DOAC-STOP REVERSAL OF FACTOR XIa INHIBITORS

EVALUATION OF DOAC-STOP TO REVERSE THE EFFECTS OF FACTOR XIa INHIBITORS ON THE ACTIVATED PARTIAL THROMBOPLASTIN TIME

By ELLA K. FITZPATRICK, B.M.Sc.

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AUTHOR:	Ella K. Fitzpatrick, B.M.Sc. (Western University)
SUPERVISOR:	Dr. Jeffrey I. Weitz, OC, M.D., FRCPC, FACP
SUPERVISORY COMMITTEE:	Dr. Bernardo Trigatti, B.Sc., Ph.D. Dr. Peter L. Gross, M.D., M.Sc., FRCPC
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LAY ABSTRACT

Blood clots that block blood vessels are a major cause of death. Anticoagulants are drugs that prevent and treat blood clots but can cause bleeding. New anticoagulants are being developed that target factor (F) XI, a component in blood, because they may be equally effective but cause less bleeding. Clotting assays test how the blood clots and help diagnose blood disorders. Anticoagulants block clotting and therefore interfere with these test results. An activated charcoal-based product called DOAC-Stop™ (DS) is designed to remove drugs from blood samples.

The goal of this thesis was to determine whether DS can reverse the effects of the FXI(a) inhibitors asundexian, milvexian, and abelacimab. We demonstrate that DS reverses the effects of asundexian and milvexian but not abelacimab. Therefore, DS is useful to reverse the effects of asundexian and milvexian on critical medical tests.

ABSTRACT

Factor (F) XI is a serine protease involved in the intrinsic pathway of coagulation. This protein is an emerging target for anticoagulation due to its presumed minor role in hemostasis, but major role in thrombosis. FXI and activated FXI (FXIa) inhibitors are promising alternatives to traditional anticoagulants like vitamin K antagonists and heparins, and newer therapies like direct oral anticoagulants (DOACs), which target thrombin or FXa.

Coagulation tests, like the activated partial thromboplastin time (APTT), are critical for evaluating the clotting pathways. Notably, the APTT is used to diagnose antiphospholipid syndrome (APS), which is an autoimmune disease that increases the risk of thrombosis. Since DOACs selectively inhibit coagulation, they prolong the APTT and can potentially mimic patterns characteristic of an underlying coagulation disorder like APS. As a consequence, in patients receiving DOAC therapy, it becomes challenging to distinguish between the inhibitory effects of DOACs and indicators of a pathological condition.

Current strategies to address DOAC interference have significant disadvantages. DOAC-Stop™ (DS) is an activated charcoal-based compound that adsorbs molecules such as DOACs. Studies show that DS reverses the effects of dabigatran, apixaban, edoxaban, and rivaroxaban on the APTT. However, it remains unknown whether DS is capable of reversing the effects of new FXI(a) inhibitors.

This thesis aims to determine whether DS reverses the effects of asundexian, milvexian, and abelacimab on the APTT. We first establish that the FXI(a) inhibitors prolong the APTT. We then show that DS reverses the effects of asundexian and milvexian on the APTT, but not abelacimab. Additionally, we show that DS distinguishes between the effects of milvexian and

heparin. These findings suggest that DS may serve as a potential method to reverse the effects of asundexian and milvexian on the APTT and to distinguish their effects from that of heparin.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	Apple
ABE	Anion-binding site
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Atrial fibrillation
ANOVA	Analysis of Variance
APC	Activated protein C
APS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
Arg	Arginine
ASO	Antisense oligonucleotide
AT	Antithrombin
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CD	Catalytic domain
Cys	Cysteine
Da	Dalton
DNA	Deoxyribonucleic acid
DOAC	Direct oral anticoagulant
DS	DOAC-Stop™
DS-L	DOAC-Stop™-Liquid

DVT	Deep vein thrombosis
EPRC	Endothelial protein C receptor
F	Factor
GP	Glycoprotein
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HB	HEPES buffer
HK	High-molecular-weight kininogen
Ig	Immunoglobulin
Ile	Isoleucine
INR	International Normalized Ratio
K_d	Dissociation constant
K_i	Inhibition constant
LMWH	Low-molecular-weight heparin
PAGE	Polyacrylamide gel electrophoresis
PE	Pulmonary embolism
PK	Prekallikrein
PT	Prothrombin time
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor

TM	Thrombomodulin
t-PA	Tissue-type plasminogen activator
UFH	Unfractionated heparin
u-PA	Urokinase plasminogen activator
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
vWF	von Willebrand Factor

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Ella K. Fitzpatrick, declare that all the experiments detailed in this thesis were conducted independently by myself under the supervision of Dr. Jeffrey I. Weitz. No part of this thesis has been submitted or published for the award of any other degree.

My supervisor, Dr. Jeffrey I. Weitz, and the members of my supervisory committee, Dr. Bernardo Trigatti and Dr. Peter L. Gross, provided guidance and support throughout all stages of this project.

All requirements for successful completion of this degree prior to the defence of this thesis have been met.

CHAPTER 1 - INTRODUCTION

1.1 OVERVIEW OF HEMOSTASIS

Hemostasis describes a complex, tightly regulated balance between pro- and anticoagulant processes that operates under a basal state in the absence of injury, and ultimately leads to the termination of bleeding upon vessel injury (1). This process depends on the interactions between endothelial cells, platelets, and coagulation proteins that (a) maintain blood in a fluid state within the intact endothelium in the absence of vascular injury; (b) allow for the appropriate cessation of bleeding to prevent hemorrhage after vascular injury; and (c) resolve blood clots after successful repair of the vasculature. Therefore, defects in hemostasis can manifest as thrombosis or bleeding. Thrombosis represents a pathological process where the body forms excess or abnormal clots within the intravascular space. In a hypocoagulable state, the body cannot form effective clots, resulting in excessive bleeding. Both thrombosis and bleeding represent leading causes of morbidity and mortality (1,2). Hemostasis is generally defined by three phases.

1.1.2 Primary, Secondary and Tertiary Hemostasis

Primary hemostasis occurs shortly after vascular wall damage to create the initial platelet plug (1). This process involves vasoconstriction and platelet adhesion, activation, and aggregation. Vasoconstriction occurs not only to decrease blood flow to prevent further blood loss, but also to allow for platelets to begin adhering to the site of injury. Platelet adhesion occurs when the disruption of the intact endothelium exposes the blood to various adhesive proteins, such as collagen and von Willebrand factor (vWF). Platelets interact with these proteins through constitutively expressed platelet surface receptors that bind collagen such as α2β1 and glycoprotein (GP) VI, and those that bind vWF, such as GPIbα and GPIIb/IIIa. Under low shear conditions, such as in venous circulation, collagen alone can facilitate platelet adhesion and activation at the

site of injury. In contrast, under high shear conditions like in the arterial circulation, collagen and vWF function together to promote efficient platelet adhesion and activation. Here, vWF acts as the molecular glue that tethers platelets to the damaged endothelium. Once platelets adhere to the exposed proteins residing in the matrix, they undergo activation. Platelet activation involves morphological changes and release of granule contents including platelet agonists like thromboxane A2 and adenosine diphosphate (ADP), and coagulation proteins. Thromboxane A2 and ADP stimulate vasoconstriction, and once bound to their respective receptors on platelets, lead to further platelet activation and recruitment to the injury site. Activated platelets provide an anionic phospholipid surface onto which coagulation proteins can form complexes that enhance coagulation protein activation, thrombin generation, and ultimately fibrin formation. This burst of thrombin also enhances platelet recruitment and activation, causing further platelet expansion around the site of injury to effectively seal or plug the damaged vasculature. Platelet aggregation is the final step of primary hemostasis and involves platelet-to-platelet linkages mediated by GPIIb/IIIa on activated platelets. Platelet activation is critical for platelet aggregation due to the conformational activation of GPIIb/IIIa, which enhances its affinity for fibrinogen and vWF. Fibrinogen and vWF then interact with conformationally activated GPIIb/IIIa to bridge adjacent platelets together resulting in clumps of platelets around the site of injury.

Secondary hemostasis involves activation of the coagulation cascade which results in thrombin generation and subsequent conversion of fibrinogen to fibrin (1). During this phase, small amounts of thrombin generated by primary hemostasis activate platelets and non-enzyme cofactors. Here, activated platelets promote the coagulation cascade by providing an anionic phospholipid surface for coagulation protein assembly, and they enhance fibrin formation and

stabilization by releasing coagulation factors (F) V, XI, XIII and fibrinogen. Therefore, secondary hemostasis stabilizes the initial platelet plug produced from primary hemostasis.

Tertiary hemostasis, or fibrinolysis, involves breakdown of the fibrin clot to restore a patent vascular system as part of tissue repair (1,3). The fibrinolytic system revolves around plasminogen, and its active form, plasmin, which digests fibrin (1). The two activators of plasminogen are tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) (1,3). t-PA is synthesized by vascular endothelial cells as a single-chain polypeptide and once converted to its active form by plasmin, it mediates fibrin degradation by cleaving plasminogen to create plasmin (1). Similar to t-PA, u-PA is synthesized as a single-chain polypeptide, but is predominantly produced by inflammatory and renal epithelial cells (1,3). When bound to its cell surface receptor, plasmin converts single-chain u-PA to its active form to which it can cleave plasminogen to plasmin. Once plasmin is produced, it cleaves fibrinogen to produce soluble fibrin degradation products (1). Together, u-PA and t-PA promote fibrin degradation. Therefore, hemostasis involves three phases that facilitate (i) the cessation of bleeding through formation of an initial platelet plug; (ii) reinforcement of the initial plug through activation of the coagulation cascade; and (iii) resolution of the plug to restore the vasculature once repair is complete. The coagulation cascade is key to the diagnosis and treatment of thrombosis.

1.2 OVERVIEW OF COAGULATION

1.2.1 Coagulation Cascade

In 1957, two separate pathways, named the extrinsic and intrinsic pathways of coagulation were proposed (4). Following this discovery, two research groups proposed that these pathways occurred as a series of stepwise proteolytic cleavages of procoagulant zymogens (5,6). This led to the development of the “cascade” or “waterfall” model of coagulation.

1.2.1.1 Extrinsic Pathway

The extrinsic pathway is activated upon damage to endothelial vessel walls and requires a transmembrane glycoprotein called tissue factor (TF) (7,8). TF is constitutively expressed by specific extravascular cells, like fibroblasts, and cells within the vessel wall, like smooth muscle cells. Endothelial cells and leukocytes also express TF under pathological or inflammatory conditions. As such, TF forms a "hemostatic envelope" surrounding highly vascularized tissues and is readily available as a potent initiator of coagulation.

In this pathway, disruption to the endothelial cell wall exposes blood components to TF in the subendothelial tissue (7,8). Once exposed, TF forms a complex with FVII in the presence of calcium ions (Ca^{2+}) on the phospholipid surface of activated platelets, which facilitates the activation of FVII to its active form, FVIIa (Figure 1.1). The resultant TF-FVIIa complex, termed the extrinsic tenase, activates FX to FXa in the presence of Ca^{2+}. The TF-FVIIa complex also contributes to thrombin production through the intrinsic pathway by facilitating the conversion of FIX to FIXa.

1.2.1.2 Intrinsic and Contact Pathway

The intrinsic pathway is activated when blood encounters negatively charged activator molecules like polyanions and denatured proteins (5,6). This pathway involves the serine proteases, FXI, FIX, and the cofactor, FVIII (Figure 1.1). Feeding into the intrinsic pathway of coagulation exists a smaller pathway called the contact system of coagulation (9). In the contact system, when prekallikrein (PK) or FXII are exposed to negatively charged activator molecules, they undergo conformational changes which results in autoactivation to produce kallikrein and FXIIa, respectively. Kallikrein activates FXII to FXIIa, which can in turn activate more PK to kallikrein, creating a positive feedback loop. FXIIa also converts FXI to FXIa, which propagates the remainder of the intrinsic pathway (5,6). Here, high-molecular-weight kininogen (HK)

functions as a cofactor for activation of FXII and PK. FXIa then activates FIX to FIXa using Ca^{2+} as a cofactor. As a final step of the intrinsic pathway, FIXa associates with its cofactor, FVIIIa, to create the intrinsic tenase complex, which, in the presence of Ca^{2+}, activates FX to FXa on the phospholipid surface of activated platelets.

1.2.1.3 Common Pathway

The extrinsic and intrinsic pathways of coagulation converge at the production of FXa (5,7). Here, thrombin generated by the extrinsic pathway and FXa activate FV to FVa in the presence of Ca^{2+} on the phospholipid surface of activated platelets. Then, FXa and its cofactor FVa associate to form the prothrombinase complex and convert prothrombin to thrombin. Thrombin then cleaves soluble fibrinogen to create insoluble fibrin. The production of fibrin accelerates FXIII activation by thrombin to produce FXIIIa in the presence of Ca^{2+}. As a final step of the coagulation cascade, FXIIIa stabilizes the initial hemostatic plug in a calcium-dependent manner by forming covalent bonds between fibrin molecules to create a cross-linked fibrin meshwork. FXIIIa also cross-links small amounts of the fibrinolytic inhibitor, α2-antiplasmin, to the fibrin meshwork. The resulting fibrin meshwork is capable of withstanding physical strain and is resistant to premature fibrinolysis.

Positive feedback loops within the coagulation cascade are crucial for clot formation as the initial levels of thrombin produced by the extrinsic and intrinsic pathways are generally insufficient for clot formation alone (7). Once thrombin is generated during the initiation phase, it can further activate platelets and feed back to the coagulation cascade where it activates FXI, FVIII, and FV. Feedback activation of coagulation factors by thrombin thereby propagates the coagulation cascade and results in amplified thrombin generation.

