscript has been submitted and is under review.

Ivan Stevic performed the research and drafted the manuscript. Howard Chan provided scientific discussion and analyzed the results. Ankush Chander helped with flow cytometry and ELISA experiments. Leslie Berry provided scientific input, analyzed the results and revised the manuscript. Anthony Chan oversaw the work.

Covalently linking heparin to antithrombin enhances prothrombinase inhibition on activated platelets

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Running Title: Inhibition of platelet-prothrombinase by covalent antithrombin-heparin

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ABSTRACT:

Factor Xa within the prothrombinase complex is protected from inhibition by unfractionated heparin (UFH), enoxaparin and fondaparinux. We have developed a covalent antithrombin-heparin complex (ATH) with enhanced anticoagulant activity. We have also demonstrated that ATH is superior at inhibiting Xa within the prothrombinase complex when assembled on synthetic vesicles. The objective of the present study is to determine the ability of ATH vs AT+UFH to inhibit Xa within the prothrombinase complex when the enzyme complex is assembled on the more native platelet system. Discontinuous inhibition assays were performed to determine final k_2 -values for inhibition of Xa, Xa within the platelet-prothrombinase, or Xa within prothrombinase devoid of various components. Thrombin generation was also assayed in the presence of inhibitors. Protection of Xa was not observed for ATH, where as a moderate 60% protection was observed for AT+UFH. Relative to intact prothrombinase, rates for Xa inhibition by AT+UFH in prothrombinase complexes devoid of either prothrombin, activated platelets or factor-Va were higher. However, inhibition by AT+UFH of prothrombinase devoid of prothrombin yielded slightly lower rates compared to free Xa inhibition. Thrombin generation was inhibited more by ATH compared to AT+UFH, thus suggesting an overall enhanced anticoagulant activity of ATH against platelet-bound enzyme complexes.

Keywords: Coagulation, covalent antithrombin-heparin, heparin, platelets, prothrombinase complex

INTRODUCTION:

Prothrombinase complex is a large enzymatic complex comprised of the catalytically active component factor Xa (Xa), its cofactor factor Va (Va), and the zymogen prothrombin (II) assembled on a phospholipid surface in the presence of calcium ions [1;2]. The function of prothrombinase is to convert II to the active product and key player of coagulation, thrombin (IIa). The importance of Xa incorporation in the prothrombinase complex becomes significant when dealing with thrombosis. Incorporation of this proteinase in the prothrombinase complex enhances the conversion of II to IIa by an excess of 300,000-fold [3-5]. Thus, the amplification of IIa enables it to feedback at specific points in the coagulation cascade to cause propagation of coagulation and subsequent thrombosis [6].

Antithrombin (AT) is a serine protease inhibitor that variably inhibits coagulation factors, including Xa and IIa [7;8]. Unfractionated heparin (UFH) is a glycosaminoglycan of which 1/3 of its molecules contain a high affinity pentasaccharide sequence capable of interacting with AT [9-11]. Binding of UFH to AT accelerates the reaction of AT with many of the serine proteases over 1,000-fold [12]. UFH has long been used for the treatment and prophylaxis of thromboembolic diseases, however numerous limitations has arisen over the seven decades of its clinical use [11;13]. Some of these limitations include non-specific protein binding, a short dose-dependent intravenous half-life, an unpredictable anticoagulant response, only 1/3 of the molecules contain the high affinity pentasaccharide sequence, and complications such as heparin-induced thrombocytopenia or severe bleeding can occur [11;14-16]. More specific to the

present project is the fact that UFH is unable to inhibit Xa when the enzyme is complexed in the prothrombinase complex [17;18]. Prothrombinase protects factor Xa from inhibition by AT and heparin complex [19]. The degree of protection is not accurately known due to the complexity of the assay system. Numerous investigators have shown a resistance of Xa to inhibition when within prothrombinase that varies anywhere from orders of magnitude to a few-fold protection [17-22]. We have also demonstrated that Xa is moderately protected from inhibition by AT+UFH when Xa is complexed in a prothrombinase complex comprised of synthetic vesicles [23].

To overcome some of these limitations, our laboratory has developed a covalent complex of antithrombin and heparin (ATH) with high anticoagulant activity.[24] Numerous *in-vitro* and *in-vivo* investigations involving ATH have led to results showing potential clinical advantages of ATH over heparin [24-29]. More specifically, we have demonstrated that ATH inhibits Xa within a prothrombinase complex much better than AT+UFH. However, the prothrombinase in the latter example utilized a synthetic vesicle system and not a native system, such as platelets [23]. Therefore, the objective of this study is to determine the ability of ATH vs AT+UFH to inhibit Xa within the prothrombinase complex when the enzyme complex is formed on the more physiological platelet system.

MATERIALS and METHODS:

Chemicals: All chemical reagents were of analytical grade. Sodium chloride and Tris (tris (hydroxymethyl) amino-methane) were purchased from Bioshop (Burlington, ON).

Polyethylene glycol 8000, ethylene-diamine tetraacetic acid-disodium salt (Na_2EDTA) and calcium chloride dihydrate (CaCl_2) were from BDH Inc. (Toronto, ON). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Human factor Xa, II and thrombin were purchased from Enzyme Research Laboratories Ltd. (South Bend, IN). Human factor Va was obtained from Haematologic Technologies Inc. (Essex Junction, VT). Purified fibrinogen was purchased from American Diagnostica Inc (Stamford, CT). Substrates for Xa (S-2222; N-benzoyl-isoleucyl-glutamyl-glycylarginyl-p-nitroanilide-hydrochloride and its methyl ester) and thrombin (S-2238; H-D-phenylalanyl-pipecolyl-arginyl-p-nitroanilide-dihydrochloride) were from DiaPharma Group Inc. (Westchester, OH). AT was purchased from Affinity Biologicals (Ancaster, ON, Canada). Unfractionated sodium heparin (UFH), Prostacyclin (PGI_2), Apyrase from potato Grade VII, and Calcium Ionophore A23187 were obtained from Sigma (St. Louis, MO). Thrombin-specific inhibitor, Pefabloc-TH (Na -(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine piperidide acetate (NAPAP)) was purchased from Centerchem (Norwalk, CT). Purchased human coagulation factors (prothrombin, thrombin, factor Va and factor Xa) as well as antithrombin were analyzed for purity by SDS-PAGE under reducing conditions, and all proteins were found to be >95% pure.

Preparation of ATH: ATH was synthesized as described by Chan *et al.* In brief, 1052 mg of human AT and 64 g of UFH was separately dialyzed against 2 M NaCl followed by Phosphate Buffered Saline (PBS) and mixed to a volume of 900 ml, followed by

incubation at 40°C for 14 d. The reaction mixture was then mixed with 0.5 M NaBH₃CN reducing agent and incubated for another 5 h at 37°C. A two-step purification process was used, which involved Butyl-Sepharose hydrophobic chromatography (Amersham, Uppsala, Sweden) and DEAE-Sepharose anion exchange chromatography (Amersham, Uppsala, Sweden) for the removal of any unbound heparin or AT, respectively. ATH was analyzed for purity using SDS-PAGE under reducing conditions and was found to be > 95 % pure (data not shown). We have previously shown that the AT content (amino acid analysis) and heparin content (by 3 different mass analysis methods) of ATH preparations are in a mole ratio of 1:1 [30].

Preparation of fresh human platelets: With approval from the Hamilton Health Sciences/McMaster Research Ethics Board, human platelets were obtained from healthy volunteers (who have not taken antiplatelet agents for at least 2 weeks) on the day of experiments. Patient consent was obtained from all donors prior to blood donation. Twenty five mL of blood was drawn from the antecubital vein of healthy donors into 2.78 mL of acid-citrate/dextrose (ACD) anticoagulant solution (0.085 M sodium citrate + 0.079 M citric acid + 0.18 M glucose) + 2 µg/mL of prostacyclin (PGI₂) + 0.5 µM Apyrase in a syringe connected to a 19 G butterfly needle (Venisystems, Hospira Inc, Lake Forest, IL, USA). The blood was transferred to a 50 mL polypropylene tube and centrifuged at 150 x g for 20 min at 22 °C to remove contaminating white and red blood cells and to obtain platelet rich plasma (PRP). The PRP (top layer) was transferred to another 15 mL tube and spun at 1000 x g for 15 min at 22 °C to form a pellet and remove

the supernatant (plasma) layer. The pellet was resuspended with Tyrode's buffer (127 mM NaCl, 10 mM HEPES, 5 mM dextrose, 5 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.5 mM Na₂HPO₄; pH 7.4) + 0.2 µg/mL of PGI₂ + 0.5 µM Apyrase. Using a 3 mL transfer pipette, 1 mL of buffer was added and lightly mixed up and down several times with avoidance of touching the pellet, followed by an additional 4 mL of buffer for full resuspension. The platelets were then spun at 1000 x g for 10 min at 22 °C and the supernatant discarded. Platelets were washed again with buffer as described above and spun at 800 x g for 10 min at 22 °C. Platelets were finally resuspended in PGI₂-free Tyrode's buffer + 0.5 µM Apyrase. Experiments requiring active platelets utilized a similar protocol as above except without PGI₂/Apyrase in the ACD solution. The platelet number was adjusted to 1.0×10^8 platelets/mL. Platelets were stored undisturbed at room temperature and used in experiments within 2-6 hours of purification.

Determination of second-order rate constants (k_2 values): k_2 values for inhibition of Xa ± platelet-prothrombinase complex by AT+UFH or ATH were determined by performing discontinuous second-order rate constant assays under pseudo first-order conditions, as previously described [23]. All rate experiments were performed at physiological temperature (37°C) and a minimum inhibitor:enzyme ratio of 10:1 was maintained for all reactions. Also, we have previously determined that maximal inhibition of Xa by AT occurs in the presence of 10 fold mole ratio of heparin to AT, or 30 nM AT + 300 nM UFH [23]. For rate experiments involving the inhibition of free-Xa by AT+UFH or ATH, the reaction protocol was as follows: First, 10 µl aliquots of TSP buffer (0.02 M

Tris HCl + 0.15 M NaCl + 0.6 % polyethylene glycol, pH = 7.4) was added to 6 separate wells of a 96-well flat bottom microtiter plate (Fisher, Nepean, ON, Canada), followed by additional 10 μ L aliquots containing 24 nM Xa, 32 mM CaCl_2 and 320 μ M Pefabloc-TH in TSP buffer into all the wells and incubation for 3 min. To well number 6, 40 μ L of TSP buffer was added to represent the control reaction for the experiment. Then, 20 μ L of 4.8 μ M II in TSP was simultaneously added to all six wells with a multichannel pipette, followed within 2 seconds by sequential addition of 40 μ L aliquots of inhibitor at time intervals ranging from 2–6 sec in wells 1 through 5. After 2-6 sec, the reactions were neutralized by the simultaneous addition of 120 μ L of developing solution containing 1.25 mg/ml of polybrene + 0.53 mM Xa-specific substrate S-2222 + 1.14 mM of Na_2EDTA in TSP buffer.

For platelet-prothrombinase reactions, the protocol was as follows: Ten μ L of freshly prepared platelets was added to 6 wells in a 96-well plate, followed by addition of 10 μ L of solution containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 and 320 μ M Pefabloc-TH in TSP buffer and incubated for 3 min. Next, 20 μ L of 4.8 μ M II was added to all six wells with a multichannel, followed by addition of 40 μ L of inhibitors at specific time intervals, and then termination of reactions by the developing solution in the manner described above. For experiments requiring activated platelets, 100 μ L of freshly isolated platelets were activated with 5 μ M calcium ionophore A23187 + 4 mM Ca^{2+} for 15 min prior to reactions described above.

After terminating the inhibition reactions, residual Xa activity for each well was determined by continuous absorbance measurement at 405 nm (SpectraMax Plus 384

plate reader) over 10-30 min. To calculate the pseudo first-order rate constant k_1 , plots of $\ln V_t/V_o$ (where V_t represents the enzyme activity at time t , and V_o is the initial enzyme activity in well number 6) versus time were constructed and the slope determined. The negative value of the slope gave k_1 (i.e. $k_1 = -\text{slope}$). The k_2 value was then calculated by dividing k_1 by the concentration of inhibitor (i.e. AT(H)) used ($k_2 = k_1/[\text{AT(H)}]$). Averages from at least 5 experiments were used to generate the final k_2 value.

To investigate the roles of individual components of the platelet-prothrombinase complex on the anticoagulant effects of ATH versus AT+UFH, additional experiments were performed where components of the complex (II, activated platelets or Va) were excluded prior to reaction with the inhibitors.

Flow cytometry: Resting and activated platelet samples were analyzed for activation and microparticle formation using a flow cytometer. Briefly, 25 μL containing 1×10^6 resting or activated platelets in Tyrodes buffer were incubated for 15 min in the dark with 5 μL of 1mg/ml mouse monoclonal Anti human CD-41 (Integrin alpha 2b) antibody. The reactions were stopped by the addition of 400 μL of PBS+1% formaldehyde fixing buffer. Flow cytometric analyses were performed using a FACSCalibur flow cytometer (San Jose, California) with a 488 nm argon ion laser and a 635 nm red diode laser. The light scatter and fluorescent parameters were set at logarithmic gain, and platelets were identified according to their characteristic forward and side scatter properties. Plots were then gated to determine CD-41 positive microparticle hits for each condition.

Thrombin generation and its inhibition: Thrombin-specific substrate S-2238 was used to assess the effect of inhibitors on thrombin generation by the platelet-prothrombinase system in the presence of 300 nM II and 30 nM AT + 300 nM UFH or 30 nM ATH. We have previously shown that 300 nM II [23] was an optimal concentration required to determine differences between the inhibitors and it also may give thrombin generation levels representative of local microbursts of thrombin that can be formed during thrombosis. Concentrations of the prothrombinase components were the same as above. Briefly, 10 μ L of activated platelets was added to 8 wells of a 96-well plate, followed by addition of 10 μ L of the solution mixture containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 in TSP buffer and incubation for 3 min. Sixty μ L of a mixture containing 400 nM II + 40 nM AT + 400 nM UFH or 400 nM II + 40 nM ATH in TSP buffer was then added to the 8 wells, one at a time, at 10 s intervals. Ten s after addition of the last II/inhibitor mixture, 120 μ L of developing solution containing polybrene, thrombin-specific substrate S-2238 and Na_2EDTA was simultaneously added to all wells and absorbance at 405 nm immediately read in the plate reader for 10 min. Linear portions of the V_{max} curves were compared against a standard curve to determine the concentration of thrombin being generated. Plots of thrombin concentration versus time were produced to compare the effect of inhibitors on thrombin generation by the prothrombinase complex, and the area under the curve was used to determine thrombin potential for the system.

Plasma turbidometric analysis: Plasma clotting in the presence of activated platelets and inhibitors was assessed using a turbidometric assay. Briefly, 25 μ L of activated platelets

were added to a well of a 96-well plate, followed by addition of 25 μL of 40 mM CaCl_2 containing 120 nM AT + 1200 nM UFH or 120 nM ATH. The control contained no inhibitors. Addition of 50 μL of adult pooled platelet poor plasma (3.2 % v/v sodium citrate) started the reaction, and the reaction absorbance at 350 nm was immediately read in the plate reader for 3 hours. The time to $\frac{1}{2}$ -max, which represents time to clot formation, was then compared for each condition.

Data analysis: The inhibition rate experiments were performed at $n=5$ and turbidity experiments at $n=3$. Statistical analysis for multiple groups was performed using ANOVA. In the case of comparison between groups, a student t-test was used. Values with a $p<0.05$ were considered to be statistically significant.

RESULTS:

Comparison of k_2 values for inhibition of free Xa or prothrombinase on resting platelets:

Discontinuous assays were performed to examine inhibition of Xa within a prothrombinase complex that utilized a surface from non-active, resting platelets. There were no differences between the free Xa and resting platelet-prothrombinase k_2 -values for both AT+UFH and ATH (figure 3.1).