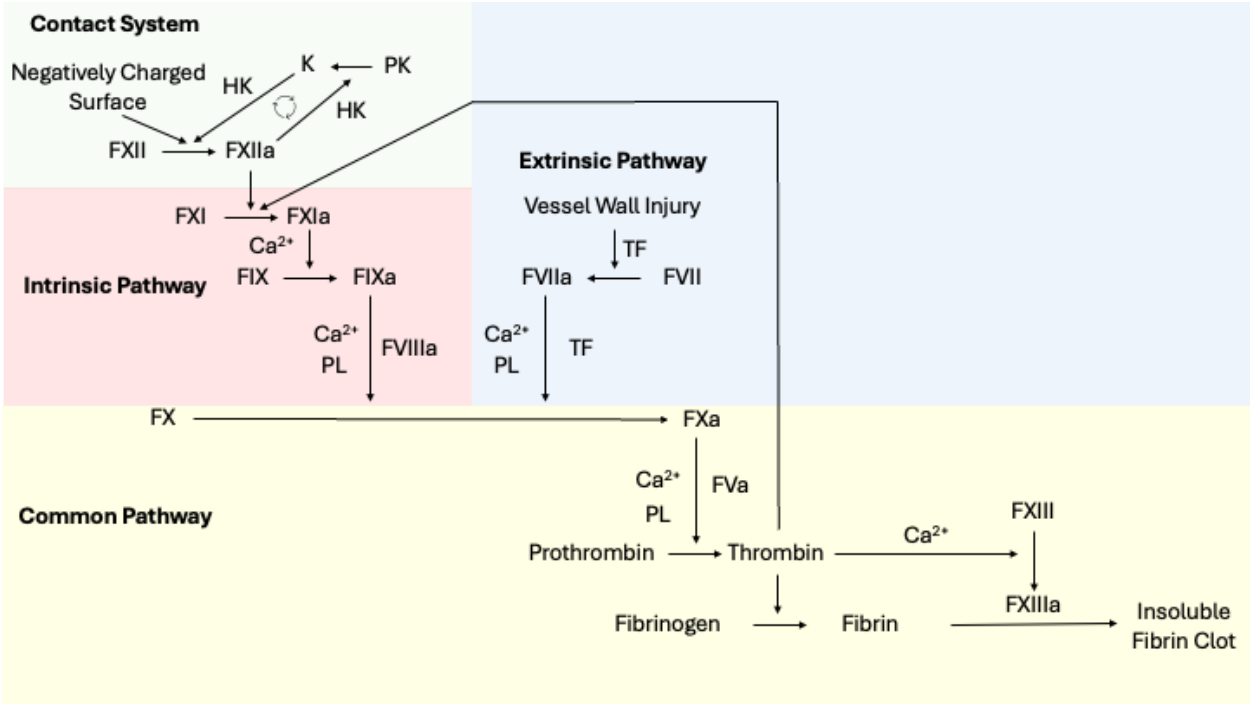


Figure 1.1: Coagulation cascade.

The intrinsic and extrinsic pathways are initiated by contact between blood and negatively charged activator molecules and vessel wall injury, respectively. The intrinsic and extrinsic pathways merge at the common pathway of coagulation which involves the activation of factor (F) X to its activated (a) form, FXa. The final product is the formation of a stable fibrin clot. PK = prekallikrein, K = kallikrein, HK = high-molecular-weight kininogen, TF = tissue factor, PL = phospholipid. This figure was adapted from Davie EW, Ratnoff OD Waterfall Sequence for Intrinsic Blood Clotting. Science. 1964;145:1310–2; Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. Nature. 1964;202:498–9; Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. Biochemistry. 1991;30:10363–70; and Mackman N, Tilley RE, Key NS. Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27:1687–93.

1.2.2 Cell-Based Model of Coagulation

The cell-based model of coagulation offers a more detailed view on coagulation by providing cellular context of where coagulation occurs (10,11). This model shifts the focus from the coagulation proteins as regulators of coagulation, to the idea that coagulation is largely influenced by cellular components. Additionally, this model proposes that coagulation functions as distinct, but overlapping, phases that incorporate the cascade model: initiation, amplification, and propagation (Figure 1.2). These phases conclude with a termination phase that acts to regulate coagulation and maintain hemostasis.

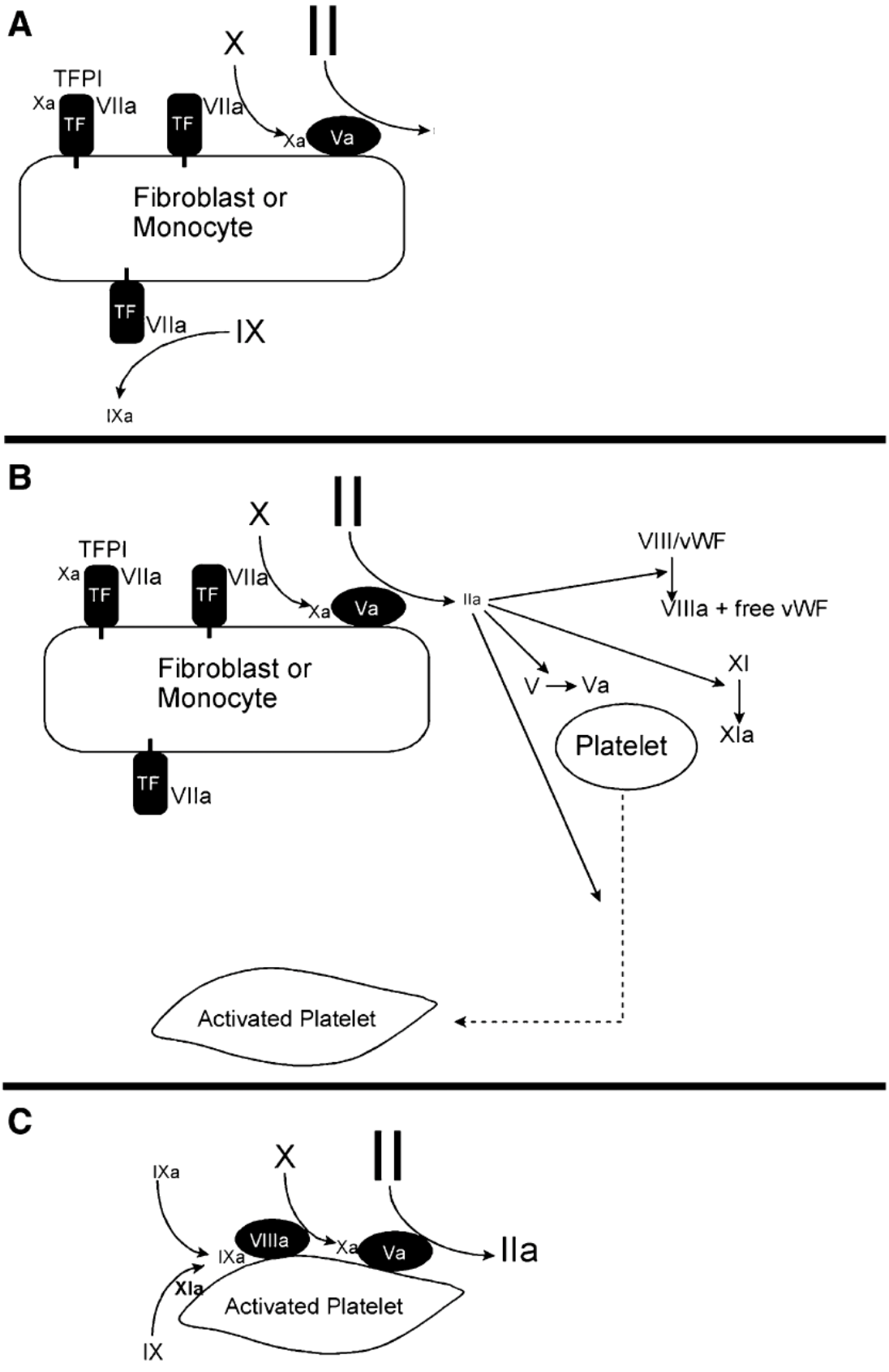
Initiation occurs on cells that express TF, like fibroblasts and epithelial cells, and begins in response to vessel wall injury (10,11). The TF-FVIIa complex formed on TF-bearing cells creates the extrinsic tenase that initiates coagulation through activation of FIX and FX. Once FXa is produced, the prothrombinase complex can then form and produce trace amounts of thrombin. The levels of FXa and thrombin during this phase are insufficient for stable thrombus formation but are critical to stimulate amplification.

Amplification involves platelet activation and coagulation protein generation on the TF-bearing cell (10,11). This phase sets the stage for the generation of large amounts of thrombin by boosting levels of active coagulation factors through positive feedback loops. Thrombin produced by the prothrombinase complex on or near TF-bearing cells contributes to platelet activation and recruitment to the thrombus site. Simultaneously, thrombin also activates FXI, FVIII, and FV.

Propagation occurs on the surfaces of activated platelets and results in the production of large amounts of thrombin (10,11). Here, activated platelets provide a surface rich with anionic phosphatidylserine which allows for the assembly of coagulation proteins required for the coagulation cascade. During this phase, FXI converts FIX to FIXa, which can then bind to platelet-

bound FVIIIa. During this phase, the intrinsic tenase and prothrombinase complexes are formed on the surface of activated platelets which result in the generation of large amounts of FXa and thrombin, respectively. Thrombin then cleaves fibrinogen to produce fibrin monomers which can be covalently cross-linked in the presence of FXIIIa to create an insoluble fibrin clot.

Termination occurs after fibrin clot formation and functions to prevent excessive coagulation and to localize clotting reactions to the site of injury (10–12). Regulation of clotting is achieved through the natural anticoagulant pathways which is mainly supported by the vascular endothelium.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Xa
Va
IIa
VIII/vWF
VIIIa + free vWF
XI
XIa
V
Va
Platelet
Fibroblast or
Monocyte
TF
VIIa
Activated Platelet
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IXa
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VIIIa
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Xa
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IIa
XIa
Activated Platelet
IX

Figure 1.2: Cell-based model of coagulation.

The cell-based model involves three distinct, but interdependent phases: initiation, amplification and propagation. (A) Initiation occurs on tissue factor (TF)-bearing cells such as fibroblasts or monocytes and begins shortly after vessel injury. Injury exposes the blood to TF, and results in low levels of thrombin generation. (B) Amplification involves platelet activation and boosts levels of thrombin through activation of the coagulation cascade. (C) Propagation occurs on the surface of activated platelets and involves the generation of large amounts of thrombin which results in the production of a stable fibrin clot. This figure is from Hoffman M, Monroe D. Coagulation 2006: a modern view of hemostasis. Hematol Oncol Clin North Am 2007;21:1-11.

1.2.3 Anticoagulant Mechanisms

Within the vasculature, several players exist to serve as natural anticoagulants to regulate coagulation. The most common physiological anticoagulants include antithrombin (AT), tissue factor pathway inhibitor (TFPI), and the protein C pathway.

1.2.3.1 Antithrombin

AT is plasma glycoprotein produced by the liver that belongs to the serine protease inhibitor (serpin) family (13,14). Thus, AT inhibits its targets by irreversibly binding to the catalytic site of coagulation proteins. AT exerts inhibitory effects on both the intrinsic and extrinsic pathways of coagulation, with its primary targets being thrombin and FXa. In addition, AT also inactivates other coagulation proteins such as kallikrein, FXIIa, FXIa, FIXa, and FVIIa. Alone, AT has relatively low reactivity with the coagulation proteins and therefore depends on the presence of endogenous glycosaminoglycans like heparan sulfate to enhance its catalytic efficiency. Since heparan sulfate is found on endothelial cells, it provides an antithrombotic shield within the vasculature. To enhance the interaction of AT with thrombin, the glycosaminoglycan must bind simultaneously to both AT and thrombin to create a bridge between them. In contrast, binding interactions between the glycosaminoglycan and FXa are not required for FXa inhibition as the interaction between AT and glycosaminoglycans induces a conformational change that exposes its protease-interactive exosite, which is otherwise hidden in a cryptic conformation. The ability of glycosaminoglycans to act as catalytic cofactors to enhance the inhibitory activity of AT forms the basis of anticoagulation therapy targeted towards AT. Specifically, heparin, which is a glycosaminoglycan derived from mast cells, is indispensable for effective treatment and prevention strategies for thrombosis.

1.2.3.2 Tissue Factor Pathway Inhibitor

TFPI is a Kunitz-type protease inhibitor that is constitutively expressed by endothelial cells and activated platelets (15,16). TFPI primarily inhibits the extrinsic and common pathways of coagulation, as it targets the TF-FVIIa complex and the FXa-FVa complex. Inhibition of the TF-FVIIa complex involves simultaneous, reversible binding between TFPI and FXa and high affinity binding between TFPI and FVIIa. Protein S acts as a cofactor in the TFPI and FXa interaction by bringing TFPI closer to the phospholipid surface of activated platelets where it can more readily interact with FXa. TFPI also inhibits the prothrombinase complex by binding to FVa to render it in an inactive procofactor conformation.

1.2.3.3 Protein C Pathway

The protein C pathway plays a critical role in controlling clot formation in response to thrombin generation (17,18). The main players of this pathway include thrombin, thrombomodulin (TM), the endothelial protein C receptor (EPCR), protein C, and protein S. This pathway results in the enzymatic degradation of FVa and FVIIIa, thereby downregulating coagulation. As levels of thrombin rise, it binds to its membrane-bound cofactor, TM. At the same time, circulating zymogen protein C binds to its receptor, EPCR. The formed thrombin-TM complex then activates protein C bound to EPCR to produce activated protein C (APC). APC can then dissociate from EPCR and inactivate FVa and FVIIIa by proteolytic cleavage on the surface of activated platelets. Inactivation of FVa by APC requires protein S as a cofactor. As a downstream effect, inactivation of FVa by APC inhibits its ability to act as a cofactor for FXa. In contrast, inactivation of FVIIIa involves both protein S and FV (19). Protein S serves to bring APC in close proximity to the phospholipid surface where it can more easily interact with FVa or FVIIIa. FV cleavage enhances the ability of APC to inactivate FVIIIa.

The importance of the protein C pathway is highlighted in patients with FV Leiden or APC resistance (18). The FV Leiden mutation results in an impaired protein C pathway and is the most prevalent hereditary risk factor for VTE. Specifically, the FV variant negatively impacts FVa degradation as it confers resistance of FVa to inactivation by APC. Furthermore, this variant renders FV a poor cofactor to APC for FVIIIa proteolysis, thereby impairing FVIIIa inactivation.

1.3 HEMOSTATIC DYSFUNCTION

When the tightly regulated mechanisms that maintain hemostasis are disrupted, it can quickly lead to hemostatic dysfunction, resulting in either excess bleeding or clotting.

1.3.1 Bleeding

Bleeding occurs when there is failure to seal or repair vascular damage due to improper formation of hemostatic plugs or premature breakdown of the plugs (1). Bleeding disorders can result from dysfunction in primary, secondary or tertiary hemostasis. Primary hemostasis involves initial platelet plug formation in response to injury. Thus, disorders of primary hemostasis involve either inherited or acquired defects in platelet levels, platelet functionality, or in the vessel itself. An example of a primary hemostatic disorder is von Willebrand disease, which involves defective or low levels of vWF (20). Secondary hemostasis involves reinforcement of the initial platelet plug through activation of the coagulation system and production of thrombin and fibrin (1). Disorders of secondary hemostasis therefore involve inherited or acquired deficiencies of coagulation factors. Examples of secondary hemostatic disorders include hemophilia A and B, which are deficiencies of FVIII and FIX, respectively (21). Tertiary hemostasis involves fibrinolysis which facilitates the breakdown of the clot formed during primary and secondary hemostasis as part of tissue repair (1). Therefore, disorders of tertiary hemostasis result from defects in any of the fibrinolytic factors, like fibrinogen, and can result in premature degradation of hemostatic plugs.