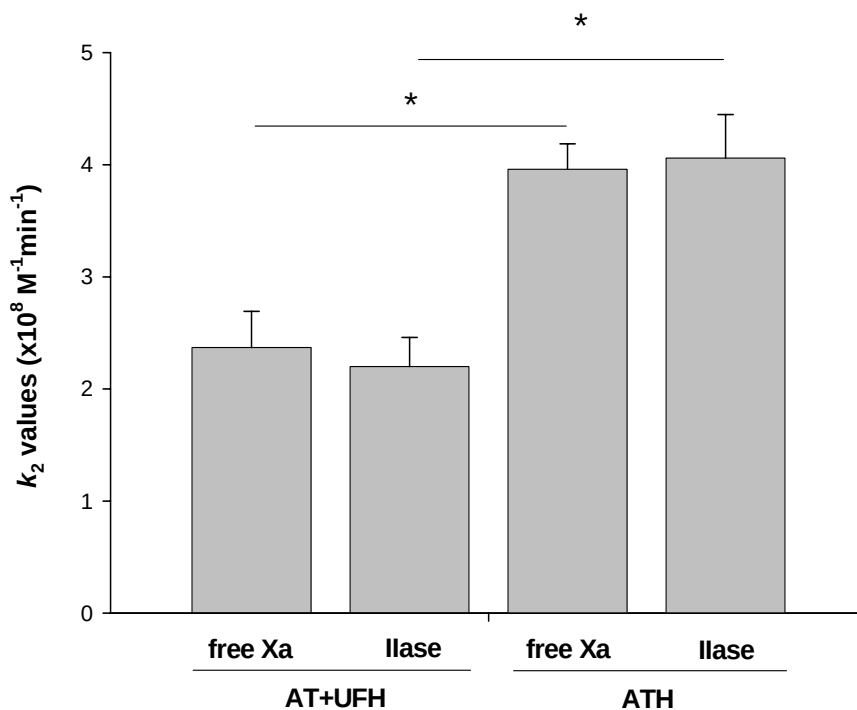


Figure 3.1: Inhibition of Xa $\pm$ prothrombinase on resting platelets. There is no difference between free and prothrombinase Xa for AT+UFH or ATH. However, the k_2 -values for ATH reactions were approximately two-fold higher compared to AT+UFH * $p < 0.01$.

Effect of calcium ionophore A23187 on resting platelets: Resting platelets were activated with calcium ionophore A23187 + calcium prior to being tagged with a platelet specific anti-CD-41 antibody conjugated to fluorescein isothiocyanate (FITC). Activation of platelets for formation of microparticles was evaluated using a flow cytometer (figure 3.2). An apparent shift in the size and density for the overall population of the platelets was observed after activation. Gating revealed that the quantity of platelet-microparticles was higher in the activated group compared to resting platelets, thus confirming that sub-populations of activated platelets also contain microparticles, as would be seen *in-vivo* during thrombosis.

Comparison of k_2 values for inhibition of free Xa or prothrombinase on activated platelets: Discontinuous inhibition assays were performed to determine the k_2 -values for inhibition of Xa $\pm$ activated platelet-prothrombinase by AT+UFH versus ATH (figure 3.3). Maximal k_2 -values for the non-conjugated AT+UFH occurred when reacted with Xa alone, whereas the k_2 -values significantly decreased when Xa was incorporated into the platelet-prothrombinase complex. Inhibition of both free Xa and Xa within the platelet-prothrombinase by ATH resulted in significantly higher values compared to those of AT+UFH. Protection of Xa by the components of the platelet-prothrombinase was not observed for ATH, whereas a significantly high protection of 60% was observed for AT+UFH (figure 3.4).

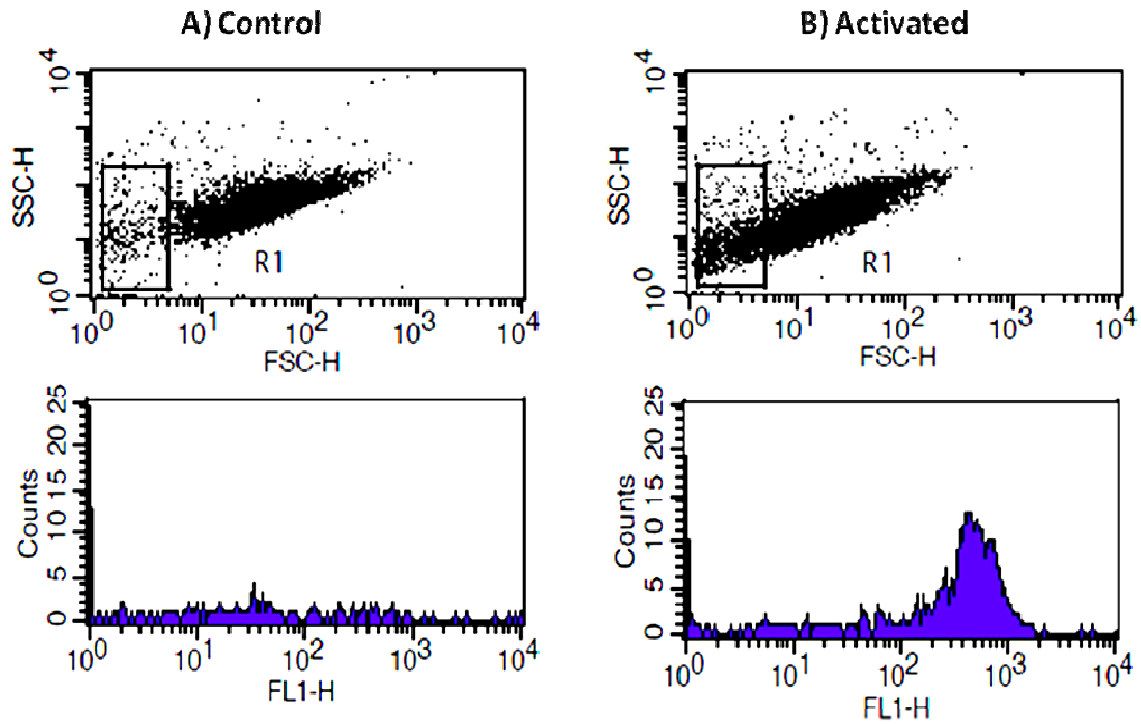


Figure 3.2: Flow cytometry for resting and activated platelets. About 1×10^6 resting platelets (A) or platelets activated with calcium ionophore A23187 + CaCl_2 (B) were incubated with a mouse monoclonal anti-human anti CD-41 antibody and evaluated for forward/side scatter profile. A general shift in the overall forward/side scatter plot exists in panel B compared to panel A. When the plots were gated for microparticles, there appears to be more CD-41 positive microparticles in the gated region of panel B compared to panel A, thus confirming platelet activation was achieved with calcium ionophore A23187.

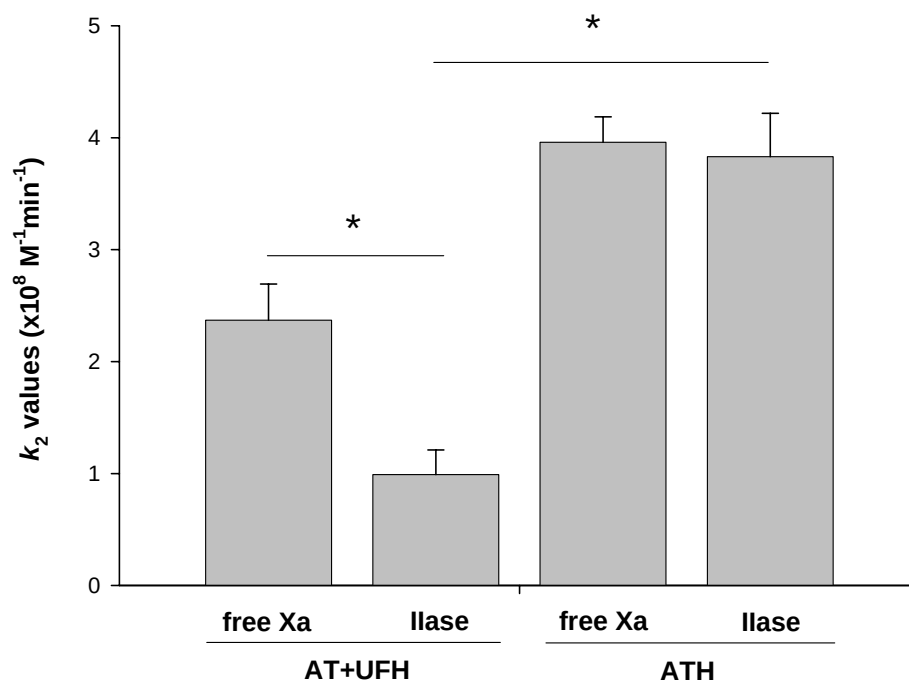


Figure 3.3: Inhibition of Xa $\pm$ prothrombinase on activated platelets. Resting platelets were activated with calcium ionophore A23187 + CaCl_2 for 15 minutes prior to reaction with prothrombinase and inhibitors. When activated platelets were used to generate the prothrombinase complex, there was a apparent difference in the rate of Xa inhibition for AT+UFH reactions. Incorporation of factor Xa in the activated platelet-prothrombinase caused a moderate decrease in Xa inhibition by the non-cojugated inhibitor, with no change observed for ATH reactions. A consistent 2-fold greater Xa inhibition was evident for ATH compared AT+UFH even on activated platelets * $p < 0.001$.

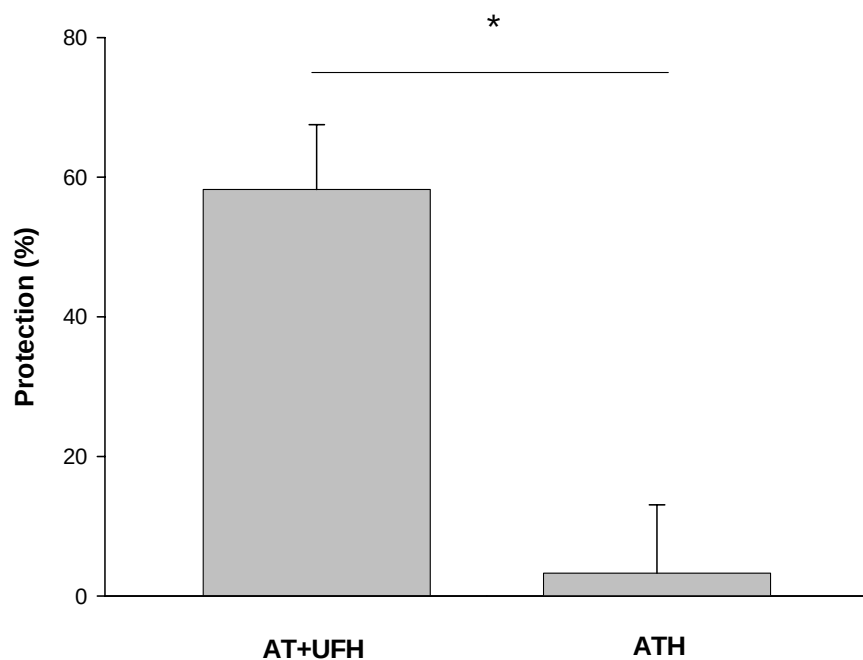


Figure 3.4: Protection of Xa by the IIase complex for AT+UFH and ATH. Inhibition of Xa decreased by 60% for AT+UFH when Xa was assembled in the IIase complex, whereas an insignificant decrease of 3% was observed for ATH. A 20-fold change in the protective effect was noticed between the two inhibitors * $p < 0.0001$.

Comparison of k_2 values for inhibition of Xa by combining/excluding components of the activated platelet-prothrombinase system: To examine the roles of prothrombinase components on mechanisms of Xa inhibition by AT+UFH versus ATH, discontinuous inhibition assays were also performed to compare the inhibition of the full platelet-prothrombinase to a prothrombinase where various components (II, activated platelets or Va) were omitted prior to reaction with inhibitors (figure 3.5). For AT+UFH reactions (figure 3.5A), relative to the intact prothrombinase, there was a significant increase in Xa inhibition when II, activated platelets and Va were omitted from the reactions. As for ATH reactions (figure 3.5B), no net effect was observed when the components were omitted from the assay system.

Inhibition of thrombin generation by a prothrombinase complex on activated platelets: Thrombin generation was performed to examine the effect of AT+UFH versus ATH on functionality of the intact prothrombinase complex system using 300 nM II (figure 3.6A). Both inhibitors decreased thrombin generation compared to control, with ATH having a greater effect. When the results were converted to inhibition of thrombin potential (area under the curve), ATH significantly reduced the thrombin potential compared to AT+UFH (figure 3.6B).

Inhibition of plasma clotting: Addition of activated platelets to plasma decreased the time at $\frac{1}{2}$ -max compared to a system with no activated platelets, thus suggesting an enhanced clotting effect by the activated platelets. The effect of inhibitors was also tested in this activated platelet/plasma clotting system. Relative to the controls, the time to $\frac{1}{2}$ -max was significantly prolonged for ATH but not for AT+UFH (figure 3.7).

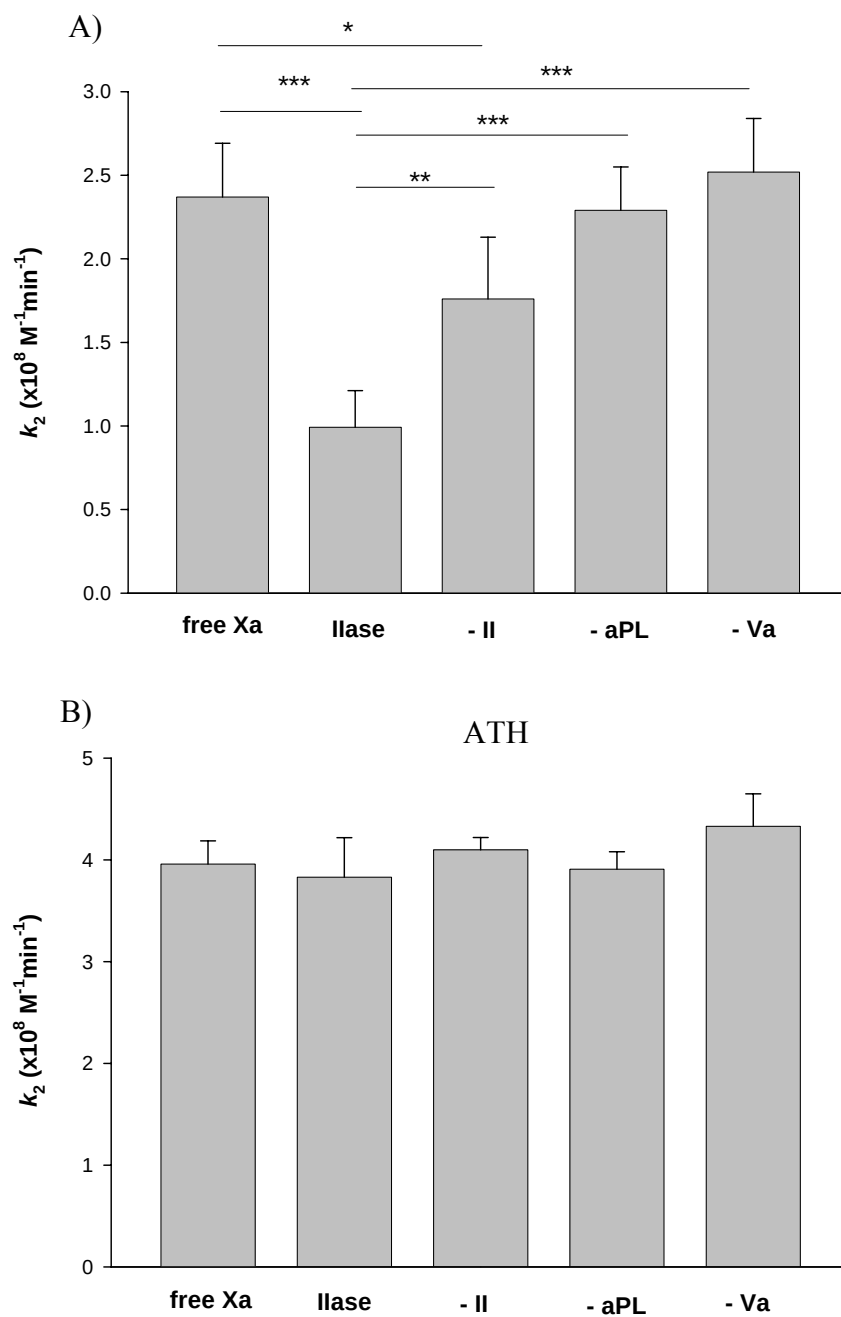


Figure 3.5: Inhibition of prothrombinase by combining/excluding components. This figure represents inhibition of Xa within the prothrombinase when the complex was devoid of II, activated platelets or Va by AT+UFH (**A**) versus ATH (**B**). There appears to be no differences for any of the ATH reactions. However, for AT+UFH, exclusion of the factors and activated platelets caused a significant increase in factor Xa inhibition. When II was excluded from the complex, the increase in Xa inhibition was not to the same extent as the free Xa results * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