Although disorders of primary, secondary, and tertiary hemostasis disrupt distinct stages of hemostasis, they all can result in bleeding.

1.3.2 Thrombosis

Thrombosis is defined as excessive formation of blood clots or thrombi within the arterial or venous circulation (1). Arterial thrombi are established at the site of disrupted or ruptured atherosclerotic plaques. Upon disruption or rupture of the plaque, thrombogenic material contained within the plaques, like TF, vWF, and collagen, is exposed to the blood. Exposure of the blood to thrombogenic material then triggers platelet aggregation and fibrin formation which can lead to occlusion. Arterial thrombosis is a common underlying cause of acute coronary syndrome (ACS) or myocardial infarction and ischemic stroke, which result from coronary artery and cerebral occlusion, respectively. Furthermore, thrombi formed within the left atrial appendage in association with atrial fibrillation (AF) can dislodge or embolize and travel to the brain to cause ischemic stroke. Venous thrombi are commonly associated with deep vein thrombosis (DVT) where thrombi form in avascular valve cusps of deep veins. A potentially fatal complication occurs when these thrombi embolize and travel to the lungs causing pulmonary embolism (PE). Collectively, DVT and PE are termed venous thromboembolism (VTE) (22).

Understanding the pathogenesis and underlying causes of thrombosis are crucial for developing and improving strategies for treatment and prevention. Three key interrelated factors contributing to thrombosis are defined by Virchow's triad: (i) endothelial damage or dysfunction; (ii) hypercoagulability; and (iii) reduced blood flow and stasis (22,23). Endothelial damage contributes to thrombosis by triggering inflammatory pathways and exposing blood proteins to surrounding tissues. It can be caused by inflammation, as seen in atherosclerosis, trauma, surgery, and by localized physical trauma. A hypercoagulable state increases the risk of thrombosis,

specifically VTE, due to changes in the hemostatic system. Acquired hypercoagulability can result from obesity, increased age, medication, trauma, and chronic inflammatory conditions. Inherited hypercoagulability includes deficiencies in natural anticoagulants such as AT, protein C, and protein S. Reduced blood flow and stasis contribute to thrombosis as sluggish blood flow can lead to local hypoxia, which in turn contributes to endothelial dysfunction. Reduction in blood flow is commonly associated with paralysis and long-haul travel where limb movement is limited.

Despite the advancements in therapeutic treatment options, thrombosis still remains one of the leading causes of mortality worldwide (2). Specifically, thromboembolic conditions account for 1 in 4 deaths. Given the significant global burden of thrombosis, there is a critical need for the continued development of both antithrombotic or antiplatelet therapies, in addition to anticoagulation therapies for prevention and treatment strategies.

1.4 TRADITIONAL ANTICOAGULANTS

Anticoagulant therapy is the mainstay for treatment and prevention of many conditions such as VTE, and works by antagonizing secondary hemostatic pathways (24). These anticoagulants include heparins, vitamin K antagonists (VKAs), and direct oral anticoagulants (DOACs).

1.4.1 Heparins and Vitamin K Antagonists

In the early 20th century, heparin was one of the first drugs utilized for VTE prevention (25–27). Unfractionated heparin (UFH) is a large, heterogenous preparation of glycosaminoglycans around 15 000 Daltons (Da) that inhibits coagulation by enhancing the efficacy of the natural inhibitor, AT. Treatment using UFH has several pharmacokinetic, pharmacodynamic, and physiological disadvantages. Once delivered via subcutaneous or intravenous administration, UFH has a short half-life of around 1-1.5 hours and there is limited

drug absorption if UFH is given subcutaneously (26). In addition, UFH treatment comes with a high associated risk of bleeding. Furthermore, due to its large molecular weight and composition, it is usually administered by intravenous infusion, which requires hospitalization. Low molecular weight heparins (LMWHs), including enoxaparin, were developed in the 1970s to produce fewer adverse side effects. In contrast to UFH, LMWH can be used to treat VTE out of hospital due to fixed subcutaneous dosing (28). In contrast to UFH, LMWHs have a mean molecular weight around 5000 Da and have a longer half-life of around 5 hours. Although LMWH confers a lower risk of bleeding compared with UFH, bleeding is still a major side effect.

VKAs, like warfarin, bind to the active site of vitamin K epoxide reductase (VKOR), which is an enzyme required for vitamin K recycling and maintenance of functional vitamin K levels the body (29). When VKAs bind to the active site of VKOR it prevents the enzyme from recycling vitamin K, thereby decreasing vitamin K levels. Decreased vitamin K levels impair a vitamin K-dependent process called gamma (γ)-carboxylation. Notably, γ-carboxylation of glutamate residues is required for calcium-dependent conformational changes that facilitate binding interactions between specific coagulation proteins and cofactors on phospholipid surfaces. As a result, VKAs impact vitamin K-dependent coagulation factors such as FVIII, FIX, FX, prothrombin, protein C, and protein S. Warfarin is a small molecule of around 300 Da (30) that is orally administered, and has a drug half-life of around 20-60 hours (24). Unfortunately, VKAs still present significant challenges with the most important being that VKAs have a high associated risk of bleeding. Further, VKA efficacy has a delayed onset of action of around 3-7 days, and its dosage and administration must be carefully monitored to ensure its effect is maintained within therapeutic range.

Despite their disadvantages and the need to develop improved anticoagulants, heparins and VKAs were highly successful in the treatment and prevention of thrombosis and continue to be used.

1.4.2 Small Molecule Inhibitors: Direct Oral Anticoagulants

In the past 15 years, development of reversible small molecule inhibitors, like DOACs, has shifted towards cardiovascular pathologies to address the clinical demand to treat conditions such as thrombosis (31). Compounds falling under this class are typically between 500 and 1000 Da and function to directly target thrombin, such as dabigatran (627.7 Da (32)), or FXa, such as rivaroxaban (435.9 Da (33)), apixaban (459.5 Da (34)), and edoxaban (548.1 Da (35)), with high selectivity and affinity (24). As a result, administration of direct thrombin or FXa inhibitors allows for specific modulation of coagulation. Due to their small size, they are often administered orally to which they are adsorbed in the gastrointestinal tract to elicit a rapid onset of action within the blood. DOACs serve as a safer treatment option for thrombosis compared with heparins and warfarin as they offer many advantages such as fixed-dose oral administration, rapid mode of action, more predictable pharmaco- dynamics and -kinetics, and reduced risk of bleeding (24). The introduction of DOACs therefore revolutionized anticoagulant treatment and prevention strategies. Despite their increasing popularity for thrombosis treatment and prophylaxis, because DOACs target thrombin and FXa, they still pose the risk of serious bleeding including gastrointestinal, intracranial, and systemic bleeding (36), especially in patients with cancer-associated thrombosis (37). As such, researchers and clinicians continue to express the need for new therapies that reach or surpass the antithrombotic efficacy of existing anticoagulant therapies with reduced or no risk of hemorrhage.

1.5 INTRINSIC PATHWAY AS A NOVEL THERAPEUTIC TARGET

Two components of the intrinsic pathway of coagulation, FXII and FXI, are emerging targets for anticoagulation due to their proposed dispensable roles in hemostasis, but major roles in thrombosis (38,39). As such, targeting FXII and FXI may address the unmet needs in anticoagulant therapy by preventing and treating thrombosis without impacting hemostasis. This idea stems from the differential processes of hemostasis and thrombosis. Hemostasis is mainly an extravascular event which depends on the TF-FVIIa complex and excludes FXII activation (40). Additionally, thrombin-mediated activation of FXI is seldom required for primary and secondary hemostasis. In contrast, thrombosis is an intravascular event and is believed to depend on FXIa as a driver of thrombus expansion beyond the site of injury. The role of FXII and FXI in hemostasis and thrombosis is supported by many epidemiological and pharmacological studies, where both coagulation proteins provide different merits of interest.

1.5.1 Factor XII

FXII initiates the contact pathway of coagulation, and is activated when blood comes into contact with negatively charged activator molecules like naturally occurring polyanions such as DNA and RNA, or medical devices such as catheters or mechanical heart valves (39).

The minor role of FXII in hemostasis is observed both in mice and humans where FXII deficiency is not associated with abnormal bleeding (41,42). The role of FXII in thrombosis is well-studied in rodent and primate models. In FXII-knockout mice, there is a reduction in clot formation compared with wild-type mice, and an overall protection from ischemic stroke (41). Similarly, FXII inhibition using monoclonal antibodies reduces clot growth in a thrombosis model using baboons (43). However, one potential caveat to FXII as a target is the weak correlation between FXII levels and thrombosis in humans (42).

1.5.2 Factor XI

FXI activation is achieved by both FXIIa and thrombin, thus playing a role in both the intrinsic/contact pathway of coagulation, and propagation of thrombin generation.

The minor role of FXI in hemostasis is highlighted in patients, particularly of Ashkenazi Jewish descent, who are FXI-deficient (hemophilia C) as they have a mild bleeding diathesis compared with patients with deficiencies of FVIII (hemophilia A) or FIX (hemophilia B) (44,45).

Unlike FXII, many studies support the link between FXI levels and thrombosis. In humans, the Leiden Thrombophilia Study concluded that patients with elevated FXI antigen concentrations have higher risks of thrombosis compared with controls (46). This study was followed by reports confirming a correlation between FXI plasma levels and incidences of VTE, myocardial infarction, and ischemic stroke, and a reduced frequency of VTE and ischemic stroke in those with FXI deficiency (47). In supporting these findings, The Longitudinal Investigation of Thromboembolism Etiology indicated elevated FXI levels are associated with an increased risk of developing VTE (48). In a mouse thrombosis model, FXI is essential for thrombus formation following injury (49). Furthermore, studies with thrombosis models in baboons highlight that when FXI is inhibited using either a FXI-directed antibody or antisense oligonucleotides (ASOs), clot growth is reduced (50,51). A clinical trial in humans (NCT01713361) (52) demonstrated the clinical benefit of FXI ASOs as reduction in FXI levels by ASO administration prevents VTE in patients undergoing knee arthroplasty (53). Similar benefit was also observed with FXI-directed antibodies, like abelacimab (EudraCT Number 2019-003756-37) (54) and osocimab (NCT03276143) (55), and the small molecule inhibitor milvexian (NCT03891524) (56) where administration of these agents is effective at preventing VTE and has minimal risk of bleeding (57–59).

Collectively, FXI has been the primary focus for anticoagulant development compared with FXII due to the stronger supporting data revolving its role hemostasis and thrombosis, and inability to bypass inhibition.

1.5.2.1 Factor XI Structure

FXI is a homodimer composed of 80 kDa subunits and each subunit contains four apple (A) domains and a single typsin-like catalytic domain (CD) (Figure 1.3) (60,61). Each A domain has designated ligand binding sites where A1 binds to thrombin, A2 binds to HK, A3 binds to FIX, heparin, and Gp1bα, and A4 has the Cys321 residue that forms the FXI dimer interface (60).

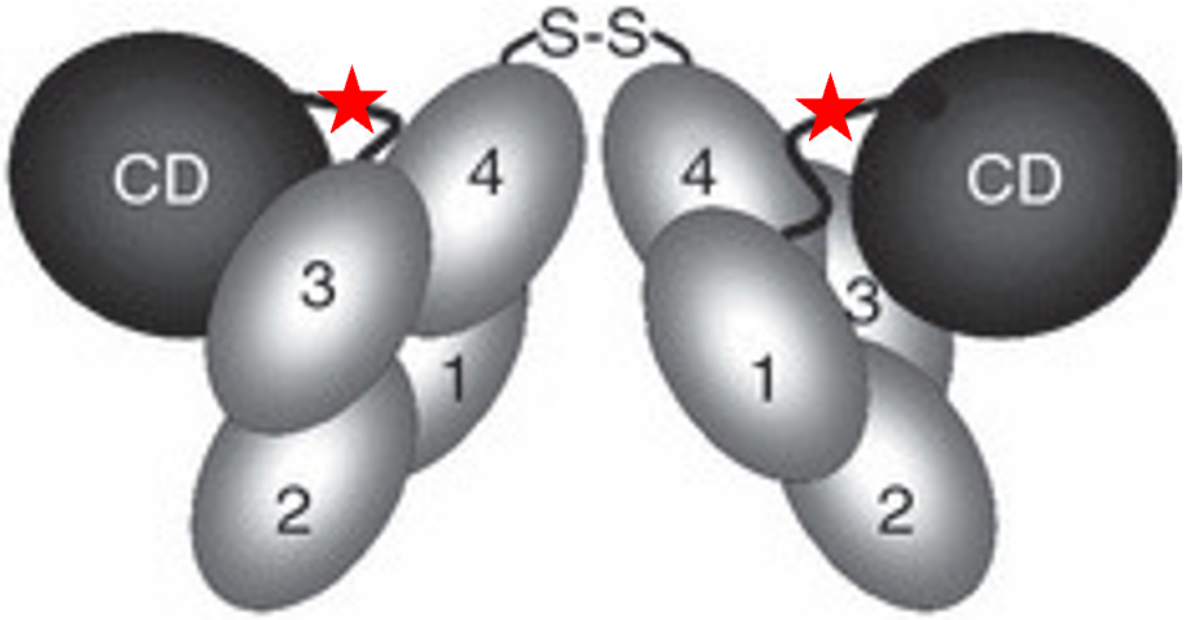

Figure 1.3: Homodimeric structure of human FXI.

Factor XI is composed of two identical dimer subunits containing four A domains (1-4) and one catalytic domain (CD) joined by disulfide bonds (S). The red stars indicate the activation site between where cleavage at Arg369-Ile370 between A domain 4 and CD generates FXIa. This figure was adapted from Gailani D, Smith SB. Structural and functional features of factor XI. J Thromb Haemost. 2009;7:75-8.

1.5.2.2 FXI Production, Activation, and Function

Once produced by the liver, FXI circulates in plasma at around 30 nM and is almost entirely bound to the glycoprotein HK (61). FXI activation occurs when the Arg369-Ile370 bond, found between the A4 and CD domains, is cleaved on each homodimer by either FXIIa, thrombin, or via autoactivation by additional FXIa in the presence of polyanions (60–62). Activation of FXI by thrombin involves binding interaction between thrombin's electropositive anion-binding sites (ABE I and ABE II) and FXI's A1 domain (60,62). This interaction is enhanced in the presence of polyanionic substances like dextran sulfate or heparin, as well as physiological molecules like DNA or RNA, which act to bridge FXI with thrombin (62). FIX activation by FXIa occurs when FIX binds to the FXIa A3 domain, followed by proteolytic cleavage (61).