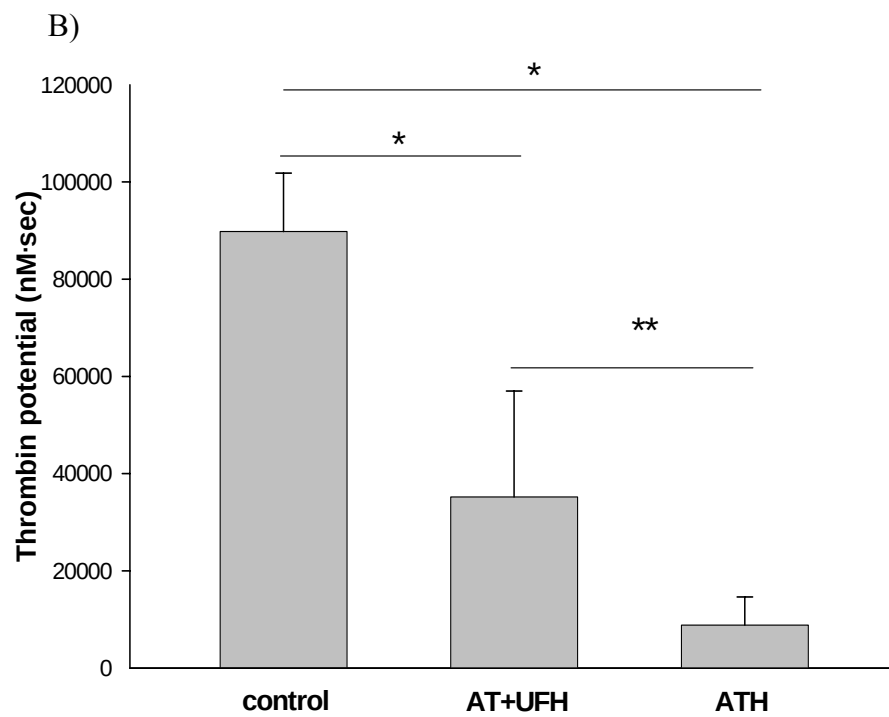
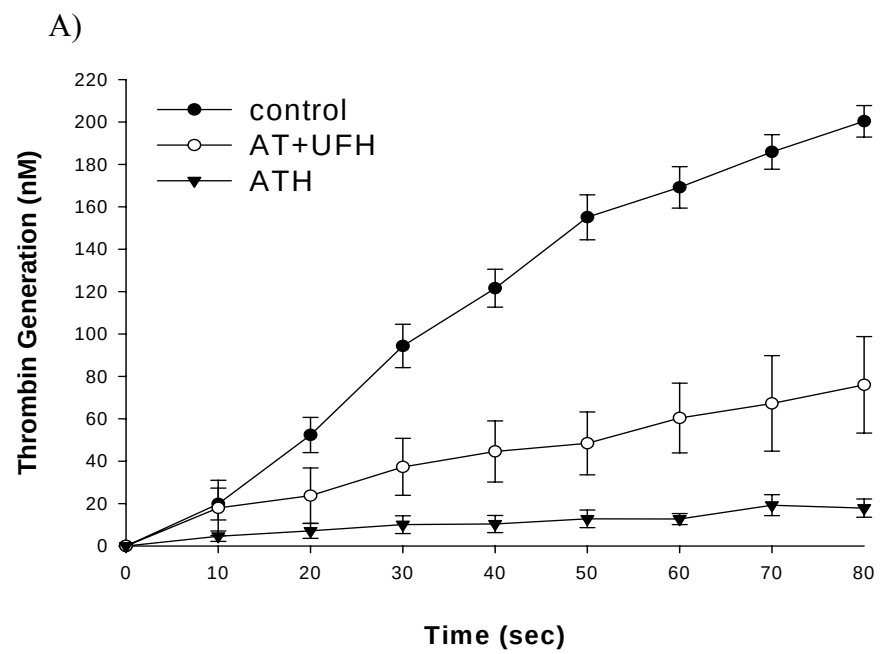


Figure 3.6: Inhibition of thrombin generation by the platelet-prothrombinase complex. Thrombin generation in the presence of inhibitors was assessed using a specific thrombin substrate S-2238 over a time course. It appears that both AT+UFH and ATH reduced thrombin generation over time compared to the control (no inhibitor), with ATH having a greater effect (**A**). However, when assessed for inhibition of thrombin potential, ATH significantly reduced the thrombin potential compared to AT+UFH (**B**). Final concentration of reactants: 3 nM Xa, 9.6 nM Va, 4 mM Ca^{2+} , 300 nM II, 40 μM Pefabloc TH, 30 nM AT, 300 nM UFH and 30 nM ATH * $p < 0.05$, ** $p < 0.001$.

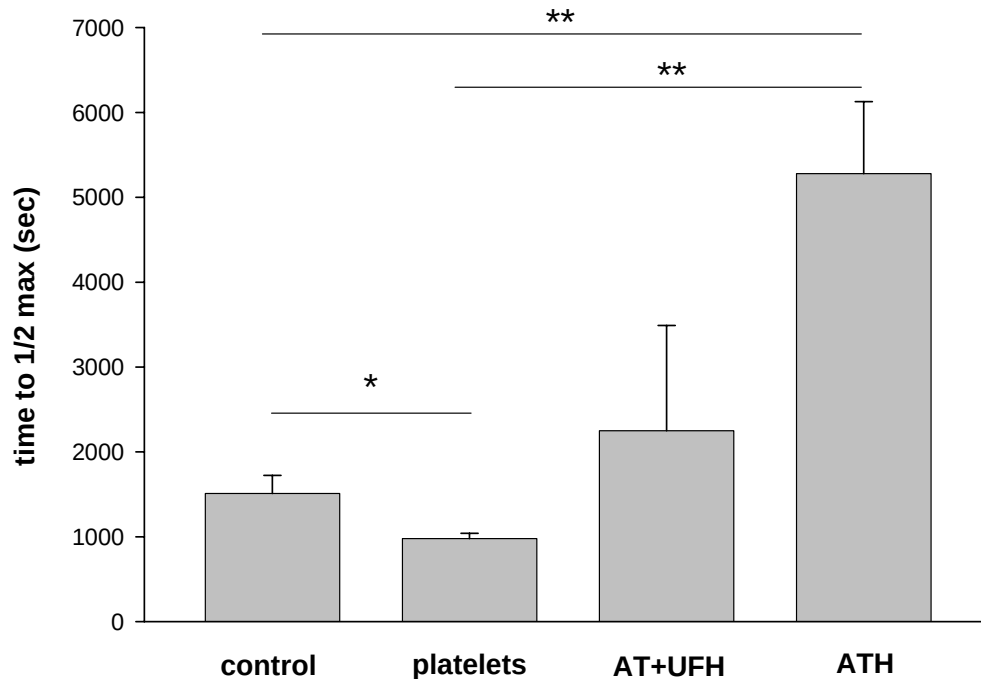


Figure 3.7: Inhibition of plasma clotting by AT+UFH vs ATH. Adult pooled platelet poor plasma was reacted with either CaCl_2 (control), activated platelets (platelets) + CaCl_2 , or activated platelets + CaCl_2 + inhibitors and analyzed for the time to reach $\frac{1}{2}$ max. Addition of activated platelets to plasma accelerated plasma clotting, as the time to $\frac{1}{2}$ -max was significantly shortened for the platelet reaction relative to control with no platelets. However, addition of ATH significantly attenuated plasma clotting compared to any of the results, as shown by a significant prolongation of the time to $\frac{1}{2}$ max. There was a trend for increased time to $\frac{1}{2}$ max for AT+UFH relative to the controls, however it was not statistically significant ($p=0.081$) $*p<0.05$, $**p<0.001$.

DISCUSSION:

Heparin has long been used clinically as the gold standard anticoagulant to treat thromboembolic diseases [11;13]. However, heparin contains numerous clinical limitations (discussed above), including its inability to inhibit fibrin-bound thrombin [31] or Xa within the prothrombinase complex (protective effect) [23], thus allowing for propagation of coagulation to occur. Chan *et al.* has developed a covalent form of AT and UFH (ATH) with superior anticoagulant responses compared to UFH alone [24]. We have previously demonstrated that ATH can inhibit some coagulation factors, such as tissue factor/VIIa, ~30-fold faster than AT+UFH [27]. In addition, we have demonstrated that ATH is capable of inhibiting Xa within a model prothrombinase complex up to 3-fold faster compared to AT+UFH [23]. However, the work in this previous study was conducted on synthetic vesicles [23]. Thus, it is pertinent to understand inhibition of the prothrombinase complex when it is formed on a more physiological surface such as platelets. Therefore, the present study examined inhibition of the prothrombinase complex on resting and activated platelets.

Numerous investigators have studied inhibition of Xa within the prothrombinase complex by heparin to determine the amount of Xa protection. These studies have reported a range of values, anywhere from orders of magnitude protection to a few-fold protection [17-23]. The reason for this vast range in results resides in the complexity of the assay. The inhibitor of the system can either react with the Xa in the prothrombinase or the product of the complex (IIa) and, since large II concentrations are used, IIa is often

in excess in the reactions. Several attempts have been made to measure inhibition of Xa in the prothrombinase using a mutant form of AT that does not bind IIa [17], or by using a mutant form of II that produces a IIa incapable of reacting with AT [18]. However, using mutant proteins could produce confounding results from distorted complex interactions, thus a preferred test system may be one where none of the reacting proteins have been altered. We developed an assay where none of the proteins have been modified, and the product of the system (IIa) is quickly extinguished by a highly selective thrombin inhibitor (Pefabloc-TH), so that Xa activity in the presence of inhibitors can be monitored using a specific Xa substrate S-2222.

We attempted to inhibit the prothrombinase complex on the surface of resting platelets. The k_2 -values for inhibition of free Xa and Xa on resting platelet-prothrombinase were similar for either inhibitor, although the overall k_2 -values for ATH were 2-fold higher than AT+UFH. Resting platelets have minimal prothrombinase function, as negatively charged phospholipids are hidden on the inner leaflet of the cell [32]. When the platelets become activated, the negatively charged lipids are exposed to the outer leaflet, which enables binding of the prothrombinase components to cause subsequent enhanced prothrombinase activity [33]. Combination of prothrombinase components with the resting platelets should eventually lead to development of enough IIa to cause activation of the platelets. However, in our inhibition assays, not enough time elapses to generate sufficient IIa for significant activation of the platelets to take place. This is due to the fact that even if ample IIa could be generated, the Pefabloc-TH reagent would quench the thrombin before it had the opportunity to act on the platelets.

We then reacted activated platelets with the prothrombinase in the presence of inhibitors and noticed that a moderate protection of Xa was evident only for AT+UFH reactions. This is consistent with previous investigations that suggested activation of platelets was necessary for the protective effect to be observed [19]. However, no differences were observed in the k_2 -values for ATH in the presence of activated platelets. Using flow cytometry and a platelet-specific antibody (anti CD-41 antibody) we established that platelet activation was achieved using the calcium ionophore A23187. Thus, activating the platelets is pertinent for prothrombinase function and for protection of Xa against non-conjugated AT+UFH complexes. We also performed a Xa:AT ELISA to confirm the formation of Xa:AT complexes in our inhibition assays (data not shown).

We have previously demonstrated that incorporation of Xa in a prothrombinase complex formed on the surface of synthetic vesicles contained a mild protective effect, approximately 30% that of free Xa [23]. In the present study, we show that on activated platelets a moderate protection of 60% was observed. Since the same assay methodology was performed in the present study as in the synthetic vesicle study, this may suggest that a surface comprised from activated platelets may cause greater protection of Xa compared to synthetic vesicles. It is known that prothrombin activation on the vesicle surface occurs through the meizothrombin pathway [34;35], whereas on platelets, the fragment 1•2 (prethrombin) pathway is preferred [33]. Thus, pathway-specific processing of prothrombinase on the two types of surfaces may be responsible for the differences observed in Xa protection. The overall difference in the protective effect between AT+UFH and ATH was 20-fold. The reason for this may stem from the ability of the

UFH from AT+UFH to dissociate and form non-productive ternary complexes with the prothrombinase components. When this happens, further non-covalent AT:UFH complexes may be repelled from reacting with the Xa by prothrombinase-bound UFH [23]. However, these non-productive ternary complexes cannot form when the UFH is covalently linked to AT as in ATH, thus enhancing the anticoagulant response. Another aspect in play may be that although binding of AT by UFH is the rate limiting step for AT+UFH reactions [33], this reaction step has been removed for ATH as all of the AT moieties are permanently activated by the covalently bound heparin chain [24].

To determine the potential role of components in the activated platelet-prothrombinase on inhibition of Xa, we performed inhibition assays where either II, activated platelets or Va were omitted from reactions prior to addition of inhibitors. Since we did not observe any difference in the k_2 -values between the free or complexed-Xa for ATH reactions, omitting the components produced no net effect. However, when we repeated the experiments with AT+UFH, we noticed that Xa inhibition was restored back to rates observed with free Xa when activated platelets or Va were absent. Although a similar effect was observed for II omission, the degree of restoration of Xa inhibition was not to the same level as free Xa. This is understandable since it has been reported that the cofactor Va provides significant protection to the proteinase [19;21;36]. Overall, these data are consistent with previous studies utilizing synthetic vesicles [23].

Utilizing prothrombinase containing moderate concentrations of II (300 nM) in the presence of inhibitors resulted in attenuation of thrombin generation by both AT+UFH and ATH. When the data was converted to thrombin potential (in essence area

under the curve for the thrombin generation), ATH inhibited thrombin generation significantly better than AT+UFH. The results from activated platelets are also consistent with previously published studies with synthetic vesicles [23].

We also attempted to show the capability of activated platelets to enhance plasma clotting in a turbidometric assay, whereby addition of inhibitors attenuated the clotting effect. We observed that both AT+UFH and ATH increased the time to $\frac{1}{2}$ -max compared to the controls, however only ATH achieved statistical significance. Since ATH significantly prolonged the time to $\frac{1}{2}$ -max compared to AT+UFH, this suggests an overall enhanced antithrombotic effect of ATH compared to AT+UFH in this more native system encompassing a procoagulant surface.