1.5.2.3 Factor XI(a) Inhibitors

Current FXI(a) inhibitors in clinical trials include ASOs, monoclonal antibodies and small molecule inhibitors. For this thesis, only monoclonal antibodies and small molecule inhibitors will be discussed.

1.5.2.3.1 Monoclonal Antibodies

Monoclonal antibodies are large heterodimeric proteins of around 150 kDa used as a therapeutic intervention for various clinical indications (63). Three humanized monoclonal antibodies used in clinical trials to inhibit FXI are abelacimab (MAA868), osocimab (BAY1213790), and xisomab, now known as gruticibart (AB023). Abelacimab, which is a human immunoglobulin G1 (IgG1) antibody, binds to the catalytic domain of both FXI and FXIa. Osocimab is another IgG1 antibody that specifically targets FXIa and neutralizes its activity by binding close to its active site. In contrast, xisomab is a recombinant humanized antibody that binds to the A2 domain of FXI, and therefore prevents FXI activation mediated by FXIIa, but does not prevent other pathways of FXI activation such as by thrombin, which binds to the A1 domain

(62). These antibodies are administered via subcutaneous or intravenous injection, have high specificity for their target, and have a rapid onset of action if given intravenously and a slow offset of action. For this thesis, only abelacimab will be discussed in depth as it is the only monoclonal antibody currently undergoing Phase 3 evaluation.

Abelacimab has high affinity for the catalytic domain of human FXI (dissociation constant (K_d) = 1.3 pM) and FXIa (K_d = 4.7 pM) (64). When abelacimab binds to FXI or FXIa, it induces conformational changes which render both proteases in an inactivate zymogen conformation. Once delivered via subcutaneous administration, the mean terminal elimination half-life ranges from 25-30 days (65).

Clinical evaluation of abelacimab began in 2021 and is currently still undergoing evaluation in ongoing trials (66). In 2021, a Phase 2 evaluation (EudraCT 2019-003756-37) (54) compared abelacimab with enoxaparin for prevention of VTE in patients undergoing total knee arthroplasty, and found that 75 and 150 mg dose regimes of abelacimab were superior to the 40 mg daily subcutaneous dose of enoxaparin (57). Following the initial investigation, abelacimab was evaluated in healthy patients (ANT-003) and in patients with AF (ANT-004) to which favourable safety profiles were established with intravenous and subcutaneous dose regimes (65). The AZALEA TIMI 71 trial (NCT04755283) (67) compared abelacimab with rivaroxaban in patients with AF at moderate-to-high risk of stroke and concluded that abelacimab reduces free levels of FXI and is associated with fewer bleeding events compared with rivaroxaban (68). The LILAC TIMI 76 trial (NCT05712200) (69) compares abelacimab with placebo in patients with AF considered ineligible for currently available oral anticoagulants and involves either a monthly 150 mg subcutaneous dose of abelacimab or placebo (70). In contrast, the ASTER and MAGNOLIA trials involve patients with cancer-associated VTE. The ASTER trial (NCT05171049) (71)

compares abelacimab with apixaban in patients eligible for a DOAC (72), while the MAGNOLIA trial (NCT05171075) (73) compares abelacimab with dalteparin in those with gastrointestinal or genitourinary cancer at high risk for bleeding (74).

1.5.2.3.2 Small Molecule FXIa Inhibitors

Small molecule FXIa inhibitors function similarly to DOACs in terms of their mechanism of action (66). As a result, these inhibitors prevent FXIa activity. Examples of FXIa small molecule inhibitors include asundexian (BAY 3433334), milvexian (JNJ70033093, BMS-986177), BMS-986209, BMS-962212, ONO-7864, and SHR2285. When administered, these inhibitors produce dose-related suppression of FXIa activity (66). For this thesis, only asundexian and milvexian, which bind to the active site of FXIa, will be discussed in depth as these two agents are the only two currently undergoing Phase 3 evaluation (Figure 1.4).

Asundexian (529.9 Da (75)) has high selectivity for human FXIa and has an inhibition constant (K_i) of 0.14 nM (76). Milvexian (626.4 Da (77)) has a K_i of 0.11 nM (78). In contrast to ASOs and monoclonal antibodies, milvexian and asundexian are orally administered, have a more rapid onset of action of around 2-4 hours, and a half-life of around 8-14 hours (66). In this thesis, only the ongoing Phase 3 clinical trials are outlined below, with the exception of one trial included for its particular significance.

In the OCEANIC stroke trial (NCT05686070) (79), asundexian is being compared with a placebo on top of standard antiplatelet therapy to prevent recurrent stroke in patients who have experienced a non-cardioembolic acute ischemic stroke or high-risk transient ischemic attack. The OCEANIC AF trial (NCT05643573) (80) compared asundexian with apixaban for stroke and systemic embolism prevention in patients with AF at high risk of stroke. While asundexian was associated with fewer major bleeding events as compared with apixaban, this Phase 2 trial was

terminated early due to inferior efficacy of asundexian in preventing stroke or systemic embolism (81). This finding suggests the 50 mg once daily dose of asundexian is insufficient for FXIa inhibition required to prevent stroke and systemic embolism, and is unlikely due to the overall inability of asundexian to inhibit FXIa. Therefore, the early termination of the OCEANIC AF trial highlights the need for further research to determine the optimal dose of asundexian. In the LIBREXIA stroke trial (NCT05702034), milvexian is being compared with a placebo in the same patient population as the OCEANIC stroke for the prevention of recurrent ischemic stroke. The LIBREXIA ACS trial (NCT05754957) (82) compares milvexian with a placebo in conjunction with dual or single antiplatelet therapy in ACS to determine whether FXIa inhibition with antiplatelet therapy reduces cardiovascular events without compromising hemostasis (83). In contrast, the LIBREXIA AF trial (NCT05757869) (84) compares milvexian with apixaban in AF for stroke and systemic embolism prevention (85). In brief, both asundexian and milvexian are being assessed in multiple Phase 3 trials that are enrolling tens of thousands of individuals over the next few years.

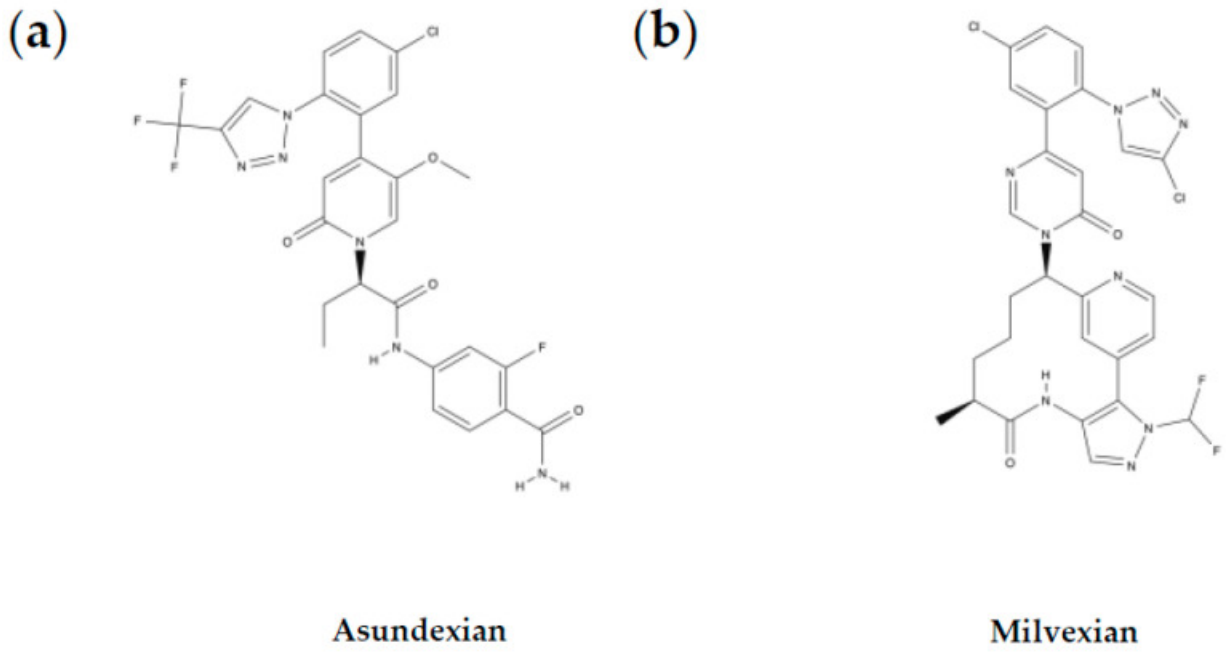

Figure 1.4: Chemical structure of asundexian and milvexian.

Structures of (a) asundexian and (b) milvexian. This figure was retrieved from Buckley GT, Crowley MP, Harte JV. Laboratory Evaluation of Interferences Associated with Factor XIa Inhibitors Asundexian and Milvexian in Routine Coagulation Assays. Diagnostics (Basel). 2024;14:1991.

1.7 COAGULATION TESTS

Coagulation tests are not only used as a cornerstone for testing or "screening" for coagulation factor deficiencies, but also as a tool for anticoagulant monitoring (86), particularly for perioperative procedures (87). For this thesis, the clot-based coagulation tests named the prothrombin time (PT) and activated partial thromboplastin time (APTT) will be focused on.

1.7.1 Prothrombin Time and Activated Partial Thromboplastin Time

The PT test investigates abnormalities in the extrinsic and common pathways and is sensitive to reductions in TF, FVII, FX, FV, prothrombin and fibrinogen (86). The PT is performed by adding thromboplastin, which is a mixture of TF, calcium, and phospholipids to citrated plasma, and measuring the time it takes to form a clot. The PT ranges from 10 to 14 seconds. This test is used to monitor VKA therapy by measuring the international normalized ratio (INR) and ensuring that it remains within the therapeutic range. The INR is calculated by first measuring the PT, which is then divided by the mean normal PT and adjusted based on the sensitivity of the PT reagent for detecting reduction in the vitamin K-dependent clotting factor levels.

The APTT investigates abnormalities in the intrinsic and common pathways and is sensitive in reductions to HK, PK, FXII, FXI, FIX, FVIII, FV, FX, prothrombin and fibrinogen (86). The APTT is performed by adding an artificial contact pathway activator, such as kaolin, silica or ellagic acid, phospholipids, and calcium to citrated plasma, and measuring the time it takes to form a clot. The key differences between the PT and APTT are that an incubation step is required before adding calcium to ensure activation of contact factors, and the phospholipids in the APTT reagent lack TF. The APTT ranges from 22 to 40 seconds. In contrast to the PT, the APTT is used to monitor UFH therapy.

1.8 COAGULATION TEST INTERFERENCE

1.8.1 Interference by Direct Oral Anticoagulants

DOACs are the mainstay of anticoagulant therapy due to their ability to attenuate thrombus formation through directly inhibiting either thrombin or FXa. In contrast with heparin therapy, the APTT is not used for monitoring DOACs. However, administration of these anticoagulants prolongs the APTT in a concentration-dependent manner, with dabigatran, rivaroxaban, and edoxaban eliciting more potent effects on the APTT compared with apixaban (88,89).

Assessment of the anticoagulant effect elicited by DOACs is not routine practice, however it becomes important in certain settings, such as surgery, or for those with critical conditions like impaired renal function (90,91). DOAC monitoring for these circumstances is therefore necessary to primarily assess hemostasis prior to surgery or procedures, but also to determine the appropriate dose regimen to avoid the risk of developing blood clots or uncontrolled bleeding as the therapeutic range for DOACs is not well-established.

The interference elicited by DOACs on the APTT is a major concern when investigating for a prothrombotic disorder called antiphospholipid syndrome (APS) (92,93). APS, characterized by the presence of lupus anticoagulant and antiphospholipid antibodies, is one of the major acquired risk factors for thrombosis. The recommended tool for diagnosing APS is to evaluate for lupus anticoagulant by performing the APTT, where a prolonged APTT is one criterion for APS (92,94). While the APTT is a more sensitive test for diagnosing APS, the PT may also be prolonged. In addition, patients under investigation for APS are often on an anticoagulant regimen. As such, the effect of DOAC therapy could potentially lead to a false positive test for a lupus anticoagulant.

1.8.1.1 Potential Solutions and Their Pitfalls

To eliminate DOAC interference on coagulation assays, several methods have been proposed, including those that require modifications to either patient treatment or laboratory procedures.

Firstly, clinicians may cease anticoagulant therapy prior to testing to avoid performing coagulation assays on plasma containing therapeutic levels of DOACs (95). The first drawback of this method is that discontinuance of anticoagulant therapy imposes an unnecessary risk of thrombosis. Additionally, there is no standardized duration between cessation of treatment and coagulation testing due to variability of DOAC pharmacokinetics and routes of clearance (96). A second approach that has been proposed is to utilize DOAC-specific neutralizing agents. Idarucizumab is a humanized Fab antibody fragment that directly binds to and inactivates dabigatran (97). Originally, idarucizumab was developed for prompt reversal of dabigatran in patients requiring emergency surgery or experiencing life-threatening bleeding, but is also effective at reversing dabigatran when added to plasma samples (98). Unfortunately, idarucizumab is not routinely used in laboratory practice due to its high cost and therefore is limited to specific situations as mentioned above. Andexanet alfa is recombinant protein that mimics the FXa active site and reverses FXa inhibitors such as apixaban, edoxaban, and rivaroxaban (99). Similar to idarucizumab, andexanet alfa was developed for reversal of FXa inhibitors in those with major bleeding (100). Andexanet alfa is not currently approved for laboratory practice due to its high cost and therefore is reserved for the same populations as indicated for idarucizumab. Lastly, ciraparantag is a small molecule currently under development in animal studies for the reversal of heparin and DOACs, but is not currently approved for use in clinical or laboratory practice (101).

A less common approach that has gained traction is the use of DOAC-insensitive assays, such as the taipan snake venom time or ecarin time for lupus anticoagulant testing as an alternative to standardized tests like the APTT (102,103). The taipan snake venom time utilizes venom from the coastal taipan whereas the ecarin time uses venom from the saw-scaled or carpet viper (104). The rationale behind using these tests is that both assays are insensitive to the effects FXa inhibitors as both venoms activate prothrombin directly. The taipan snake venom time test is not influenced by the presence of rivaroxaban in plasma but has not yet been evaluated with the other DOACs. These assays are not used in laboratory settings due to the complexity of the procedure itself and sensitivity towards direct thrombin inhibitors like dabigatran.