In summary, we demonstrated that prothrombinase activity is enhanced on activated platelets and, unlike ATH, the rate of Xa inhibition by AT+UFH is compromised when the Xa is incorporated in the activated platelet-prothrombinase complex. Covalent linkage between AT and heparin assists in access and neutralization of complexed Xa with concomitant inhibition of prothrombinase function. Thus, unlike heparin, the covalent conjugate has enhanced anticoagulation independent of surface-complexation by the enzymes. Thus, these data indicate a clinical advantage for ATH over UFH in treatment of cell-based coagulation *in vivo*.

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Chapter 4: Manuscript Three
Inhibition of the prothrombinase complex on red blood cells
by heparin and covalent antithrombin-heparin complex

Foreword

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Ivan Stevic performed the research and drafted the manuscript. Howard Chan provided scientific discussion and analyzed the results. Leslie Berry provided scientific input, analyzed the results and revised the manuscript. Ankush Chander helped with low molecular weight heparin and fondaparinux experiments. Anthony Chan oversaw the work.

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Topic: Enzyme Inhibitors

Inhibition of the Prothrombinase Complex on Red Blood Cells by Heparin and Covalent Antithrombin-Heparin Complex

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Running Title: Inhibition of the red blood cell prothrombinase complex

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Abbreviations: AT: antithrombin, UFH: unfractionated heparin, ATH: covalent antithrombin-heparin complex, II: prothrombin, RBCs: red blood cells

ABSTRACT:

The role of red blood cells (RBCs) in coagulation is not well understood. Overt exposure of phosphatidylserine (PS) on surfaces of RBCs provide docking sites for formation of the prothrombinase complex, which further aids in amplification of coagulation leading to subsequent thrombosis. No studies to date have evaluated heparin inhibition of the RBC-prothrombinase system. Therefore, the present study examines the ability of heparin and a covalent antithrombin-heparin (ATH) to inhibit the RBC-prothrombinase system. Discontinuous inhibition assays were performed to obtain k_2 values for inhibition of free or prothrombinase-bound Xa by antithrombin and heparin (AT+UFH) versus ATH. Additionally, components of the complex (prothrombin, RBCs or Va) were excluded prior to reaction with inhibitors to investigate potential mechanisms involved. Inhibition of thrombin and fibrin generation by the RBC-prothrombinase system was also examined. Protection of Xa was observed for AT+UFH and not for ATH reactions. Inhibition rates for ATH were significantly faster compared to AT+UFH results. The greatest impact on Xa inhibition was observed from factor Va omission for both inhibitors. ATH quenched thrombin and fibrin generation better compared to AT+UFH. This study determined potential control of coagulation contributed by RBCs. Moreover, greater control of coagulation is achieved by covalently-linking heparin to AT.

Keywords: covalent antithrombin-heparin, heparin, inhibition prothrombinase complex, red blood cells

INTRODUCTION:

Erythrocytes constitute the majority of cell components in blood. For a long while it has been thought that red blood cells (RBCs) were inert with minimal function in the body, outside of their involvement with gas exchange. However, evidence exists to show that RBCs may have other important functions, such as their role in immunity and vasodilation for example (1;2). More recently, RBCs have been receiving much attention for their participation in thrombosis (3), however, the exact mechanism is not completely understood.

RBCs from patients with various hematological conditions, such as sickle cell anemia, beta-thalassemia or preeclampsia for example, have been linked with having increased procoagulant activity (4-7). Evidence suggests that alterations in RBC membrane, particularly overt exposure of phosphatidylserine (PS) on the outer leaflet or production of microvesicles rich in PS, may be a potential mechanism to explain their involvement in thrombosis (8). PS exposure on the outer surface of the membrane may provide docking sites for formation of large macromolecular enzyme complexes (tenase or prothrombinase) that help to amplify and propagate coagulation (9;10). The prothrombinase complex (which is composed of factor Xa (Xa, the catalytically active component), its cofactor Va (Va), a phospholipid surface (PCPS), and Ca^{2+} ions) is the enzyme complex responsible for converting the substrate prothrombin (II) to thrombin (11-13). Thus, overt prothrombinase activity resulting from increased PS exposure on the surface of RBCs may lead to subsequent thromboembolic complications.

In patients with acute thromboembolism, heparin may be used as the first line of treatment in these patients (14;15). Heparin interacts with an *in vivo* serine protease inhibitor called antithrombin (AT), to assist reaction with many serine proteases in the coagulation cascade (16;17). However, heparin contains numerous clinical limitations, such as reduced ability to inhibit thrombin and Xa bound to surfaces (18-20). Thus, to overcome these limitations, Chan *et al* have developed a covalent antithrombin-heparin complex (ATH) with high anticoagulant activity compared to heparin (21;22). Recently, we have investigated comparison of non-covalent versus covalent antithrombin-heparin complexes to inhibit Xa in prothrombinase composed of synthetic vesicles (23) or platelets (24) and have noticed greater inhibition of Xa by the ATH. Much of the work with prothrombinase and use of anticoagulants has been performed on the native platelet system or synthetic vesicles (25;26). However, very little is known regarding heparin's efficiency to pacify the procoagulant RBC-prothrombinase system. Considering the vast amount of evidence to show participation of RBCs in coagulation, understanding the ability of heparin or other anticoagulants to pacify coagulant complexes on the surface of RBCs is pertinent. Therefore, the present study examines the ability of AT and unfractionated heparin (AT+UFH), compared to ATH, to inhibit the RBC-prothrombinase system.

MATERIALS and METHODS:

Chemicals: All chemical reagents were of analytical grade. Sodium chloride and Tris (tris (hydroxymethyl) amino-methane) were purchased from Bioshop (Burlington, ON).

Polyethylene glycol 8000, ethylene-diamine tetraacetic acid-disodium salt (Na_2EDTA) and calcium chloride dihydrate (CaCl_2) were from BDH Inc. (Toronto, ON). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Phosphatidic acid (PA; 1,2-Dioleoyl-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) Human factor Xa, II and thrombin were purchased from Enzyme Research Laboratories Ltd. (South Bend, IN). Human factor Va was obtained from Haematologic Technologies Inc. (Essex Junction, VT). Purified fibrinogen was purchased from American Diagnostica Inc (Stamford, CT). Substrates for Xa (S-2222; N-benzoyl-isoleucyl-glutamyl-glycylarginyl-p-nitroanilide-hydrochloride and its methyl ester) and thrombin (S-2238; H-D-phenylalanylpipecolyl-arginyl-p-nitroanilide-dihydrochloride) were from DiaPharma Group Inc. (Westchester, OH). Antithrombin (AT) and adult pooled plasma was purchased from Affinity Biologicals (Ancaster, ON, Canada). Unfractionated sodium heparin (UFH) was obtained from Sigma (St. Louis, MO). Thrombin-specific inhibitor, Pefabloc-TH ($\text{N}\alpha$ -(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine piperidide acetate (NAPAP)) was purchased from Centerchem (Norwalk, CT).

Preparation of ATH: ATH was synthesized as described by Chan *et al.* (21) In brief, 1052 mg of human AT and 64 g of UFH was separately dialyzed against 2 M NaCl followed by Phosphate Buffered Saline (PBS) and mixed to a volume of 900 ml, followed by incubation at 40°C for 14 d. The reaction mixture was then mixed with 0.5 M NaBH_3CN reducing agent and incubated for another 5 h at 37°C. A two-step purification

process was used, which involved Butyl-Sepharose hydrophobic chromatography (Amersham, Uppsala, Sweden) and DEAE-Sepharose anion exchange chromatography (Amersham, Uppsala, Sweden) for the removal of any unbound heparin or AT, respectively. ATH was analyzed for purity using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and was found to be > 95 % pure (21). We have previously shown that the AT content (amino acid analysis) and heparin content (by 3 different mass analysis methods) of ATH preparations are in a mole ratio of 1:1 (27).

Preparation of Red Blood Cells: With approval from the Hamilton Health Sciences/McMaster Research Ethics Board, 10 mL of human blood was drawn from the antecubital vein from healthy donors using 10% acid-citrate/dextrose (ACD) anticoagulant solution (0.085 M sodium citrate + 0.079 M citric acid + 0.18 M glucose) in a syringe and a 19 G butterfly needle (Venisystems, Hospira Inc, Lake Forest, IL, USA) on the day of each experiment. The blood was then transferred to a 15 mL round bottom polypropylene tube and was centrifuged at 150 x g for 15 min at 22 °C. The platelet rich plasma and buffy coat layers were removed after spinning. The RBCs (taken from the middle of the packed RBCs) were then transferred to another 15 mL round bottom polypropylene tube and were resuspended with PBS (1 mM KH_2PO_4 , 154 mM NaCl, and 3 mM Na_2HPO_4 ; pH 7.4) and washed three times, twice with PBS and once with Tris buffer (15 mM Tris·HCl, 150 mM NaCl, 5 mM KCl, and 1 mM MgCl_2 ; pH 7.4). RBCs were resuspended to 1.0×10^8 RBCs/mL in Tris buffer for use in experiments

within a 6 h time period. The final concentration of RBCs used in all experimental reactions was 1.0×10^6 RBCs/mL.

Determination of second-order rate constants (k_2 values): k_2 values for inhibition of Xa $\pm$ RBC-prothrombinase complex by AT+UFH vs ATH were determined by performing discontinuous second-order rate constant assays under pseudo first-order conditions, as previously described (23). All rate experiments were performed at physiological temperature (37°C) and a minimum inhibitor:enzyme ratio of 10:1 was maintained for all reactions. Also, we have previously determined that maximal inhibition of Xa by AT occurs in the presence of 10 fold mole ratio of heparin to AT, or 30 nM AT + 300 nM UFH (23). For rate experiments involving the inhibition of free-Xa by AT+UFH and ATH, the reaction protocol was as follows: First, 10 μ l aliquots of TSP buffer (0.02 M Tris HCl + 0.15 M NaCl + 0.6 % polyethylene glycol, pH = 7.4) was added to 6 separate wells of a 96-well flat bottom microtiter plate (Fisher, Nepean, ON, Canada), followed by additional 10 μ L aliquots containing 24 nM Xa, 32 mM CaCl₂ and 320 μ M Pefabloc-TH in TSP buffer were added to all the wells and incubated for 3 min. Then 20 μ L of 2.75 μ M II in TSP was simultaneously added to all six wells with a multichannel pipette and allowed to incubate for another 3 min. To well number 6, 40 μ L of TSP buffer was added to represent the control reaction for the experiment. At time intervals ranging from 2–5 s, a 40 μ L aliquot of 60 nM AT+600 nM UFH or 60 nM ATH was added in wells 1 through 5. Following another 2-5 s, the reactions were neutralized by the simultaneous addition of

120 μL of developing solution containing 1.25 mg/ml of polybrene + 0.53 mM Xa-specific substrate S-2222 + 1.14 mM of Na_2EDTA in TSP buffer.

For RBC-prothrombinase reactions, the protocol was as follows: freshly isolated RBCs were activated with 20 μM phosphatidic acid (PA) and 4 mM Ca^{2+} for 15 min prior to subsequent reactions. Ten μL of the activated RBCs were added to 6 wells in a 96-well plate, followed by addition of 10 μL of solution containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 and 320 μM Pefabloc-TH in TSP buffer and incubated for 3 min. Next, 20 μL of 2.75 μM II was added to all six wells with a multichannel pipette and further incubation allowed. Inhibitors were added and the reactions terminated by the developing solution in the manner described above.

After terminating the inhibition reactions, residual Xa activity for each well was determined by continuous absorbance measurement at 405 nm (SpectraMax Plus 384 plate reader) over a period of time (10-30 min). To calculate the pseudo first-order rate constant k_1 , plots of $\ln V_t/V_o$ (where V_t represents the enzyme activity at time t , and V_o is the initial enzyme activity in well number 6) versus time and the slope determined. The negative value of the slope gave k_1 ($k_1 = -\text{slope}$). The k_2 value was then calculated by dividing k_1 by the concentration of inhibitor (AT(H)) used ($k_2 = k_1/[\text{AT(H)}]$). Averages from at least 5 experiments were used to generate the final k_2 value.

To investigate the roles of individual components of the RBC-prothrombinase complex on the anticoagulant effects of ATH versus AT+UFH, additional experiments were performed where components of the complex (II, activated RBCs or Va) were excluded prior to reaction with the inhibitors.

Thrombin generation and inhibition of thrombin generation: Thrombin-specific substrate S-2238 was used to assess the effect of inhibitors on thrombin generation by the RBC-prothrombinase system. Inhibition of RBC-prothrombinase activity by 30 nM AT + 300 nM UFH versus 30 nM ATH was assessed. The concentration of the prothrombinase components were the same as above. Briefly, 10 μ L of activated RBCs were added to 7 wells of a 96-well plate, followed by addition of 10 μ L of the solution mixture containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 in TSP buffer and incubated for 3 min. Sixty μ L of a mixture containing 1.6 μ M II + 40 nM AT + 400 nM UFH or 1.6 μ M II + 40 nM ATH in TSP buffer was then added to the 7 wells, one at a time, at 10 s intervals. Ten s after addition of the last II/inhibitor mixture, 120 μ L of developing solution containing thrombin-specific substrate S-2238 was simultaneously added to all wells, and immediately read in the plate reader at 405 nm, for 10 min. Linear portions of the $V_{\max}$ curves were compared against a standard curve to determine the concentration of thrombin being generated. Plots of thrombin concentration versus time were produced to compare the effect of inhibitors on thrombin generation by the prothrombinase complex, and the area under the curve was used to determine thrombin potential for the system.

Fibrinogen turbidometric analysis: Conversion of purified fibrinogen to fibrin by the RBC-prothrombinase system in the presence of inhibitors was assessed by a clotting turbidity assay at $A_{350\text{nm}}$. Briefly, 10 μ L of activated RBCs were added to a well of a 96-well plate, followed by addition of 10 μ L of solution containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 in TSP-2 buffer (0.02 M Tris HCl + 0.15 M NaCl + 0.1 % polyethylene

glycol, pH = 7.4) and allowed to incubate for 3 min. Then, 60 μ L of solution containing 1.6 μ M II, 4 μ M fibrinogen, 6.67 mM CaCl_2 , 40 nM AT+400 nM UFH or 40 nM ATH in TSP-2 buffer was added and immediately read in the plate reader for 60 min at $A_{350\text{nm}}$. The time to $\frac{1}{2}$ max was then compared for each condition.

Plasma turbidometric analysis: Plasma clotting was assessed using a similar turbidity protocol outlined above. Briefly, 25 μ L of activated RBCs were added to a well of a 96-well plate, followed by addition of 25 μ L of 40 mM CaCl_2 containing 120 nM AT + 1200 nM UFH or 120 nM ATH. The control contained no inhibitors. Addition of 50 μ L of adult pooled platelet poor plasma (3.2 % v/v sodium citrate) started the reaction, and the reaction absorbance at 350 nm was immediately read in the plate reader for 3 h. The time to $\frac{1}{2}$ max was then compared for each condition.

Data analysis: The inhibition rate experiments were performed at n=5, fibrinogen and plasma turbidometric analyses were at n=5 and at least n=3, respectively, since previous work using these assays showed this number of replicates is sufficient to show statistical significance between groups. Statistical analysis for multiple groups was performed using ANOVA. In the case of comparison between groups, a student t-test was used. Values with a $p < 0.05$ were considered significant.

RESULTS:

Thrombin generation by the RBC-prothrombinase system: Results from Noh et al. were

recapitulated using our thrombin generation method, thus confirming the functionality of the PA-induced RBC-prothrombinase system (figure 4.1).

Comparison of k_2 values for inhibition of Xa $\pm$ RBC-prothrombinase complex:

Discontinuous second-order rate constant assays (28) were performed to determine the effect of RBC-prothrombinase complexation on k_2 values for inhibition of Xa by AT+UFH versus ATH and the underlying mechanisms involved. Results for inhibition of Xa alone or Xa within the prothrombinase complex are shown in table 4.1. Maximal k_2 values for non-conjugated AT+UFH occurred when reacted with Xa alone, whereas the k_2 value significantly decreased when Xa was incorporated into the RBC-prothrombinase complex. Inhibition of both free Xa and Xa within the RBC-prothrombinase by ATH resulted in significantly higher values compared to those of AT+UFH. Protection of Xa by the components of the RBC-prothrombinase complex was not observed for ATH, whereas a moderate protection of approximately 60% that for free Xa was observed for AT+UFH (figure 4.2). Overall, there was a >15-fold protective effect for AT+UFH compared to ATH.