Due to their drawbacks, there is an unmet need for a standardized method to reverse the effect of DOACs in laboratory settings. Thus, advancements are required to find a simple way to abolish DOAC interference in assays.

1.8.2 Interference by Factor XI(a) Inhibitors

Like DOACs, FXI(a) inhibitors also impact the coagulation assays. Preliminary studies utilizing human-derived plasma spiked with the direct FXIa inhibitors, asundexian and milvexian, in assays such as the APTT and PT show that they prolong FXIa-dependent assays like the APTT in a concentration-dependent manner (105). In contrast, they have no impact on FXIa-independent assays like the PT. Further, the presence of direct FXIa inhibitors may appear as an apparent FXI deficiency. Thus, the potential for FXI(a) inhibitors as therapeutic agents, coupled with the prolongation they elicit on coagulation tests, highlights the necessity for clinicians to understand not only the effects that FXI(a) inhibitors have on these assays, but also how to interpret test results and reverse the effect they cause on the tests themselves.

1.9 DOAC-STOP™

DOAC-Stop™ (DS) is a commercially available activated charcoal-based compound developed to simply and effectively eliminate DOAC interference on specialized laboratory assays like the APTT (106).

1.9.1 How DS Works

While the exact mechanism by which DS works is not disclosed by the manufacturer, it appears to function like other commercially available activated charcoal-based compounds. In doing so, it works by adhering to anticoagulants which can be removed from plasma samples without interacting with coagulation proteins (107,108).

1.9.1.1 Overview of Activated Charcoal Compounds

Activated carbon or charcoal is a highly porous material made from graphite that has many small, low-volume pores which increase its surface area for adsorption (109). It is composed of multiple layers of carbon arranged in a hexagonal pattern which are held together by the weak van der Waals forces within the carbon-carbon bonds.

Adsorption by activated charcoal depends on pore size distribution and non-covalent interactions. The pore size distribution determines which molecules the activated charcoal can successfully interact with and adsorb as the molecule must fit within the pore to be successfully removed (110). The second factor that determines whether a molecule is a candidate for activated charcoal adsorption is the overall polarity. Adsorption occurs through non-covalent interactions and is reinforced by forces like London dispersion forces (van der Waals forces) and liquid-phase adsorption (109). With these two factors in mind, adsorption by activated charcoal compounds is mainly limited to small, mainly hydrophobic compounds. However, while most activated charcoal compounds only interact with non-polar substances, activated charcoal compounds that have undergone specific activating processes can be utilized to successfully remove small proteins from

aqueous solutions through hydrophobic interactions between the protein and modified functional groups (111).

Due to their adsorptive properties, activated charcoal compounds are often used in medical circumstances such as hemoperfusion (112), and to prevent the absorption of various medications, like aspirin and paracetamol, into the gastrointestinal tract (113,114).

1.9.2 FXI(a) Inhibitors as a Potential Candidate for Reversal by DS

FXI is an emerging target for anticoagulant therapy due to its dispensable role in hemostasis, but critical role in thrombosis (39). As such, targeting FXI addresses the need for new anticoagulants that surpass the efficacy in preventing and treating thrombosis of traditional anticoagulants without imposing a risk of bleeding. Due to the advancements in clinical trial development of FXI(a) inhibitors, it is important to improve our understanding of how the incorporation of these inhibitors into clinical practice impacts the results and interpretation of laboratory coagulation tests. In attenuating thrombosis though inhibiting components of the intrinsic pathway of coagulation, FXI(a) inhibitors, like asundexian, milvexian, and abelacimab, prolong the APTT in a concentration-dependent manner (65,105). As such, patients undergoing treatment with FXI(a) inhibitors may appear as though they have a FXI deficiency. Therefore, treatment using FXI(a) inhibitors may complicate coagulation screening tests which aim to monitor anticoagulant therapy or investigate defects in the coagulation system.

DS is a simple laboratory tool that effectively eliminates DOACs from plasma. When both spiked and patient samples containing DOACs are treated with DS, significant reduction in apixaban, dabigatran, edoxaban, and rivaroxaban concentrations are observed (108,115,116). Additionally, DS is effective in reversing the effect of DOACs on coagulation tests like the APTT

(108,115,116). Further studies also evaluated the efficacy of DS compared with idarucizumab, where DS performs as efficaciously (117).

In 2020, the International Society of Thrombosis and Hemostasis released guidelines which expressed the need for further investigations on DOAC-removal agents, like DS, for lupus anticoagulant screening (118,119). The increasing interest in FXI(a) inhibitors highlights opportunity to evaluate the capacity of DS to reverse these agents. To our knowledge, DS has not yet been tested with asundexian, milvexian, or abelacimab, which begs the question whether DS will perform as efficaciously.

CHAPTER 2 – HYPOTHESIS AND OBJECTIVES

2.1 OVERVIEW

The overall aim of this thesis is to evaluate the efficacy of the commercially available activated charcoal-based compound, DS, to reverse the effects of the FXI(a) inhibitors asundexian, milvexian, and abelacimab on the APTT.

2.2 HYPOTHESIS

Based on previous evaluations of DS as outlined in Chapter 1 and the chemical and physical characteristics of the FXI(a) inhibitors, asundexian, milvexian, and abelacimab, we hypothesize that (a) DS will reverse the effects of asundexian and milvexian on the APTT; (b) DS will not reverse the effect of abelacimab on the APTT; and (c) the liquid formulation of DS, DOAC-Stop™-Liquid (DS-L), will reverse the effect of milvexian on the APTT.

2.3 OBJECTIVES

The specific objectives of this thesis are as follows:

Objective 1: To evaluate the ability of DS to reverse the effects of asundexian and milvexian on the APTT, where abelacimab serves as a negative control.

Objective 2: To evaluate whether DS can distinguish between the effects of milvexian and UFH on the APTT.

Objective 3: To determine the capacity of DS for milvexian reversal.

Objective 4: To evaluate the ability of DS-L to reverse the effect of milvexian on the APTT.

CHAPTER 3 - DOAC-STOP™ REVERSES THE ANTICOAGULANT EFFECT OF ASUNDEXIAN AND MILVEXIAN

Authors: Ella K. Fitzpatrick[1,2], James C. Fredenburgh[1,3], Jeffrey I. Weitz[1-4]*

[1]Thrombosis and Atherosclerosis Research Institute and Hamilton Health Sciences, Hamilton, Ontario, Canada.

[2]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada.

[3]Department of Medicine, McMaster University, Hamilton, Ontario, Canada.

[4]Department of Medical Sciences, McMaster University, Hamilton, Ontario, Canada.

***Corresponding author:** Dr. Jeffrey I. Weitz, Thrombosis and Atherosclerosis Research Institute. 237 Barton St. E., Hamilton, Ontario, Canada L8L 2X2. Email: weitzj@taari.ca.

Foreword: The ability of DOAC-Stop™ to reverse the FXI inhibitors asundexian and milvexian are described in Chapter 3. We demonstrate that DOAC-Stop™ reverses the effect of asundexian and milvexian, but not abelacimab, on the APTT.

Short Title: DOAC-Stop™ reverses asundexian and milvexian

Author Contributions: E.K.F. designed and performed experiments, analyzed data, and wrote the manuscript; and J.C.F. and J.I.W. designed experiments, analyzed data, and wrote the manuscript.

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References for this manuscript have been incorporated into the Bibliography (Chapter 8) at the end of this thesis.

3.1 KEY POINTS

- DS reverses the effects of asundexian and milvexian, but not abelacimab, on the APTT.
- DS distinguishes between the effects of milvexian and UFH.
- One DS tablet is able to reverse up to 10 μM milvexian.

3.2 ABSTRACT

Background: Asundexian and milvexian are small molecule factor (F) XIa inhibitors, whereas abelacimab is an antibody that binds FXI and blocks its activation and activity. All three FXI inhibitors are currently undergoing Phase 3 evaluation. Like heparin and dabigatran, asundexian, milvexian, and abelacimab prolong the activated partial thromboplastin time (APTT) in a concentration-dependent manner. DOAC-Stop™ (DS) is an activated charcoal-based compound that adsorbs direct oral anticoagulants such as dabigatran, but not heparin, from plasma. It is unknown whether DS also neutralizes asundexian and milvexian.

Objective: To determine whether DS reverses the prolongation of the APTT caused by asundexian or milvexian. Dabigatran was used as a positive control, while abelacimab and heparin served as negative controls.

Methods: The APTT in human plasma, with or without asundexian, milvexian, abelacimab, dabigatran, or heparin, was determined before and after DS treatment.

Results: As expected, all drugs produced concentration-dependent prolongation of the APTT. DS returned the APTT to baseline values with asundexian, milvexian, and dabigatran, but not with abelacimab or heparin. The APTT was prolonged in a more than additive manner with the combination of 2.5 μM milvexian and 0.125 U/mL heparin. However, DS only reversed the APTT prolongation induced by milvexian, but not that by heparin.

Conclusion: DS reverses the effect of asundexian and milvexian on the APTT and distinguishes between the APTT effects of milvexian and heparin.

Keywords: anticoagulation, factor XI, factor XI inhibitors, activated charcoal

3.3 INTRODUCTION

Factor (F) XI/XIa inhibitors have the potential to produce less bleeding than currently available direct oral anticoagulants (DOACs) because FXI is essential for thrombosis but mostly dispensable for hemostasis (120). Phase 3 trials are now exploring this possibility. Asundexian and milvexian, small molecule inhibitors of FXIa, are under evaluation in the ongoing OCEANIC and LIBREXIA programs (66). Abelacimab, an antibody that binds FXI and blocks its activation and activity (57), also is being evaluated (68).

In the OCEANIC stroke trial (NCT05686070), asundexian is being compared with a placebo in addition to standard antiplatelet therapy in non-cardioembolic acute ischemic stroke or high-risk transient ischemic attack (79). Milvexian is being compared with a placebo in the same patient population in the LIBREXIA Stroke trial (NCT05702034) (121). In the LIBREXIA ACS trial (NCT05754957), milvexian is being compared with a placebo in conjunction with dual or single antiplatelet therapy in acute coronary syndrome (ACS) (82). In contrast, the LIBREXIA AF trial (NCT05757869) compares milvexian with apixaban in atrial fibrillation (AF) (84). The LILAC TIMI 76 trial (NCT05712200) compares abelacimab with a placebo in patients with AF considered ineligible for currently available anticoagulants (69). In contrast, the ASTER trial (NCT05171049) compares abelacimab with apixaban in patients with cancer-associated venous thromboembolism deemed suitable for direct oral anticoagulant (DOAC) therapy (71), while the MAGNOLIA trial (NCT05171075) compares abelacimab with dalteparin in those deemed

unsuitable for DOAC therapy (73). Therefore, FXI inhibitors are being evaluated in trials that will enroll over 70,000 participants.

FXI is positioned in the intrinsic pathway of coagulation (120). Consequently, all FXI or FXIa inhibitors prolong the activated partial thromboplastin time (APTT) (105). Among the currently available DOACs, dabigatran, which inhibits thrombin, produces the greatest prolongation of the activated partial thromboplastin time (APTT). DOAC-Stop™ (DS) is an activated charcoal compound developed to reverse the anticoagulant effects of DOACs such as dabigatran in plasma. We hypothesized that DS would reverse the anticoagulant effect of asundexian and milvexian but not abelacimab due to their structural characteristics. We also postulated that DS could distinguish between the impact of milvexian and heparin on the APTT, which may be helpful in patients undergoing percutaneous coronary interventions in the LIBREXIA ACS or AF trials. To test these hypotheses, milvexian, asundexian, or abelacimab was added to plasma, and the APTT was determined with and without DS treatment. Dabigatran was used as a positive control, and studies were also performed with milvexian and heparin alone and in combination.

3.4 METHODS

3.4.1 Materials

Dabigatran, the active form of dabigatran etexilate, was a gift from Dr. J van Ryn (Boehringer Ingelheim, Biberach, Germany). Unfractionated heparin (UFH) was purchased from Sigma-Aldrich (St. Louis, MI). Asundexian, which was purchased from MedChemExpress (Monmouth Junction, NJ), exhibited over 99% purity by high performance liquid chromatography analysis. Milvexian was provided by Dr. M. Chintala of Janssen Research and Development (Spring House, PA). Dr. Dan Bloomfield, Anthos Therapeutics, Inc. (Cambridge, MA) provided

abelacimab. DOAC-Stop™ (Haematex Research, Hornsby, Australia) was generously provided by Jennifer J. Kiblinger (DiaPharma Group, Inc., West Chester, OH). Costar clear, round-bottom 96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). Liquid HemosIL® APTT-SP was purchased from Instrumentation Laboratory (Bedford, MA).

Dabigatran, asundexian, and milvexian were dissolved to 10 mM in dimethyl sulfoxide and diluted in 20 mM HEPES buffer, pH 7.4 (HB). Abelacimab was diluted in HB, whereas UFH was dissolved to a concentration of 1 mg/mL (180 U/mg) in distilled water before dilution in HB.

3.4.2 Preparation of Plasma

Blood (30 mL) was collected from each of 8 to 10 healthy volunteers into syringes containing 3 mL of 3.2% buffered sodium citrate. The plasma was isolated by two centrifugation steps at 4°C for 10 minutes at 3000 X g, pooled, and stored frozen in aliquots at -80°C (122).

3.4.3 Plasma Treatment with Anticoagulants and DOAC-Stop™

To two 950-µL plasma aliquots, 50 µL of HB or anticoagulant at varying concentrations was added. Dabigatran, UFH, asundexian, milvexian, and abelacimab were added up to 848 nM, 0.7 U/mL, 10 µM, 10 µM, and 100 nM, respectively. Asundexian, milvexian, and abelacimab concentrations were chosen to span and exceed the expected on-treatment concentrations. The expected on-treatment level of asundexian is approximately 1.2 µM with the 50 mg once daily dose (123). For milvexian, the expected on-treatment range is 0.7 µM with the 25 mg twice daily dose and 3 µM with the 100 mg twice daily dose (59). For abelacimab, the expected on-treatment concentration is 0.35 µM with the 150 mg once monthly dose (65). After the addition of a DS tablet to one plasma aliquot, samples were incubated for 5 min at room temperature on a rotator (Adams Nutator, Parsippany, NJ) and then subjected to centrifugation at room temperature for 2 min at 5000 X g using a benchtop microfuge. The supernatants were removed and subjected to a

second centrifugation step under the same conditions. Samples without or with DS treatment were then subjected to APTT determination.

3.4.4 APTT Determination

A diluted APTT was used to enhance the assay's sensitivity to detect low levels of the drugs. To the wells of a 96-well plate, 50-µL plasma aliquots with or without DS treatment, 10 µL of HemosIL APTT-SP (1:10 final dilution), and 30 µL of HB were added. After 10 minutes of incubation at 37°C, clotting was initiated by adding 10 µL of a 260 mM $CaCl_2$ solution to each well, and absorbance was measured at 405 nm using a THERMOmax plate reader (Molecular Devices, Sunnyvale, CA). The clotting time was calculated as the time to achieve the half-maximum increase in absorbance, as determined by the instrument software. Unless otherwise stated, the APTT was measured twice, each in triplicate.

3.4.5 Effect of DS on Samples Containing Milvexian and/or UFH

The APTT was determined as described above in plasma samples containing 2.5 µM milvexian, 0.125 U/mL UFH, or both milvexian and UFH, without and with DS treatment.

3.4.6 Capacity of DS for Milvexian Reversal

To determine the maximum concentration of milvexian that could be adsorbed with DS, milvexian was added to plasma at concentrations up to 100 µM. The APTT was measured in triplicate, both without and with treatment of 1-mL samples with DS.

3.4.7 Statistical Analysis

Data are presented as means ± standard deviations (SD). Dose-response analysis was determined by non-linear regression using SigmaPlot version 11 (Systat Software, San Jose, CA). The significance of the difference between groups ± DS was determined by one- or two-way

analysis of variance (ANOVA) followed by the Tukey comparison test using Prism 10 (GraphPad, Boston, MA). For all analyses, P values < 0.05 were considered statistically significant.

3.5 RESULTS

3.5.1 Effect of DS on the APTT with Dabigatran and Heparin

To confirm that DS neutralizes DOACs, the APTT was measured with or without DS treatment of plasma containing dabigatran. Without DS, dabigatran prolonged the APTT in a concentration-dependent manner (Figure 3.1). In contrast, after DS treatment, the APTT values returned to baseline levels, and the dose-response relationship was abrogated ($P < 0.0001$). These data confirm previous reports that DS reverses the APTT effect of dabigatran (108,115,116,124).

UFH was used as a negative control. Like dabigatran, UFH prolonged the APTT in a concentration-dependent manner. However, in contrast to the findings with dabigatran, DS treatment had no effect on the APTT with UFH (Figure 3.2).

3.5.2 Effect of DS on the APTT with Asundexian, Milvexian, and Abelacimab

Asundexian (Figure 3.3), milvexian (Figure 3.4), and abelacimab (Figure 3.5) prolonged the APTT in a concentration-dependent manner, with milvexian producing greater prolongation than asundexian or abelacimab. DS treatment abrogated the dose-response with asundexian ($P < 0.05$) and milvexian ($P < 0.0001$), but not with abelacimab.

3.5.3 Effect of DS on the APTT with Milvexian and/or UFH

Subsequent experiments examined whether DS selectively neutralizes milvexian in samples containing both milvexian and UFH. Whereas milvexian and UFH both prolonged the APTT, DS only neutralized ($P < 0.0001$) the effect of milvexian (Figure 3.6). When both milvexian and UFH were present, the APTT was prolonged in a greater than additive manner (Table 3.1). With DS, the APTT was significantly reduced ($P < 0.01$) to that obtained with UFH alone ($P =$

NS). Collectively, these results indicate that DS abrogates the anticoagulant effect of milvexian, but not that of UFH, when both are present in plasma.

3.5.4 Capacity of DS to Reverse Milvexian

To evaluate the adsorptive capacity of DS, plasma samples containing up to 100 μM milvexian were treated with one DS tablet. One DS tablet reversed milvexian up to 10 μM, but not higher concentrations (Figure 3.7). While DS did not adsorb milvexian concentrations exceeding 10 μM, it successfully reversed the maximum milvexian concentrations likely to be achieved with the milvexian dose regimens evaluated in the Phase 3 LIBREXIA program (125). Our results show that a DS tablet can readily reverse milvexian concentrations within the therapeutic range.

3.6 DISCUSSION

In this study, we show that (a) DS reverses the effect of asundexian and milvexian on the APTT, but not the effect of abelacimab, (b) DS reversal can be used to distinguish between the combined effects of milvexian and UFH on the APTT, and (c) the maximum adsorptive capacity of a DS tablet for milvexian is 10 μM. Dabigatran and UFH were used as positive and negative controls, respectively, due to their potent effect on the APTT and their characteristics, such as size, molecular weight, and polarity.

In plasma samples containing milvexian and UFH, DS only reversed the effect of milvexian, allowing for a distinction between the effects of the two anticoagulants on the APTT and highlighting the specificity of activated charcoal compounds, such as DS. Distinguishing between the effects of milvexian and UFH on the APTT may be useful in patients receiving both anticoagulants, such as during percutaneous coronary interventions.

In plasma containing milvexian concentrations up to 100 μM, one DS tablet reversed concentrations up to 10 μM. These findings are relevant because plasma milvexian concentrations

of 3 to 5 μM are likely to be achieved with the milvexian dosing regimens being evaluated in the Phase 3 trials (59).

Molecular size and polarity both play essential roles in activated charcoal adsorption, where the pores within the activated charcoal structure must accommodate the adsorbate molecule for successful removal (126). The forces involved in adsorption are hydrophobic or van der Waals forces (126). Both factors explain why DS interacts with and eliminates the effect of small, non-polar anticoagulants, such as asundexian and milvexian, but not large or polar anticoagulants like abelacimab, an antibody, and UFH, a sulfated polysaccharide. The capability of DS to successfully reverse dabigatran, despite its polarity, suggests that molecular weight may be more substantial than polarity in determining the susceptibility of a compound to DS adsorption.

A limitation of this study is that the anticoagulants were added to plasma instead of using samples from patients who had received the various anticoagulants. However, previous reports demonstrated that DS similarly reversed dabigatran in plasma samples to which it was added (108,124) and plasma samples from patients taking dabigatran (115,116).

In conclusion, the results of this study indicate that DOAC-Stop™ reverses the effects that asundexian and milvexian elicit on the APTT and can distinguish between the effects of milvexian and UFH.

3.7 FIGURES AND TABLES

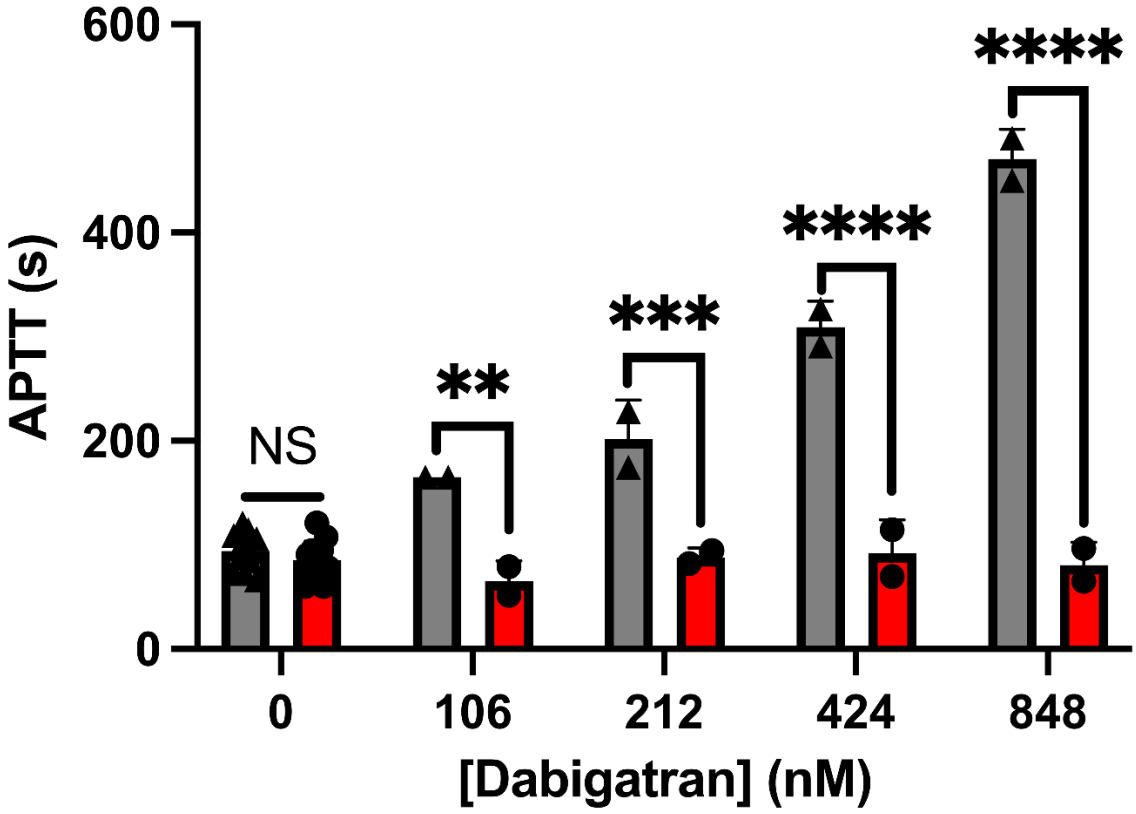

Figure 3.1: DS reverses the effect of dabigatran on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing dabigatran at the indicated concentrations. Bars represent the mean, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical significance annotations indicate pairwise comparison of DS-untreated control samples with DS-treated samples (above pair brackets). NS, not significant compared with the control. There were no significant differences between the DS-treated samples and the control without dabigatran (two-way analysis of variance, Tukey method).

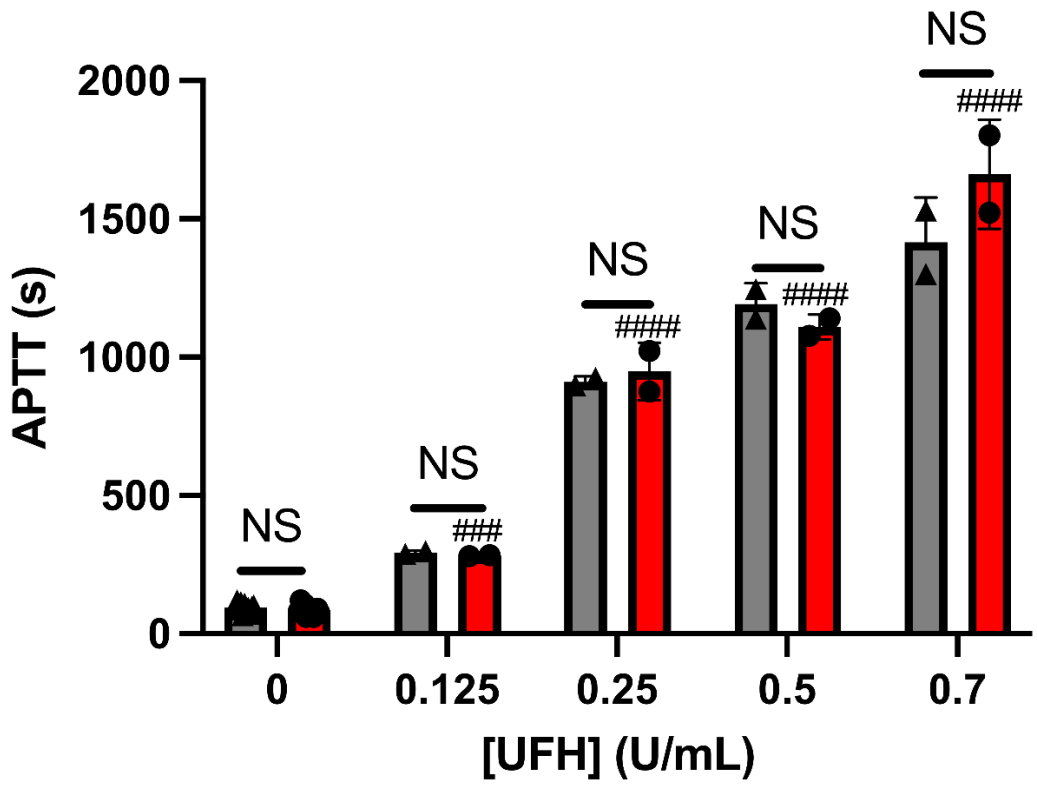

Figure 3.2: DS does not reverse the effect of UFH on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing UFH at the indicated concentrations. Bars represent the means, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. ###P < 0.001, ####P < 0.0001. Statistical significance annotations indicate a pairwise comparison of (a) DS-untreated control samples with DS-treated samples (above the horizontal lines) or (b) a comparison of heparin- and DS-treated samples with the control DS-treated samples lacking heparin (above the red bars). NS, not significant compared with the control (two-way analysis of variance, Tukey method).

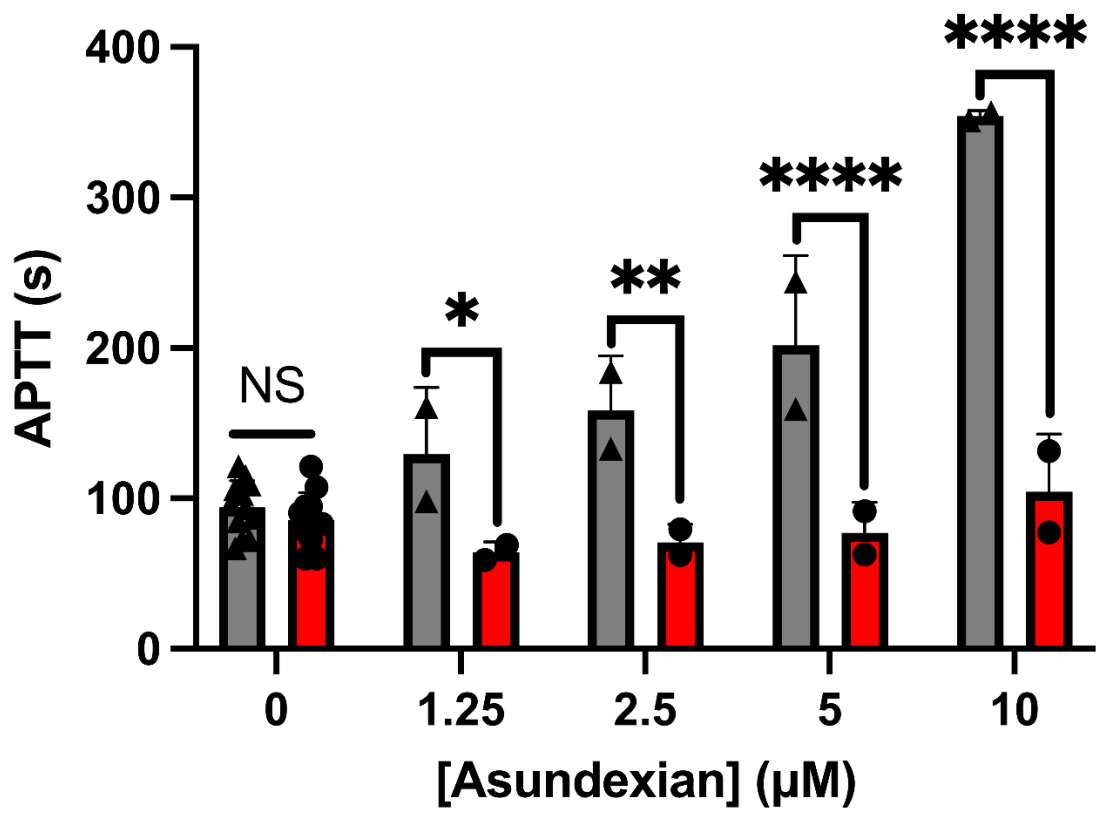

Figure 3.3: DS reverses the effect of asundexian on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing asundexian at the indicated concentrations. Bars represent the means, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. *P < 0.05, **P < 0.01, ****P < 0.0001. Statistical significance annotations indicate (a) pairwise comparison of DS-untreated control samples with DS-treated samples containing asundexian (above pair brackets). NS, not significant compared with the control. There were no significant differences between the DS-treated samples and the control without asundexian (two-way analysis of variance, Tukey method).