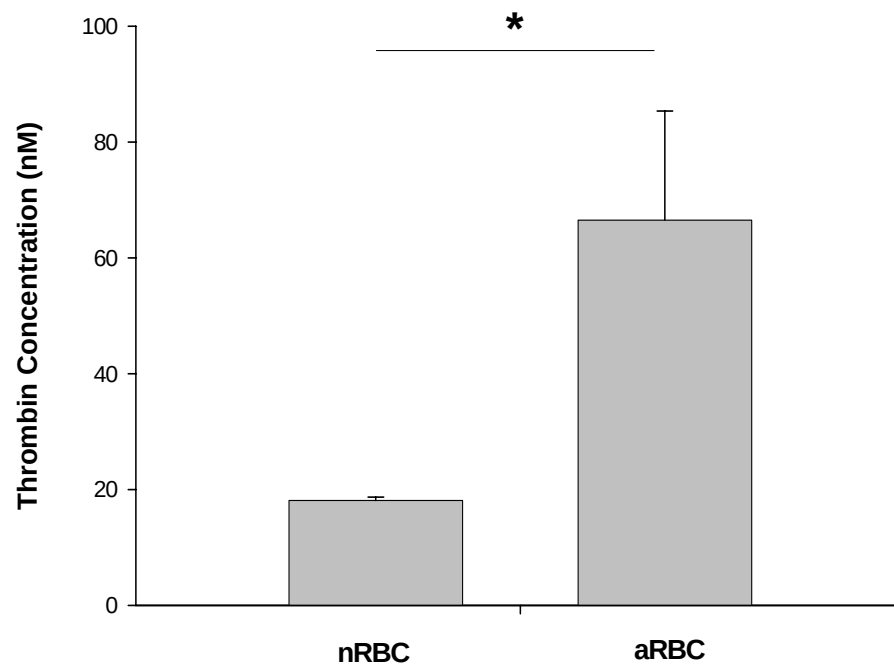


Figure 4.1: Thrombin generation. A single time point comparison of thrombin generation by non-activated red blood cells (nRBC) to those activated with PA and Ca^{2+} (aRBC) for 15 min prior to reaction with prothrombinase components. These data suggests that aRBCs contained enhanced prothrombinase activity compared to nRBCs $*p < 0.006$.

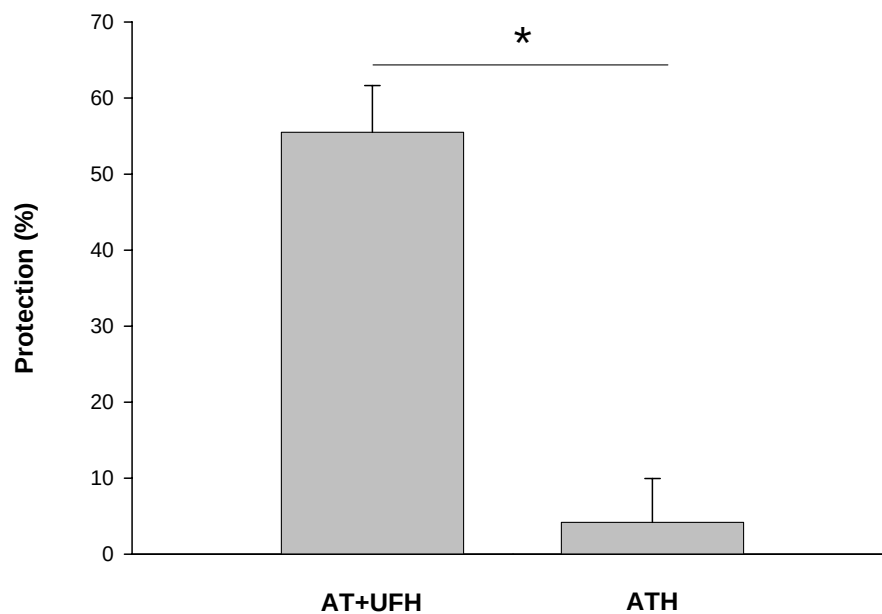


Figure 4.2: Protection of Xa by the IIase complex for AT+UFH and ATH.

Approximately 45% of Xa was inhibited by AT+H compared with 96% inhibition by ATH under similar reaction conditions. An overall > 15-fold change in the protective effect was noticed between the two inhibitors $*p < 0.0001$.

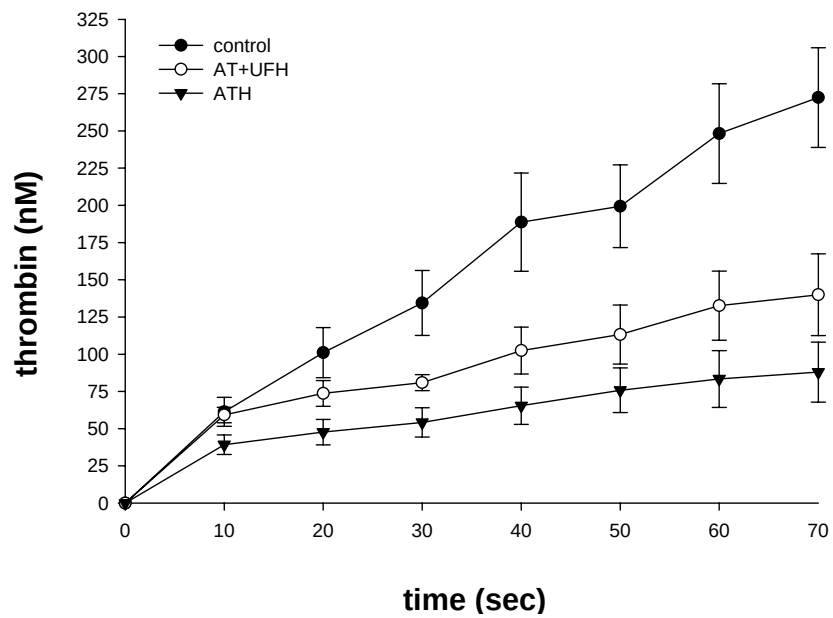
Comparison of k_2 values for inhibition of Xa by combining/excluding components of the RBC-prothrombinase system: To examine the roles of prothrombinase components on mechanisms of Xa inhibition by AT+UFH versus ATH, discontinuous inhibition assays were also performed to compare the inhibition of the intact RBC-prothrombinase to a prothrombinase where various components were combined or omitted prior to reaction with inhibitors (table 4.1). For AT+UFH reactions, relative to the intact prothrombinase, there was a significant increase in Xa inhibition when the substrate II was added to the system, a drastic increase almost to the level of free Xa when activated RBCs were omitted, and a further decrease in Xa inhibition upon Va exclusion. As for ATH reactions, a decrease in Xa inhibition was observed only for Va omission, whereas no change was observed for the other conditions.

Inhibition of thrombin generation: Thrombin generation was performed to examine the effect of AT+UFH versus ATH on functionality of the intact RBC-prothrombinase system using physiological levels of II (figure 4.3). Both inhibitors decreased thrombin generation compared to control, with ATH having a greater effect (figure 4.3A). When the results were converted to inhibition of thrombin potential (area under the curve), ATH significantly reduced thrombin potential compared to AT+UFH (figure 4.3B).

Table 4.1: Inhibition of Xa within the prothrombinase complex by AT+UFH versus ATH

Condition	AT+UFH (k_2 values $\pm$ SD)	ATH (k_2 values $\pm$ SD)
Free Xa	$3.14 \times 10^8 \pm 0.27 \times 10^{8**}$	$5.12 \times 10^8 \pm 0.60 \times 10^8$
Prothrombinase	$1.40 \times 10^8 \pm 0.19 \times 10^8$	$4.47 \times 10^8 \pm 1.09 \times 10^8$
Prothrombinase + II	$2.10 \times 10^8 \pm 0.57 \times 10^{8*}$	$4.85 \times 10^8 \pm 0.53 \times 10^8$
Xa+Va+Ca ²⁺ +II	$2.95 \times 10^8 \pm 0.59 \times 10^{8**}$	$4.99 \times 10^8 \pm 1.01 \times 10^8$
Activated RBCs+Xa+Ca ²⁺ +II	$4.63 \times 10^7 \pm 0.82 \times 10^{7*}$	$2.86 \times 10^8 \pm 0.32 \times 10^{8*}$

A)



B)

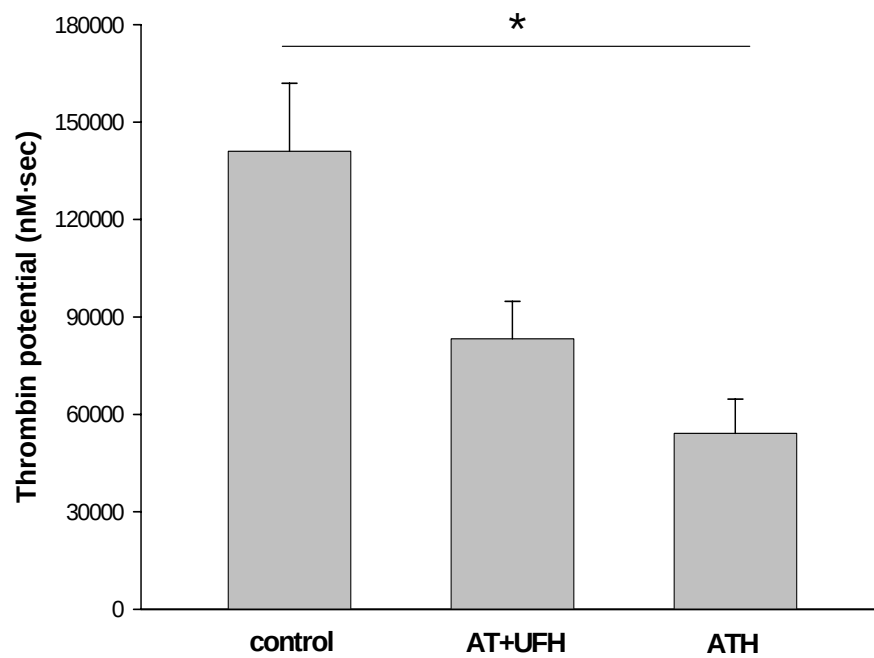


Figure 4.3: Inhibition of thrombin generation by the RBC-prothrombinase complex.

Thrombin generation in the presence of inhibitors was assessed using a specific thrombin substrate S-2238 over a time course. It appears that both AT+UFH and ATH reduced thrombin generation compared to the control (no inhibitor), with ATH having a greater effect **(A)**. However, when assessed for inhibition of thrombin potential, ATH significantly reduced the thrombin potential whereas AT+UFH did not, compared to the control **(B)**. Final concentration of reactants: 3 nM Xa, 9.6 nM Va, 4 mM Ca^{2+} , 1.2 μM II, 40 μM Pefabloc TH, 30 nM AT, 300 nM UFH and 30 nM ATH $*p < 0.005$.

Inhibition of fibrinogen conversion by the RBC-prothrombinase: A turbidity assay was performed to determine the effect of the RBC-prothrombinase system on fibrinogen conversion in the presence or absence of inhibitors (figure 4.4). Both AT+UFH and ATH significantly increased the time to $\frac{1}{2}$ max (a measure of time to clot) compared to a control assay with no inhibitors. Moreover, fibrinogen conversion by the RBC-prothrombinase was inhibited much better with ATH compared to AT+UFH.

Inhibition of plasma clotting: Addition of activated RBCs to plasma increased the time at $\frac{1}{2}$ max compared to a system with no activated RBCs (data not shown). The effect of inhibitors was tested in this activated RBC/plasma clotting system. Relative to a control containing activated RBCs in plasma with no added inhibitor, the time to $\frac{1}{2}$ max was significantly prolonged for ATH but not for AT+UFH. These data suggested that ATH may be a more efficient inhibitor of plasma clotting (figure 4.5), consistent with results for purified fibrinogen.

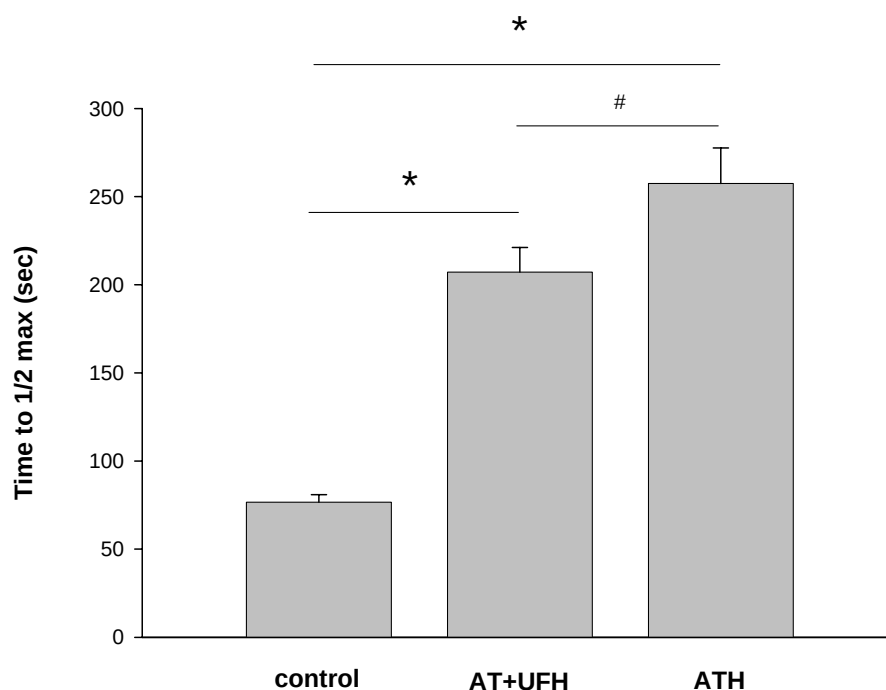


Figure 4.4: Inhibition of fibrinogen conversion by the RBC-prothrombinase system.

Using a turbidity assay, the ability of thrombin (generated by the RBC-prothrombinase complex) to convert fibrinogen to fibrin in the presence or absence of inhibitors was assessed. Both inhibitors increased the time to $\frac{1}{2}$ max (in essence time to clot) compared to the control. However, ATH caused a further increase in the time to $\frac{1}{2}$ max compared to AT+UFH, suggesting that ATH suppressed fibrinogen conversion better than AT+UFH. Final concentration of reactants: 3 nM Xa, 9.6 nM Va, 4 mM Ca^{2+} , 1.2 μM II, 3 μM fibrinogen, 40 μM Pefabloc TH, 30 nM AT, 300 nM UFH and 30 nM ATH * $p < 0.0001$, $p < 0.05$

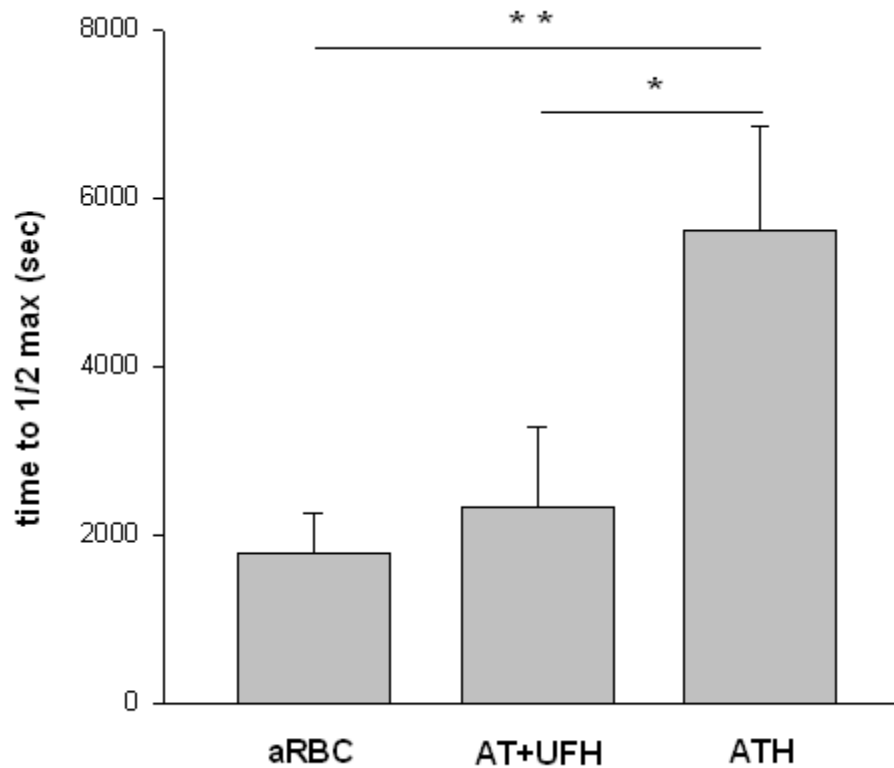


Figure 4.5: Inhibition of plasma clotting by AT+UFH vs ATH. Adult pooled plasma was reacted with activated RBCs (aRBCs) + CaCl_2 with or without inhibitors and analyzed for the time to reach $\frac{1}{2}$ max. ATH significantly attenuated plasma clotting compared to the control (plasma + aRBCs with no inhibitors added) and AT+UFH as shown by a significant prolongation of the time to $\frac{1}{2}$ max. There was a trend for increased time to $\frac{1}{2}$ max for AT+UFH relative to the control, however it was not statistically significant $*p<0.01$, $**p<0.001$.

DISCUSSION:

Heparin, heparin derivatives and other anticoagulants are clinically used for the treatment and prophylaxis of thromboembolic diseases (15). In the past few decades, much of the work on heparin's ability to inhibit the prothrombinase complex has been performed on systems comprised of vesicles and platelets (25;26). This is understandable because platelets are the primary cells in the body that participate in clotting and formation of coagulant complexes. On the other hand, since synthetic vesicles readily reproduce platelet results, are easier to maintain and are facile to make, they are a very useful platform for studying coagulation.

Various investigations have reported that RBC counts are closely related to bleeding time and occurrence of thrombosis (29). For example, transfusion of RBCs abolishes bleeding in patients with prolonged hemorrhagic complications (30). Conversely, individuals with polycythemia vera (having abnormally high blood cell counts) often experience thrombotic complications (31;32). In both of these examples RBC levels were a predictive marker for thrombosis (8). Furthermore, although venous thrombi contain a large proportion of fibrin-entrapped RBCs with relatively few platelets (33), venous thrombi have a high occurrence rate of thromboembolic events. Even though RBCs constitute the highest proportion of the cell component in blood, they have been regarded as inert with respect to thrombosis and hemostasis (8). While the exact mechanism for RBCs participation in thrombosis is still under investigation, a large body of evidence suggests that PS exposure on the outer surface of RBCs may be a major contributor, as PS allows for formation of enzyme complexes (such as prothrombinase)

that aid with amplification and propagation of the coagulation cascade (7). To better understand treatment and prophylaxis of thromboembolic events, it is important to examine inhibition of surface-complexed enzymes when the surface is contributed by a major cell type such as RBCs.

Previous investigations have revealed that intracellular signaling through an increase in Ca^{2+} , Ca^{2+} -dependent activation of phosphokinase C- α (PKC- α)(34) and ATP depletion (35) can mediate PS exposure in RBCs. Additionally, other signaling factors, such as arachadonic acid (3), prostaglandins (36), and platelet-activating factor (37), can all induce PS exposure on RBCs. However, the extent of this exposure is minute, ranging from 3-5% of the total lipids on the surface (9). As demonstrated by a recent study, RBCs treated with PA responded quickly by decreasing intracellular ATP levels, increasing Ca^{2+} and activating PKC- α , which further resulted in activation of the scramblase and attenuation of flippase enzymes (9). Cumulatively, this caused exposure of PS on the surface of RBCs and accelerated thrombin generation through enhanced prothrombinase activity (9). We modified the protocol from Noh *et al.* and adapted it with our well established discontinuous inhibition assays (28) in order to examine inhibition of prothrombinase on the RBC surface. Prior to starting the discontinuous inhibition assays, we wanted to prove through reproduction of the results by Noh *et al.*, that our protocol for pre-treatment of RBCs with PA and Ca^{2+} also yielded elevated prothrombinase activity (figure 4.1).

Many studies on prothrombinase inhibition by AT + heparin, or heparin derivatives such as low molecular weight heparin (LMWH) and pentasaccharide

(fondaparinux), have been performed using platelets and vesicles (18;19;23). In these previous studies, it was noticed that UFH yielded the highest inhibition rates, followed by LMWH then fondaparinux. In our study we utilized activated RBCs instead and were able to show inhibition of RBC-prothrombinase complex by AT+UFH versus ATH (table 4.1). We observed that ATH inhibited Xa in the prothrombinase complex far better than AT+UFH. We also observed a significant reduction in Xa inhibition when factor Xa was incorporated in the prothrombinase complex for AT+UFH reactions. This is consistent with previous findings using vesicle or platelet surfaces (18;19;23). The decrease in Xa inhibition is often referred to as the protective effect, where certain components of the complex protect the catalytically active enzyme from inhibition by anticoagulants (38). Conversely, protection of Xa was not observed for ATH, as inhibition of both free Xa and Xa complexed within prothrombinase yielded similar k_2 values for all of the ATH reactions. Moreover, AT+UFH displayed a greater than 15-fold difference in the protective effect over ATH (figure 4.2). One simple explanation for ATH's enhanced inhibitory effect is that covalent linkage prevents dissociation of the heparin moiety and, thus, prohibits potentially non-productive interactions with prothrombinase components observed with the UFH from AT+UFH mixtures (23). Other detailed structural features may moderate these mechanisms. We have recently obtained preliminary data on inhibition of the RBC-prothrombinase system by LMWH and fondaparinux. Although protection of Xa inhibition by prothrombinase on activated RBCs was not observed, the measured inhibition rates were one and two orders of magnitude lower for LMWH and fondaparinux, respectively, relative to UFH and, especially, ATH. When we repeated this

work with fondaparinux using synthetic vesicles in place of RBCs, protection of Xa was restored to levels reported in the literature (26). We intend to further investigate these findings using low molecular weight fractions of ATH and RBC membrane components to determine the heparin chain length dependence on anticoagulant interactions of serpin-heparinoids with activated RBCs.

Previous investigations with vesicle and platelet surfaces determined that II is the key player protecting factor Xa from inhibition by AT + heparins (UFH, LMWH or fondaparinux) (18;19;23;26). However, these systems utilized a prothrombinase where the substrate for the enzyme was part of the complex, which does not fall under the definition of prothrombinase in this study (11). For AT+UFH reactions, we determined that inhibition of prothrombinase in the absence of substrate II yielded a significant 60% reduction in the inhibition of complexed-Xa compared to free Xa. However, when the substrate was included with the prothrombinase system, inhibition rates increased. This is contrary to previous findings with vesicle systems (23). In our previous study with synthetic phospholipid vesicles, when a prothrombinase without the presence of its substrate was reacted with AT+UFH or ATH, the rate of Xa inhibition increased relative to a prothrombinase in the presence of its substrate (although not reaching the rates observed for free Xa) (23). Furthermore, omission of Va from the RBC prothrombinase caused an even further drop in the k_2 values, which is also contrasts with previous findings on the vesicle system (23). Thus, surface-based dissimilarities between a heterogeneous RBC plasma membrane and purified phosphatidylserine/choline bilayers may underlie these confounding results. Although consistent with previous observations,

when the phospholipid surface (aRBCs) was removed, the k_2 values increased almost to the same level as free Xa reactions (table 4.1). Since there is no membrane for the prothrombinase complex to form on, it makes sense to observe elevated k_2 values for these conditions. For ATH reactions, relative to the intact prothrombinase, addition of the substrate or exclusion of activated RBCs caused no net effect, but omission of Va reduced the k_2 values. Thus, in the absence of Va, the Xa/II/Ca²⁺/RBCs complex configuration resulted in a greater protection of Xa, which is opposite to results of previous studies involving different cell surfaces (23). Mechanistically, this suggests that the membrane surface required for coagulation complex interaction on activated RBCs may be different compared to vesicles or platelets, and should be subjected to further investigations in the future. The methodology employed in this study is well recognized and has been used in prior experiments with synthetic vesicles or platelets to acquire results consistent with that of other investigators (23).

Prothrombinase has been the target of past, present and novel anticoagulants, thus emphasizing the importance of targeting this enzyme for treatment and prevention of thromboembolism (33). Since prothrombinase is the enzyme complex responsible for converting II to thrombin, examining the ability of the RBC-prothrombinase to convert II to thrombin in the presence of inhibitors is important. In this study, it appeared that both AT+UFH and ATH may attenuate thrombin generation (figure 4.3A) and the thrombin potential (figure 4.3B), however further analysis revealed that inhibition by ATH was statistically greater compared to AT+UFH.

Thrombin feeds back variously in the coagulation cascade, with one of the functions involving conversion of fibrinogen to fibrin (39). In this study, we attempted to show the capability of the RBC-prothrombinase-generated thrombin to convert fibrinogen to fibrin in the presence of inhibitors using a fibrinogen turbidity (clotting) assay (figure 4.4). We observed that both AT+UFH and ATH significantly increased the time to $\frac{1}{2}$ max compared to the control with no inhibitors, suggesting overall suppression of fibrinogen conversion by both agents. Moreover, the time to $\frac{1}{2}$ max was much greater for ATH compared to AT+UFH. Similar results were observed when a plasma turbidogenic assay was employed (figure 4.5). In the plasma experiments, ATH significantly prolonged the time to $\frac{1}{2}$ max compared to AT+UFH, suggesting an overall enhanced antithrombotic effect for ATH compared to AT+UFH in this more native system.

This study sheds some light into potential control of coagulation occurring in samples containing red blood cell products. These data also reveal important findings involved in the anticoagulant effects of heparin and ATH against RBC-bound coagulation enzymes. It is clear that the prothrombinase components hinder the ability of conventional heparin to inhibit Xa. However, covalent linkage between AT and heparin assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function, as assessed through inhibition of thrombin generation and fibrinogen conversion. Enhanced inhibition of serine proteases on surfaces and within supramolecular complexes encourages further investigation of ATH for clinical application in thrombosis. Moreover, participation of the membrane surface on activated

RBCs in coagulation complex formation may be different compared to conventional surfaces studied previously. This aspect further incites continued investigation into the prothrombotic properties of RBCs.

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Chapter 5: General discussion, conclusion, future directions and limitations

5.1 General discussion

The prothrombinase complex contains the key enzyme (Xa) responsible for converting inactive II to active thrombin within the coagulation cascade. Prothrombinase is a large enzyme complex composed of factor Xa, its cofactor Va, Ca^{2+} , assembled on a phospholipid surface composed of anionic phospholipids, that converts the zymogen II to thrombin. Since thrombin is a potent clotting enzyme capable of causing positive feedback within the clotting cascade, targeting this enzyme for the control and prevention of thromboembolism has been the goal ever since its discovery. One of the major anticoagulants used clinically to down-regulate thrombin activity is heparin. As discussed in the Introduction, heparin interacts with endogenous AT to inhibit thrombin and other serine proteases in the coagulation cascade. Drs. Jay McLean and William Howell have been credited for discovering heparin in 1915, and it was not until 1935 that heparin was enrolled in clinical trials to evaluate its potential as an anticoagulant agent (1). Thus, heparin is one of the oldest drugs still currently used for the treatment and/or prophylaxis of thromboembolic complications. However, over the past seventy years of heparin use, clinicians have encountered numerous limitations that have significantly impacted patient care. This has prompted the scientific community to develop better and safer anticoagulants. Even though fractions of heparin (e.g. LMWH and pentasaccharide) have been developed and have been shown to address some of the limitations of heparin, they too contain clinical limitations that need to be addressed as well.

To help address the limitations of heparin and heparin derivatives, Chan *et al.* developed a covalent complex of antithrombin and heparin, ATH, in 1994. Ever since its synthesis, the laboratory of Chan *et al.* has shown potential clinical advantages of ATH over UFH (see introduction). One of the most striking differences between ATH and heparin is that, unlike UFH, ATH is capable of inhibiting clot-bound thrombin (2). Furthermore, ATH inhibition of coagulation factors in isolation was significantly faster compared to AT+UFH (3). For example, inhibition of TF/VIIa by ATH was approximately 30-fold greater than AT+UFH (3). Since ATH performed much better at inhibiting non-complexed coagulation factors compared AT+UFH, it was pertinent to understand inhibition of proteases when incorporated within their respective macromolecular enzyme complexes.

The assembly of the proteinase in its corresponding complex produces a double edged sword. In thrombosis, it speeds up the rate of conversion of zymogen to active enzyme. However, during anticoagulation, it protects the proteinase from inhibition by anticoagulants such as UFH. In the past several decades, variable protection of Xa by the prothrombinase components against UFH has been reported in the literature, from full protection to essentially no protection (4-9). The reason for the variability in reports arises from the complexity of the assay system. The inhibitor (AT) can either react with Xa in the prothrombinase or the product of the complex, thrombin. Studies that showed the greatest protection of Xa utilized mutant proteins in their inhibition assays (8-9). This could produce confounding results because modification in any of the proteins might disrupt critical interactions necessary for complex formation and function.

Bruffato *et al.* utilized a mutant II in which the active site Ser has been modified to a Cys that generated a thrombin incapable of reacting with the AT (8). In theory, the modified product of prothrombinase (Cys-thrombin) was incapable of reacting with the AT, which in turn is able to react and inhibit the Xa within the prothrombinase (8). Another study utilized a mutant AT which rapidly inhibited Xa but did not react with thrombin (9). However, we believe that an assay containing all native and unmodified proteins is preferred in the inhibition assays to generate results most likely to mimic *in-vivo* reactions. In order to achieve this, we utilized an assay that contained Pefabloc TH, which is a potent inhibitor of thrombin (10). Thus, in the presence of all native and purified proteins (purity of proteins found in appendix A), the product of prothrombinase (thrombin) would be immediately extinguished by Pefabloc TH, thus leaving AT to react with Xa. To ensure that AT reacts with Xa in our assay, we performed an ELISA which confirmed that Xa:AT complexes are produced at expected concentrations (appendix B).

Synthetic phospholipid vesicles comprised of 75% PC and 25% PS have long been used as the membrane surface for evaluating formation and function of the prothrombinase complex. Thus, our first investigation (chapter 2) looked at the detailed mechanism of inhibition of Xa within the prothrombinase complex by AT+UFH versus ATH when the prothrombinase was assembled on the surface of synthetic vesicles. All of the inhibition rates encompassing AT+UFH reactions produced comparable results to those described in literature. It is important to note that we did not observe a difference in the k_2 -values if the AT was mixed with the UFH for different time lengths. However, for consistency we ensured that the inhibitors were mixed together at room temperature and

incubated for 5 minutes, followed by another 3 min at 37 °C, and then the inhibitors were reacted in the discontinuous assays at appropriate 2-5 sec time intervals. Whereas, for ATH reactions, it was evident that the inhibition rates were 2-3-fold higher compared to any of the AT+UFH results. Moreover, the protective effect for ATH was not observed to the same degree as AT+UFH. There was, however, a statistically significant but mild (~25%) protection of Xa observed for ATH on the PCPS vesicles. This may be due to the excessively long heparin chains found on some ATH molecules. Our laboratory has demonstrated that ~33% of the heparin chains on ATH contain one or more high affinity pentasaccharide sequences (11), and it could be these relatively long heparin chains that are interacting with the prothrombinase in a non-productive manner to yield the small protective effect. It was further enlightening to see that when we repeated the work on activated platelets (chapter three) we observed similar results as those achieved with synthetic vesicles. Moreover, the small protective effect of Xa for ATH observed in reactions on vesicles was completely absent on activated platelets. This becomes more significant than the outcome with synthetic vesicles, because the surface in this study was provided by a more physiologically relevant cell. It is well known that prothrombinase forms on activated platelets *in-vivo*, thus the direct comparison of prothrombinase inhibition on activated platelets is of greater importance.