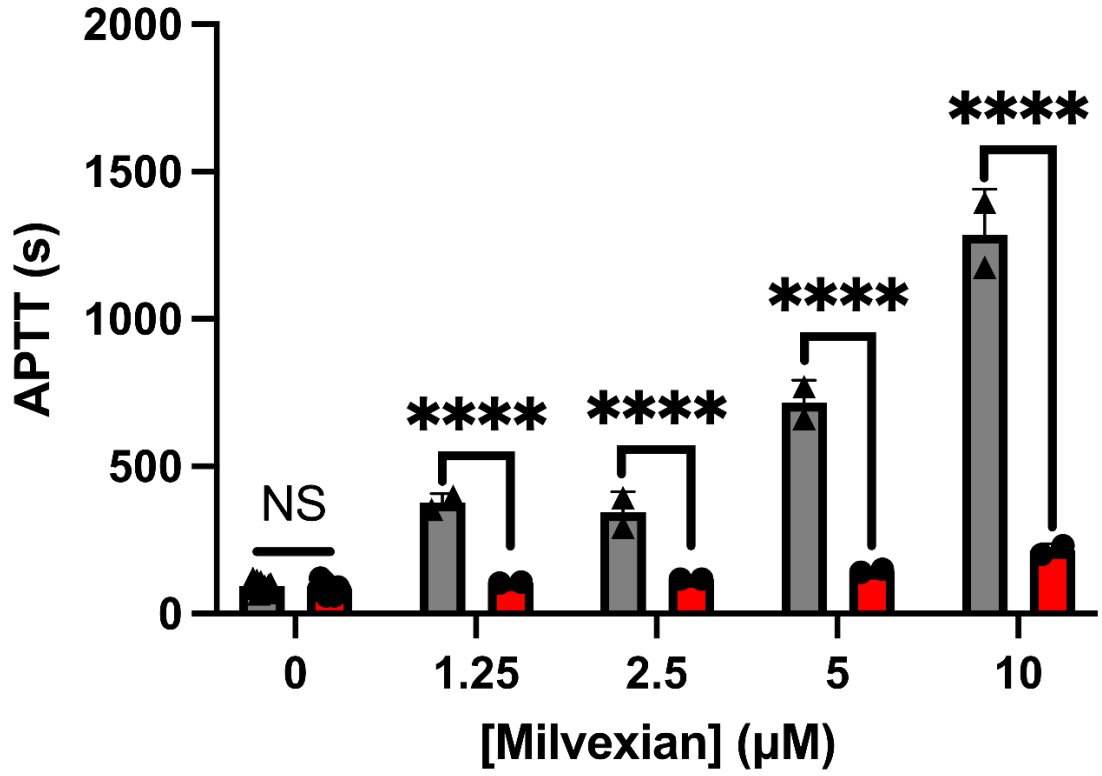

Figure 3.4: DS reverses the effect of milvexian on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing milvexian at the indicated concentrations. Bars represent the means, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. ***P < 0.01, ****P < 0.0001. Statistical significance annotations indicate (a) pairwise comparison of DS-untreated control samples with DS-treated samples containing milvexian (above pair brackets). NS, not significant compared with the control. There were no significant differences between the DS-treated samples and the control without milvexian (two-way analysis of variance, Tukey method).

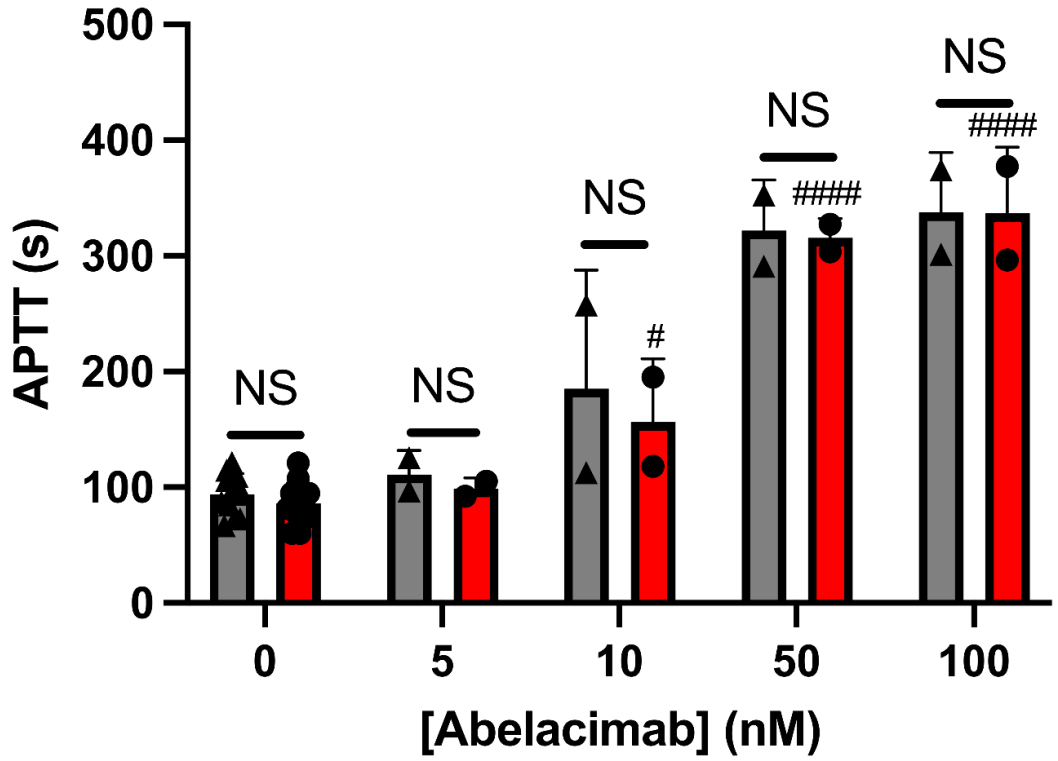

Figure 3.5: DS does not reverse the effect of abelacimab on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing abelacimab at the indicated concentrations. Bars represent the means, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. #P < 0.05, ####P < 0.0001. Statistical significance annotations indicate (a) pairwise comparison of DS-untreated control samples with DS-treated samples containing abelacimab (above the horizontal lines) or (b) comparison of abelacimab- and DS-treated samples with the control DS-treated samples lacking abelacimab (above the red bars). NS, not significant compared with the control. There were no significant differences between the DS-treated samples containing 5 nM abelacimab and the control without abelacimab (two-way analysis of variance, Tukey method).

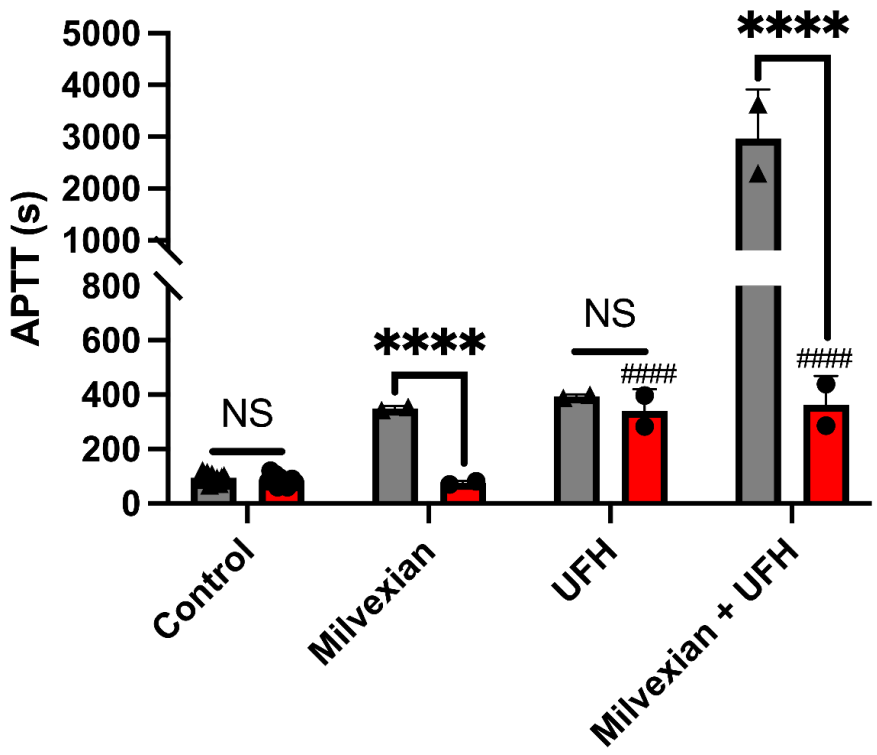

Figure 3.6: DS distinguishes between the effect of milvexian and UFH on the APTT.

The APTT was measured in control plasma (grey bars, triangles) or DS-treated plasma (red bars, circles) containing 2.5 μM milvexian and 0.125 U/mL UFH. Bars represent the means, whereas the lines above the bars indicate the standard deviation of 2-12 determinations performed in triplicate. ****P < 0.0001, ####P < 0.001. Statistical significance annotations indicate (a) pairwise comparison of DS-untreated control samples with DS-treated samples containing anticoagulant (above square brackets) or (b) comparison of anticoagulant- and DS-treated samples with control DS-treated samples lacking anticoagulant (above the red bars). NS, not significant compared with the control. There was no significant difference between the DS-treated sample containing milvexian and the control without anticoagulant (two-way analysis of variance, Tukey method).

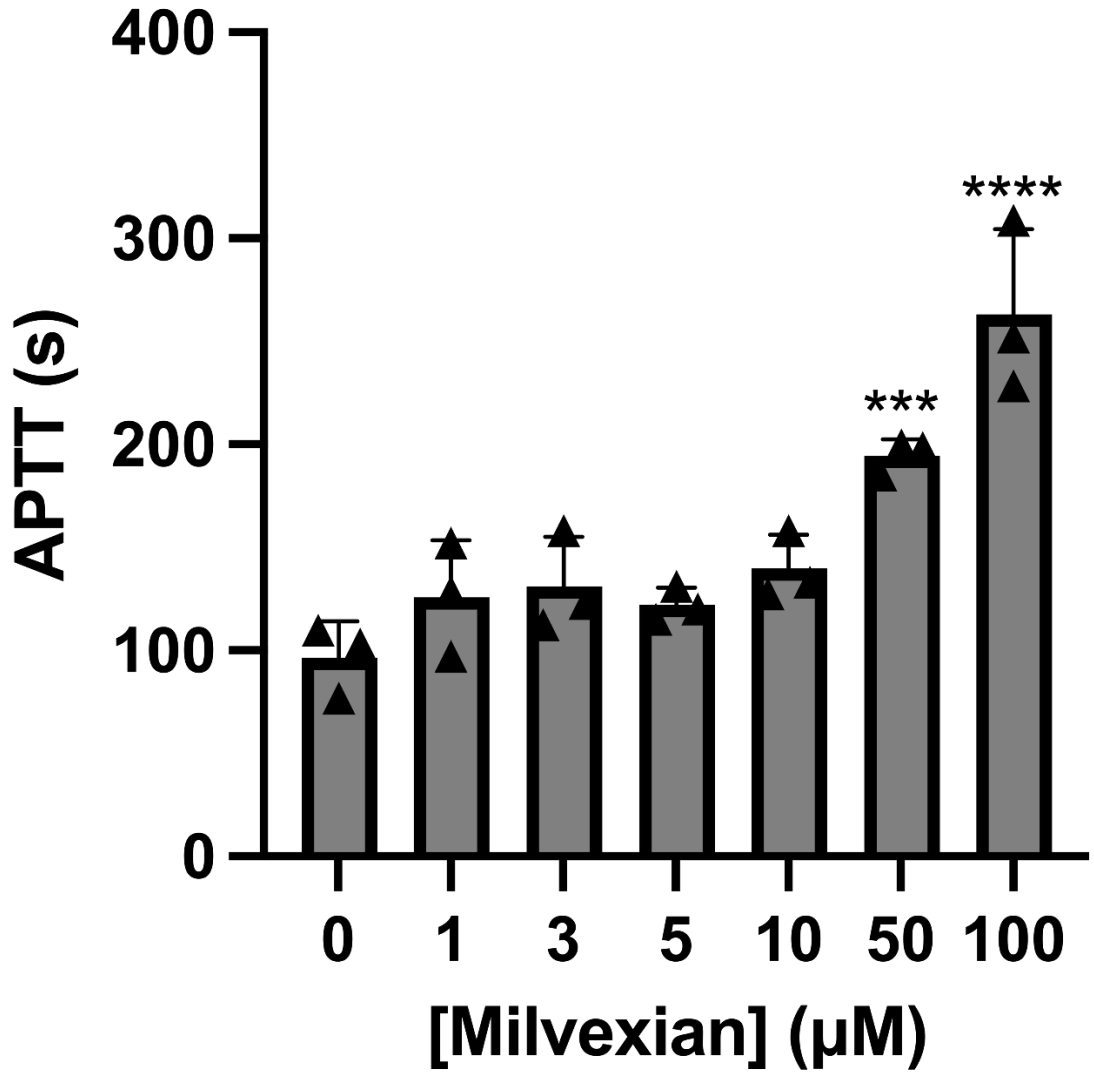

Figure 3.7: Capacity of DS for milvexian reversal.