For experiments where components of the complex (phospholipid membrane, Va or II) were excluded from the reactions prior to addition of inhibitors yielded comparable results for both synthetic vesicles and activated platelets. This confirms that vesicles, although artificially produced, readily recapitulate results from activated platelets. Our

work, which utilized the same assay system for both vesicles and platelet surfaces, confirms that vesicles are a good model to use for coagulation-based studies. Any differences in inhibition rates that may have occurred between the two surfaces could be due to the differences in the II activation pathway. For example, it has been shown that II activation occurs through the meizothrombin pathway when prothrombinase is formed on a surface artificially expressing phosphatidylserine (12,13), such as synthetic vesicles, whereas when the prothrombinase forms on activated platelets, the prethrombin-2 pathway predominates (14).

To delineate the differences in the mechanisms of inhibition of ATH versus AT+UFH, we further employed fluorescent microscopy utilizing fluorescently labelled factor Xa and inhibitors (F-UFH and F-ATH) to determine fluorescence intensity of a fully assembled prothrombinase complex when formed on synthetic phospholipid membranes. When we used an active site blocked fluorescent Xa (FPPack-Xa), the results showed that UFH or AT+UFH may displace the Xa from the prothrombinase. In the presence of UFH only, the fluorescence intensity of the prothrombinase complex was significantly reduced relative to one without the inhibitors. When repeated with the presence of AT (AT+UFH), the experiments yielded similar results. However, when ATH was introduced, the fluorescence intensity remained high and was comparable to the fluorescence intensity of the control with no inhibitors. This suggests that UFH, even in the presence of AT (as in AT+UFH), may interact with prothrombinase complex to displace the Xa from the complex. However, this was not possible when UFH was permanently conjugated to the serpin, as in ATH. This infers that one of the mechanisms

that AT+UFH may inhibit prothrombinase is through physical disruption of the complex. The findings from our fluorescent work are similar to results from previous investigations, which showed that heparin possesses the ability to displace Xa and II from the prothrombinase complex (15). Upon repeating the inhibitor/prothrombinase interactions with a fluorescently labelled Xa with an intact active site (F-Xa), similar results were observed.

When fluorescently labelled inhibitors were utilized as a probe to determine heparin interaction with prothrombinase, the fluorescence intensity was highest for F-UFH when reacted with prothrombinase and prothrombinase devoid of components. Thus, there may be a capability for UFH to form ternary complexes with various prothrombinase components. However, these interactions were limited for F-ATH. Therefore, while UFH may displace Xa from prothrombinase, Xa is detained within prothrombinase during ATH reactions. Overall the results suggest that covalent linkage between AT and heparin assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function compared to conventional heparin. The model for prothrombinase complex inhibition by AT+UFH versus ATH in Appendix C may suggest that the UFH in the non-covalent conjugate may dissociate from the AT and interact with the prothrombinase complex. As a result, two consequences occur: 1) the prothrombinase-bound UFH leaves the AT in an inactive state, consequently the AT is unable to react with the Xa in the complex. 2) The prothrombinase-bound UFH produces an anionic charge difference on prothrombinase, which results in repulsion of other incoming AT+UFH molecules and preventing inhibition of Xa.

There are no studies to date that have evaluated inhibition of the prothrombinase complex on cells other than activated platelets or synthetic vesicles. However, there is one study that looked at prothrombinase formation and function on monocytes, lymphocytes, and neutrophils. This investigation has determined that each of the three cell surfaces contained a significant capability to bind Xa and Va (16). The catalytic efficiencies of prothrombinase on monocytes and lymphocytes are comparable to platelets, whereas neutrophils possess only 25% of the catalytic efficiency (16). More recently, II activation was studied on red blood cells, and it was determined that RBCs may contribute approximately 35% to prothrombin activation *in-vivo* (13). Thus, significant prothrombinase function can be attributed to procoagulant RBCs expressing the appropriate anionic phospholipids on its surface. Moreover, this study also showed that II activation proceeds through the meizothrombin pathway when prothrombinase forms on procoagulant RBCs (13). Other investigators have shown that various hematological conditions can also produce procoagulant RBCs, *via* excessive exposure of PS on the outer membrane surface of RBCs (17-19). In contrast, inhibition of macromolecular complexes on red blood cells has never been studied. Thus, the last chapter (chapter four) of this thesis evaluated inhibition of Xa within the prothrombinase complex by AT+UFH versus ATH when the prothrombinase assembled on procoagulant RBCs.

Since the meizothrombin pathway predominates on RBCs and phospholipid vesicles, we anticipated that the results from inhibition analysis would be similar to the vesicle system. For inhibition of free Xa and prothrombinase by AT+UFH and ATH, the

results from RBCs mirrored the results from the vesicle system. However it was not until mechanistic experiments were performed, where components of the complex (II/lipid surface/Va) were excluded one at a time prior to adding the inhibitors, did striking differences become apparent. Although the vesicle and platelet systems behaved similarly, the RBC results were confounding. For example, when Va was excluded from the system, there was a modest increase in the rate of Xa inhibition for the vesicle and platelet systems. However, for the RBC system, exclusion of Va caused a significant decrease in Xa inhibition for both AT+UFH and ATH, with the greatest effect observed for AT+UFH reactions. In addition, similar trends were observed for II omission for AT+UFH (but not ATH), however not to the same degree. In the vesicle and platelet systems, omission of II caused a net increase in Xa inhibition for AT+UFH. Thus, it is apparent that the results using RBCs differ from those with vesicle and platelet systems. We attribute these changes may due to the heterogeneous membrane surface of the RBCs, which has never been studied with respect to coagulation. Other detailed structural features may moderate these mechanisms, such as heparin chain length dependence on anticoagulant interactions of serpin-heparinoids with activated RBCs. We have recently obtained preliminary data on inhibition of the RBC-prothrombinase system by LMWH and fondaparinux. In brief, although protection of Xa inhibition by prothrombinase on activated RBCs was not observed, the measured inhibition rates were one and two orders of magnitude lower for LMWH and fondaparinux, respectively, relative to UFH and, especially, ATH. When we repeated this work with fondaparinux using synthetic vesicles in place of RBCs, protection of Xa was restored to levels reported in the literature (9).

Thus far, the results with RBCs suggest that in addition to the chain length effect, membrane differences may also play a significant role. Therefore, we intend to further investigate these findings by using low molecular weight fractions of ATH and RBC membrane components.

Prothrombinase has long been the target of testing anticoagulants, thus emphasizing its importance in the treatment and prevention of thromboembolism (20). Since prothrombinase is the enzyme complex responsible for converting II to thrombin, examining the ability of the cell-based prothrombinase system to convert II to thrombin in the presence of inhibitors is important. This is especially the case for novel anticoagulants, such as ATH, which are not so clearly understood as yet. For vesicle and activated platelet systems, we utilized a prothrombinase complex that contained up to 300 nM II, because higher levels of II significantly overwhelmed the inhibitor system. Thrombin generation over time was much lower for the RBC-prothrombinase system compared to vesicle and platelets, and as a consequence, we were able use physiological concentrations of II for the RBC-prothrombinase reactions. Nonetheless, on all the surfaces tested, ATH was a better inhibitor of thrombin generation compared to AT+UFH.

Since thrombin functions to convert fibrinogen to fibrin (21), we then evaluated the effect of inhibitors on clot formation in purified and plasma turbidometric assays. Both activated platelets and RBCs were subjected to turbidometric analysis involving reactions with adult pooled platelet-poor plasma and noticed that the time to achieve half-maximum ($1/2$ -max) turbidity for clotting was much shorter than results with no cells in

the assay. This confirmed that activated platelets and RBCs contributed to clotting. However, when either inhibitor was added to the system, it was evident that ATH significantly prolonged the time to $\frac{1}{2}$ -max compared to AT+UFH, suggesting an overall enhanced antithrombotic effect for ATH compared to AT+UFH in the plasma coagulation system. For the RBC-prothrombinase system, we also evaluated the conversion of purified fibrinogen to fibrin. We observed similar results as the plasma clotting system, whereby ATH significantly prolonged the time to $\frac{1}{2}$ -max relative to AT+UFH. Overall, this confirmed the conclusion that ATH is a better inhibitor of prothrombinase than AT+UFH in cell-based systems.

5.2 Conclusion

From a global standpoint, this work provides detailed mechanistic work showing inhibition of the prothrombinase complex by ATH versus AT+UFH. It also provides insights into interactions of inhibitors with macromolecular complexes, particularly those that have been shown to provide protection of the proteinase. Additionally, this study examined a new area of coagulation involving the inhibition of procoagulant RBCs, which has never been studied before. Lastly, these data indicate a clear advantage for ATH over UFH for the treatment of cell-based coagulation *in-vivo* and for potential use of ATH as a clinical agent.

5.3 References

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5.4 Future Directions

For the most part, we have studied the interactions of inhibitors with prothrombinase in great detail. In the past, numerous investigations have shown inhibition of prothrombinase complex by heparin and heparin derivatives like LMWH and pentasaccharide when prothrombinase forms on vesicles and platelets. However, since we are the first to show inhibition of the RBC-prothrombinase system by heparin, detailed work needs to be performed to understand interactions of LMWH and pentasaccharide with this system as well. This is the target of future studies in this lab. We have obtained preliminary data to suggest that protection of Xa by prothrombinase on activated RBCs was not observed for both AT+LMWH and AT+fondaparinux. However, the measured inhibition rates were one and two orders of magnitude lower for LMWH and fondaparinux, respectively, relative to UFH and, especially, ATH. We intend to investigate these findings further by using low molecular weight fractions of ATH and RBC membrane components to determine the heparin chain length dependence on anticoagulant interactions of serpin-heparinoids with activated RBCs.

Since ATH is a novel anticoagulant, determining its performance relative to the new anticoagulants is of great importance. Moreover, ATH is in fact a direct Xa/thrombin inhibitor (unlike the heparinoids discussed above), future studies comparing inhibition of prothrombinase complex with the new direct Xa/thrombin inhibitors is very appropriate. Most of the work with the new anticoagulants did not attempt to inhibit Xa within the prothrombinase complex using discontinuous inhibition assays, but have shown Xa inhibition indirectly by inhibiting II activation by the prothrombinase complex in other

clotting systems. Therefore, it is important to perform discontinuous inhibition assays instead to make direct comparisons.

Based on the ample work with the prothrombinase complex, it is a common belief that incorporation of the proteinase in the complex protects it from inhibition by anticoagulants. Investigation of inhibition of other macromolecular complexes, such as tenase complex, is also of great importance. We have started an assay aimed at investigating inhibition of IXa in the tenase; however this assay is proving to be more difficult than the prothrombinase assay. In the tenase assay, the product of tenase, Xa, cross reacts with the IXa substrates and selective blocking agents to give false positive results. The goal is to use a molecule which is a potent inhibitor of Xa but not IXa so that the IXa substrate or the inhibitor being tested does not react with Xa. We have tried to produce a recombinant tick anticoagulant peptide (rTAP) because the commercial TAP is not available. TAP has been shown to be a highly selective inhibitor for Xa but not for other proteases, thus making it a good candidate for the tenase assay. However, TAP production is proving difficult and is still under investigation. On the other hand, our laboratory has recently purified the active ingredient from the commercially available drug, Rivaroxaban. Rivaroxaban has been shown to be a potent inhibitor of Xa that does not cross react with other serine proteases in the coagulation cascade, which is exactly what is needed for the tenase assay. We hope to investigate Rivaroxaban with the tenase assay in the near future.

5.5 Limitations

There are a few limitations to this study. Discontinuous inhibition assays requires sub-sampling of the inhibitor in the reaction mixture at relatively quick time intervals, as evident from the 2-5 s timings in parts of our study. It is difficult to achieve time intervals less than 2 s, thus the inhibition assays may prove to be difficult for faster inhibitors requiring shorter time intervals. Fortunately, we were able to achieve fast enough speeds to obtain interpretable results from these experiments.

Furthermore, if the discontinuous inhibition assay was to be used with slower inhibitors that require long time intervals, there could be sufficient cross reaction with the Xa substrate by the very low amounts of generated thrombin that is not bound to pefabloc TH, giving false positive results for enzyme activity. However, in our study, from the time it takes to add the inhibitor in the first well to the time we stop the reaction with the developing solution, only a small amount of time had elapsed (10-30 sec total) for all experiments conducted in this study. Thus, the total time that elapsed in our assay is not enough for any low level free thrombin (in equilibrium with the thrombin-pefabloc TH complex) to cross-react with the Xa substrate or inhibitor. Future studies evaluating slow inhibitors should be vigilant for this effect.

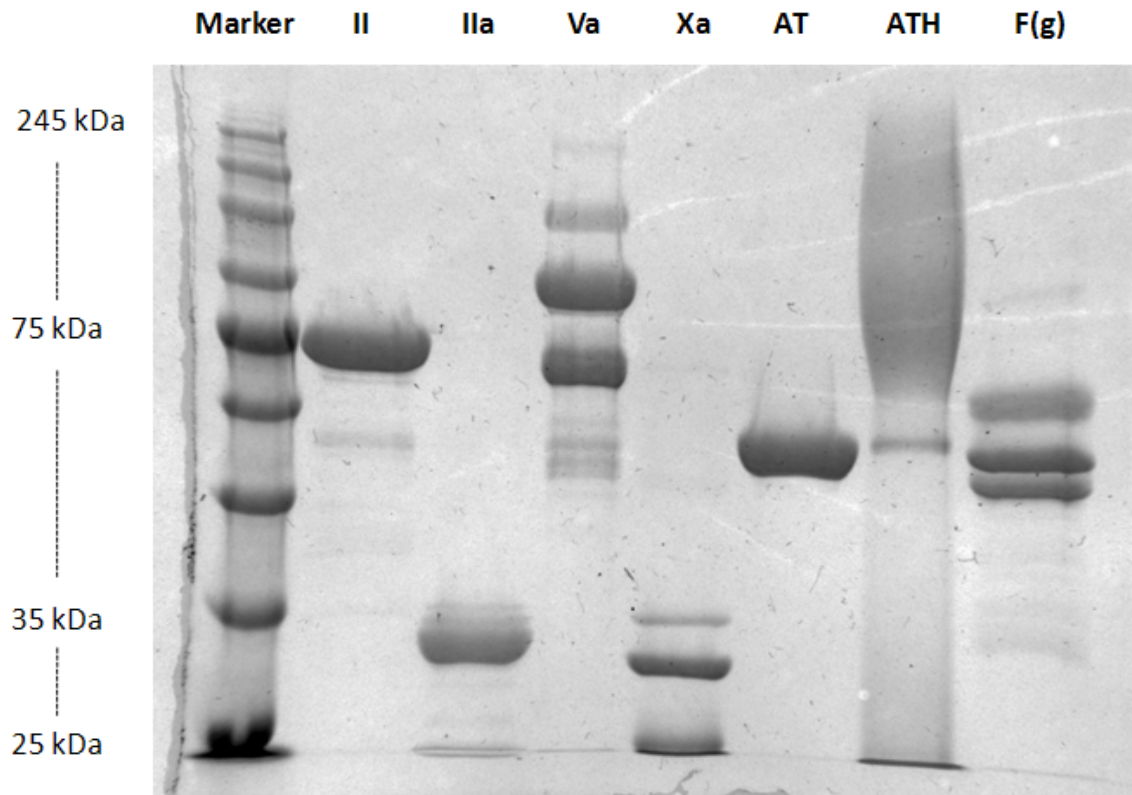
For the fluorescence experiments, the methodology used for preparation of synthetic phospholipid vesicles is often employed for developing small vesicles that are very difficult to pick up with a high powered light/fluorescent microscope. Thus, in the future, protocols for large vesicles should be used instead. Also, the experimenter should practice sanitary lab practices to protect from contaminations.

Chapter 6: Appendices

6.1 Appendix A:

Procedure for SDS-PAGE for detection of protein purity:

The BioRad Mini Protean 3 system was used to perform the gel experiments. Briefly, the BioRad system was set up as per the manufacturer's instructions. A 7.52% separating gel (1.875 mL of 30% acrylamide, 1.875 mL of 1.5 M Tris-HCl (pH 8.8), 3.7 mL of H₂O, 5 µL of TEMED, 21.25 µL of 0.1 g/mL (NH₄)₂S₂O₈) with a 3.99% stacking gel (0.65 mL of 30% acrylamide, 1.25 mL of 1.5 M Tris-HCl (pH 8.8), 2.95 mL of H₂O, 7.5 µL of TEMED, 25 µL of 0.1 g/mL (NH₄)₂S₂O₈) were prepared. The gel (between plates) was then inserted into the apparatus and filled with running buffer (30.3 g/L Tris + 14.4 g/L Glycine + 0.1% (w/v) sodium dodecyl sulphate (SDS)). Twenty µL volume containing 20 µg of protein (prothrombin, thrombin, Va, Xa, antithrombin, ATH or fibrinogen) were mixed with 10 µL of sample buffer containing SDS and β-mercaptoethanol in a microfuge tube, boiled in water for 3 min and then cooled at room temp. The microfuge tubes were centrifuged for 1 min at full speed before loading 30 µL (e.g. all of the boiled sample) of the solution on 7 wells of the gel. In the first lane, 10 µL of the molecular weight markers was loaded for reference. The gel was run at 200 V for 30 min. After running the gel, it was fixed for 1 hour with 40% methanol, 10% acetic, 50% H₂O (Destain A), stained for 30 minutes with 0.1% coomassie blue in Destain A, then destained with Destain D (10% acetic acid (no methanol)). The stained gel was dried between two permeable clear membranes and the bands were analyzed using the BioRad Gel Doc system.



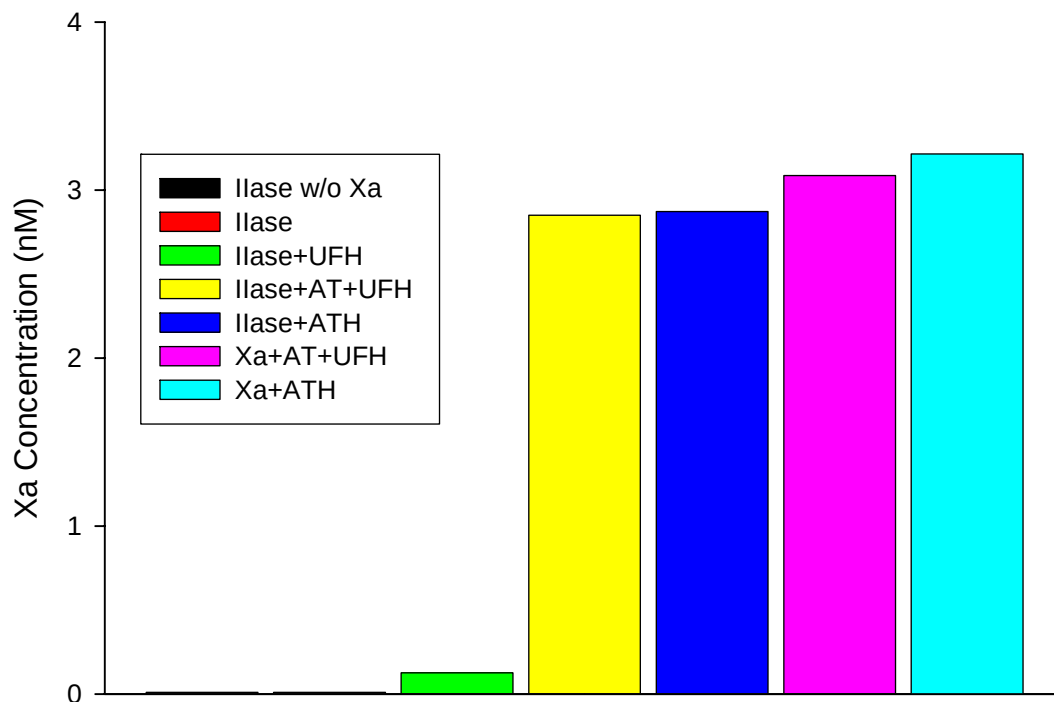
Appendix Figure A: Protein purity gel. SDS-PAGE under reducing conditions and stained with Coomassie Blue was performed to determine purity of proteins used in this study. All of the proteins are >95% pure, except for Va (>89% pure). Va contains two subunits, the A1/A2 subunit + A3/C1/C2 subunit thus appears as two bands. ATH appears as a smear because of the various covalently linked heparin chain lengths. Fibrinogen (F(g)) contains the α -/ β -/ γ -fibrinogen and appears as three bands.

6.2 Appendix B

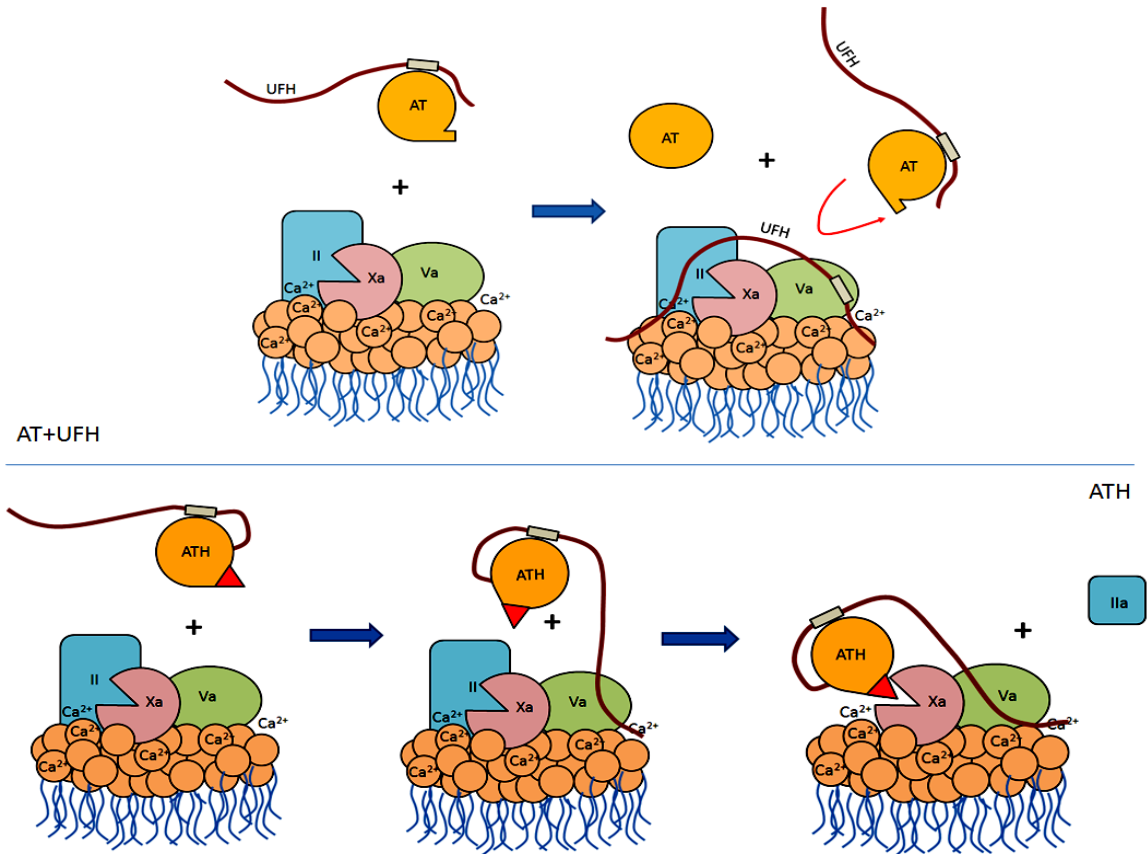
Procedure for Xa:AT ELISA:

Elisa kits (factor X and TAT) were purchased from Affinity Biologicals (Ancaster, ON). The capture antibody was from the X Elisa and the detection antibody was from the TAT Elisa. The Elisa was performed as per manufacturer's instructions, and the overall Elisa integrated materials from both kits. A 96-well flat bottom microtiter plate was coated with the capture antibody. Briefly, the capture antibody was diluted 1/100 in coating buffer (50 mM carbonate buffer, pH=9.6), and 100 μ L was immediately added to the experimental wells of the plate. The plate was incubated between 2-8 °C overnight. After overnight incubation, the contents of the plate were discarded and 150 μ L of blocking solution (2% BSA-PBS (w/v), pH=7.4) was added to each well and incubated at 22 °C for 60 min. The plates were then washed 3 X with wash buffer (PBS-Tween (0.1% v/v), pH=7.4). 100 μ L of the sample mixtures were added to each well. Briefly, sample mixture contained 50 μ L of sample diluent buffer (HBS-BSA-Tween20, pH=7.2) + 50 μ L of prothrombinase reactions (final concentrations: 3nM Xa, 9.6 nM Va, 1×10^6 /mL activated platelets, 4 mM Ca^{2+}) or free Xa + 4 mM Ca^{2+} in the presence or absence of 30 nM AT+300 nM UFH or 30 nM ATH. The standards (containing final concentrations of Xa: 0, 1.5, 3, 6 and 12 nM) were all loaded to separate wells in the presence of sample diluents buffer as above. The reactions were allowed to incubate for 2 hours at room temp, followed by washing 3X with wash buffer. The detecting antibody was diluted 1/100 with the conjugate diluent (provided by the TAT Elisa kit), then 100 μ L was applied to each well and incubated for 1 hour at 22 °C, then the plate washed 3X

with wash buffer. 100 μL of freshly made substrate (5 mg of o-Phenylenediamine-2HCl (Sigma # P-6912) + 12 mL of Citrate-Phosphate Buffer + 12 μL of 30% H_2O_2) was added to all wells. The reaction was allowed to incubate at room temp from 30 sec to 15 min, depending on rate of colour change. Observe closely, as colour change may be rapid. Once colour was generated in the wells, 50 μL of 2.5 M H_2SO_4 was added to each well, and read plate at 490 nm wavelength. The values for the samples and standard curve were obtained and the results determined.

ELISA Results:

Appendix Figure B: ELISA for detection of Xa:AT complexes. Xa:AT complexes were detected in conditions containing either free Xa or prothrombinase (Ilase)-based Xa + inhibitors. For the conditions with no inhibitors (Ilase w/o Xa or Ilase) no Xa:AT complexes were detected. In the Ilase+UFH experiment, some of the UFH may have formed ternary complexes with Ilase and antibodies used in the ELISA. The final concentrations of Xa used in the experiments were approximately 3 nM, and for AT was 30 nM. Based on these results, appropriate concentration of Xa was detected.

Appendix C:

Appendix Figure C: Summary figure of prothrombinase inhibition by AT+UFH versus ATH. In Panel **A**, the UFH can dissociate from AT and form ternary complexes with the prothrombinase complex, which leaves AT in the inactive state and unavailable to inhibit Xa. The UFH:prothrombinase complexes may also repel any other incoming AT+UFH molecules from inhibiting Xa. Panel **B** illustrates the mechanism of IIase inhibition by ATH, depicting the central role of the covalent bond in disallowing the UFH to dissociate from AT. This will reduce the low specificity binding of free UFH to prothrombinase components resulting in enhanced Xa inhibition.

6.4 Appendix D

7.4.1 Published/accepted articles:

- 1) **Stevic I.**, Berry L.R., Chan A.K.C. Mechanism of inhibition of the prothrombinase complex by a covalent antithrombin-heparin complex. *J Biochem.* 2012; 152: 139-148.
- 2) Peters S.J., Devries M.C., Samjoo I.A., **Stevic I.**, Robertshaw H.A., Tarnopolsky M.A. Lipid droplet proteins in human skeletal muscle: the effect of sex, obesity, and training. *Appl Physiol Nutr Met.* 2012; 37:724-735.
- 3) **Stevic I.**, Parmar N., Paredes N., Berry L.R., and Chan A.K.C. Binding of heparin to metals. *Cell Biochem Biophys.* 2011; 59: 171-178.
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- 6) **Stevic, I.**, Chan, H.H.W., Berry, L.R. and Chan A.K.C. Inhibition of the prothrombinase complex on red blood cells by heparin and covalent antithrombin-heparin complex. Accepted in *J Biochem* MS#: JB-2012-07-0242.

7.4.2 Submitted articles:

- 7) **Stevic, I.**, Chan, H.H.W., Chander, A., Berry, L.R. and Chan A.K.C. Covalently linking heparin to antithrombin enhances inhibition of prothrombinase on activated platelets. MS under review
- 8) **Stevic I.**, Kozenko M.A., LoStracco R., Chan A.K.C, Chan H.H.W. Clinical presentation for a novel mutation affecting a conserved Cysteine residue in exon-63 of Fibrillin-1 (Cys2633Arg). MS under review.

7.4.3 Published abstracts:

- 1) **Stevic, I.**, Chan, H.H.W., Chander, A., Berry, L.R. and Chan A.K.C. Inhibition of platelet-prothrombinase by a covalent antithrombin-heparin complex. *Blood* 2012; 120 (21): 2212

- 2) Gantioqui, J., **Stevic, I.**, Kim, P.Y., Lau, K.K., Chan, A.K.C., Chan, H.H.W. An *in-vitro* model using thromboelastography to evaluate the effects of anticoagulants on clot formation in plasma enriched with autologous platelets. *Blood* 2012; 120 (21): 1091
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7.4.4 Awards:

- 1) 2009-2013 Canadian Blood Services Graduate Fellowship Award (\$88,000)
- 2) 2012 Annual Child Health Research Day: Award for Best Poster Presentation (\$50)

- 3) 2012 Faculty of Health Sciences Graduate Program Excellence Award (certificate)
- 4) 2012 Medical Sciences Research Day Outstanding Achievement Award (certificate)
- 5) 2011 Canadian Cardiovascular Society: Have A Heart Award (all expenses paid to attend the 2011 Canadian Cardiovascular Congress, Vancouver, BC + \$200)
- 6) 2011 ISTH Young Investigator Award (\$500)
- 7) 2011 McMaster GSA Student Travel Award (\$500)
- 8) 2011 McMaster Child Health Research Day: Award for Best Oral Presentation (\$100)
- 9) 2011 Faculty of Health Sciences Research Plenary: Award for Best Poster Presentation (trophy)
- 10) 2009 Thrombosis Interest Group of Canada-Bayer: Thrombosis Research Award (\$3,000)
- 11) 2009 American Society of Hematology Abstract Achievement Award (\$500)
- 12) 2008 Keith Leppmann Teaching Award, McMaster University (\$200)
- 13) 2007 Hamilton's Inaugural Health Research in the City Award (trophy)
- 14) 2007 Canadian Obesity Network Student Research-Travel Award (\$500 and plaque)
- 15) McMaster University Graduate School Entrance Scholarship (\$3,500)
- 16) University of Guelph' Entrance Scholarship (\$2,500)