The APTT was measured in DS-treated plasma containing milvexian at the indicated concentrations. Bars represent the means, whereas the lines above the bars indicate the standard deviation of three determinations performed in triplicate. ***P < 0.001, ****P < 0.0001. Statistical annotations indicate a comparison with the control lacking milvexian (one-way analysis of variance, Tukey method).

Table 3.1: Effect of DS on the APTT in the absence or presence of 2.5 μM milvexian and 0.125 U/mL UFH alone or in combination

Inhibitors	**APTT (s)**	
	No DS	**With DS**
None	86 ± 32	85 ± 8
Milvexian	287 ± 47	98 ±21
UFH	407 ± 133	353 ±147
Milvexian + UFH	3656 ±1493	340 ± 132

CHAPTER 4 – EVALUATION OF DOAC-STOP™-LIQUID

4.1 KEY POINTS

- DS-L is a liquid formulation of DS that is composed of activated charcoal reconstituted in water.
- The recommended volume of DS-L is not as effective at neutralizing the effect of milvexian on the APTT as one DS tablet.

4.2 INTRODUCTION

In Chapter 3, we show that DS (a) reverses the effect of asundexian and milvexian on the APTT; (b) distinguishes between the effects of milvexian and UFH on the APTT; and (c) adsorbs up to 10 μM milvexian. DS (Haematex, Hornsby, Australia) has undergone many evaluations for its practicality and effectiveness in anticoagulant reversal (127). However, the effect of residual activated charcoal particles on plasma remains a question of concern due to the potential interference it has on optical clot-based detection systems. A new formulation called DS-L was released as an alternative to the tablet formulation of DS (107,127). Its liquid formulation is expected to more readily disperse in samples and facilitate faster formation of a precipitate with less residual activated charcoal left in the plasma as a smaller volume is required (127). Another advantage of DS-L over DS tablets is that DS-L can treat lower volumes of plasma. Specifically, a 0.25% (w/v) concentration of DS-L can be used, whereas one 5 mg DS tablet is used for a 1.0 mL plasma sample creating a 0.5% (w/v) concentration. Thus, DS-L may serve as a more effective laboratory tool to address the need for a standardized method to reverse the anticoagulant effect that DOACs elicit on coagulation tests.

Similar to DS, DS-L has not yet been tested with FXI(a) inhibitors. Therefore, whether it can reverse the effect they elicit on the APTT remains unknown. In the following experiments, we compare the efficacy of one 5 mg DS tablet with DS-L.

4.3 METHODS

4.3.1 Materials

Milvexian was provided by Dr. M. Chintala of Janssen Research and Development, Spring House, PA. DS tablets and DS-L (Haematex Research, Hornsby, Australia) were generously provided by Jennifer J. Kiblinger (DiaPharma Group, Inc., West Chester, OH). Costar clear, round-bottom 96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). Liquid HemosIL® APTT-SP was purchased from Instrumentation Laboratory (Bedford, MA).

Milvexian was dissolved to 10 mM in dimethyl sulfoxide and diluted in 20 mM HEPES buffer, pH 7.4 (HB). The procedure for the preparation of DS-L is to add 1.5 mL of water to the vial containing the solid DS activated charcoal and to shake vigorously, creating a 12.5% (w/v) suspension of activated charcoal (127).

4.3.2 Plasma Treatment with Milvexian and DS-L and DS Tablets

Plasma was prepared as described in Chapter 3. Milvexian was added to plasma to concentrations up to 5 μM. Therefore, the concentrations utilized in these experiments exceed the expected on-treatment concentrations evaluated in Phase 3 clinical trials where the expected on-treatment range is 0.7 μM with the 25 mg twice daily dose and 3 μM with the 100 mg twice daily dose (59). Plasma was treated with DS-L as recommended by the manufacturer guidelines (107) or with one DS tablet as outlined in Chapter 3. For DS-L, to a 1-mL aliquot of plasma containing milvexian, 20 μL of vortexed DS-L was added to create a 0.25% (w/v) concentration of DS-L and incubated on a rotator for 5 min at room temperature. After incubation, samples were centrifuged

for 10 min at 5000 X g. For DS, to a 1.0-mL aliquot of plasma containing milvexian, one DS tablet was added and incubated on a rotator for 5 min at room temperature. After incubation, samples were centrifuged for 2 min at 5000 X g, and supernatants were subjected to a second centrifugation step under the same conditions. The supernatant was used for APTT testing. Control plasma was not treated with milvexian, DS-L, or DS.

4.3.3 APTT Determination

A diluted APTT was used as described in Chapter 3. To the wells of a 96-well plate, 50-µL plasma aliquots without or with DS-L or DS treatment, 10 µL of HemosIL APTT-SP (1:10 final dilution), and 30 µL of HB were added. After 10 minutes of incubation at 37°C, clotting was initiated by adding 10 µL of a pre-warmed 260 mM $CaCl_2$ solution to each well, and absorbance was measured at 405 nm using a THERMOmax plate reader (Molecular Devices, Sunnyvale, CA). The clotting time was calculated as the time to achieve the half-maximum increase in absorbance, as determined by the instrument software. Unless otherwise stated, the APTT was measured three times, each in triplicate.

4.3.4 Comparison of DS-L and DS Tablets for Milvexian Reversal

To compare the efficacy of DS-L with DS, the APTT was performed with plasma containing 5 µM milvexian that was treated with up to 100 µL of DS-L or one DS tablet.

4.3.5 Adsorptive Capacity of DS-L for Milvexian Removal

To determine the capacity of DS-L for the reversal of milvexian, the APTT was performed with plasma containing up to 5 µM milvexian treated with 20 µL of DS-L.

4.3.6 Statistical Analysis

Data are presented as means ± standard deviations (SD). The significance of the difference between groups ± DS was determined by one-way analysis of variance (ANOVA) followed by the

Tukey comparison test using Prism 10 (GraphPad, Boston, MA). For all analyses, P values < 0.05 were considered statistically significant.

4.4 RESULTS

4.4.1 The Recommended Volume of DS-L Does Not Reach the Potency of One DS Tablet

To determine the equivalency of DS-L to one DS tablet, DS-L was titrated into plasma containing a fixed concentration of milvexian. In plasma samples spiked with 5 μM milvexian, the recommended volume of 20 μL per 1 mL of plasma was insufficient to reverse the effect of 5 μM milvexian on the APTT (P < 0.0001) (Figure 4.1). Consequently, 50 μL of DS-L, creating a concentration of 0.625% (w/v), was needed to reach the adsorptive capacity of one DS tablet, which reaches a concentration of 0.5% (w/v), to bring APTT values within range of the baseline control (P = NS).

4.4.2 The Recommended Volume of DS-L is Insufficient for Removal of Milvexian Concentrations Reaching Therapeutic Levels

To evaluate the capacity of DS-L, concentrations of milvexian up to 5 μM were added to plasma and subjected to treatment with the recommended 20 μL of DS-L. DS-L was only able to reverse the effect of up to 1 μM milvexian on the APTT (P = NS) (Figure 4.2). DS-L did not reverse the effect of 3 μM (P < 0.01) or 5 μM (P < 0.0001) milvexian on the APTT.

4.5 DISCUSSION

In these experiments, we evaluated the efficacy of DS-L, a liquid suspension of DS activated charcoal. Here, we show that the recommended volume of DS-L (a) does not reach the potency of one DS tablet; and (b) is only capable of reversing up to 1 μM milvexian.

In plasma containing 5 μM milvexian and varying amounts of DS-L, almost three times the recommended volume was required to elicit the same reversal effect as one DS tablet, highlighting the potential inefficacy of the recommended protocol and underlying differences in

formulations. DS-L was only able to reverse concentrations up to 1 μM, which is less than the concentration of milvexian likely to be achieved in Phase 3 evaluations.

Both observations are likely explained by the different amounts of activated charcoal added to the plasma. With DS treatment, a 1-mL aliquot of plasma is treated with one 5 mg activated charcoal tablet, resulting in a concentration of 0.5% (w/v). Whereas because DS-L has a starting concentration of 12.5% (w/v), the recommended volume results in a final concentration of 0.25% (w/v) (127). As such, treatment with DS-L has less activated charcoal molecules per volume compared with those in one DS tablet. This explanation is supported by our results where 50 μL of DS-L, which is 0.625% (w/v), produced roughly equivalent reductions in the APTT as one DS tablet. To note, one study reports that 0.5% (w/v) DS-L reverses the effect of 2000 ng/mL dabigatran, rivaroxaban, or apixaban on the APTT (127). While we did not explore the effect of DS-L in plasma containing dabigatran, our experiments show that DS is able to reverse the effect of around 4000 ng/mL milvexian on the APTT. It would be valuable to therefore test DS-L in plasma containing dabigatran to confirm if the capacity of DS-L is the same for different anticoagulants.

Similar to Chapter 3, the primary limitation of these experiments is that milvexian was added to pooled normal plasma, instead of using plasma from patients who had received milvexian. Further, these experiments do not evaluate the efficacy of DS-L to reverse any of the DOACs that have potent effects on the APTT, like dabigatran (127), or other FXI(a) inhibitors such as asundexian, abelacimab, or osocimab (66). However, under the assumption that the sole difference between DS tablets and DS-L is the formulation of activated charcoal, treatment of a 1-mL aliquot of plasma with at least 50 μL of DS-L to achieve a 0.625% (w/v) concentration is expected to reverse the effect of dabigatran on the APTT similar to DS. Furthermore, DS-L is expected to

reverse the effect of asundexian on the APTT. Furthermore, this revised concentration should also allow for treatment of reduced plasma volumes. Therefore, based on the data, DS-L may be able to treat smaller plasma volumes unlike DS. In contrast, due to the structural characteristics of abelacimab (65) and osocimab (128), it is not expected that any volume of DS-L will reverse their effects on the APTT.

DS-L was developed as an alternative to DS to address concerns regarding ease of use and the potential effects of DS on plasma (127). In comparison with DS, the liquid formulation of DS-L can treat smaller volumes of plasma, more easily disperses in plasma samples, and for analyses that do not use optical detections systems to monitor clot formation, DS-L does not require sedimentation (127). Our findings confirm that with the revised concentration of at least 0.625% (w/v), DS-L can treat smaller volumes of plasma. Due to the fact that both DS-L and DS were tested in the APTT using a turbidity-based assay, sedimentation of both products was required for plasma preparation. Therefore, the efficacy of DS-L in its ability to omit the requirement of sedimentation is not explored. Further, it should be noted that an incubation period of 5 min with DS-L or DS was required prior to centrifugation for 10 min or 2 min twice, respectively. Therefore, DS-L treatment required a longer sedimentation step. After centrifugation, the activated charcoal in both DS-L and DS precipitate and render the supernatant relatively free from activated charcoal. To further explore whether the activated charcoal interacts with any coagulation proteins, further experiments that evaluate potential interactions between the activated charcoal and such proteins should be performed. Another consideration is the therapeutic concentrations of DOACs compared with milvexian, where DOAC concentrations are usually less than 200 ng/mL (31), whereas current clinical trials evaluate milvexian concentrations greater than 1000 ng/mL (85). As DS-L was likely developed for DOACs, the evaluation of DS-L with milvexian therefore provides a

challenge. In conclusion, the results of this chapter indicate that DS-L functions the same as DS, but only at concentrations exceeding those recommended by the manufacturer.

4.6 FIGURES

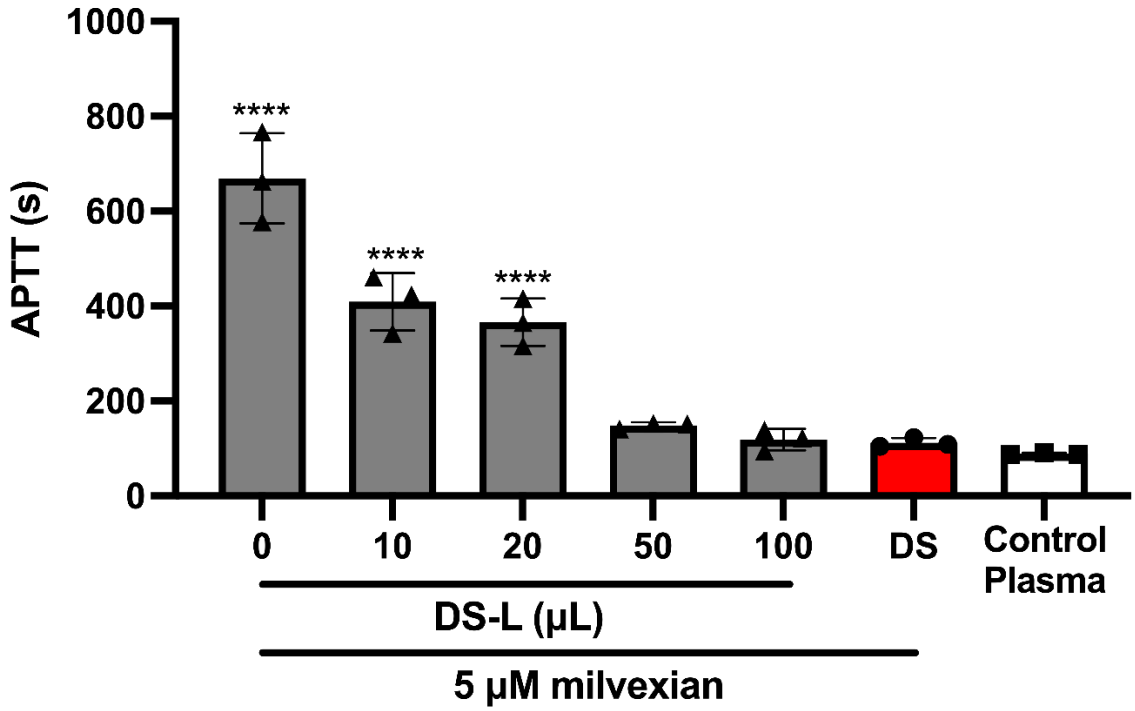

Figure 4.1: 50 µL of DS-L is required to reach the potency of one DS tablet.

The APTT was measured in plasma containing 5 µM milvexian treated with increasing volumes of DS-L (grey bars, triangles) or one DS tablet (red bar, circles). Control plasma was not treated with DS-L, DS, or milvexian (white bar, squares). Bars represent the mean, whereas the lines above the bars indicate the standard deviation of 3 determinations performed in triplicate. Statistical significance annotations indicate comparison with plasma samples treated with one DS tablet (****$P < 0.0001$). There were no significant differences between samples treated with ≥ 50 µL of DS-L and the sample treated with one DS tablet (one-way analysis of variance, Tukey method).

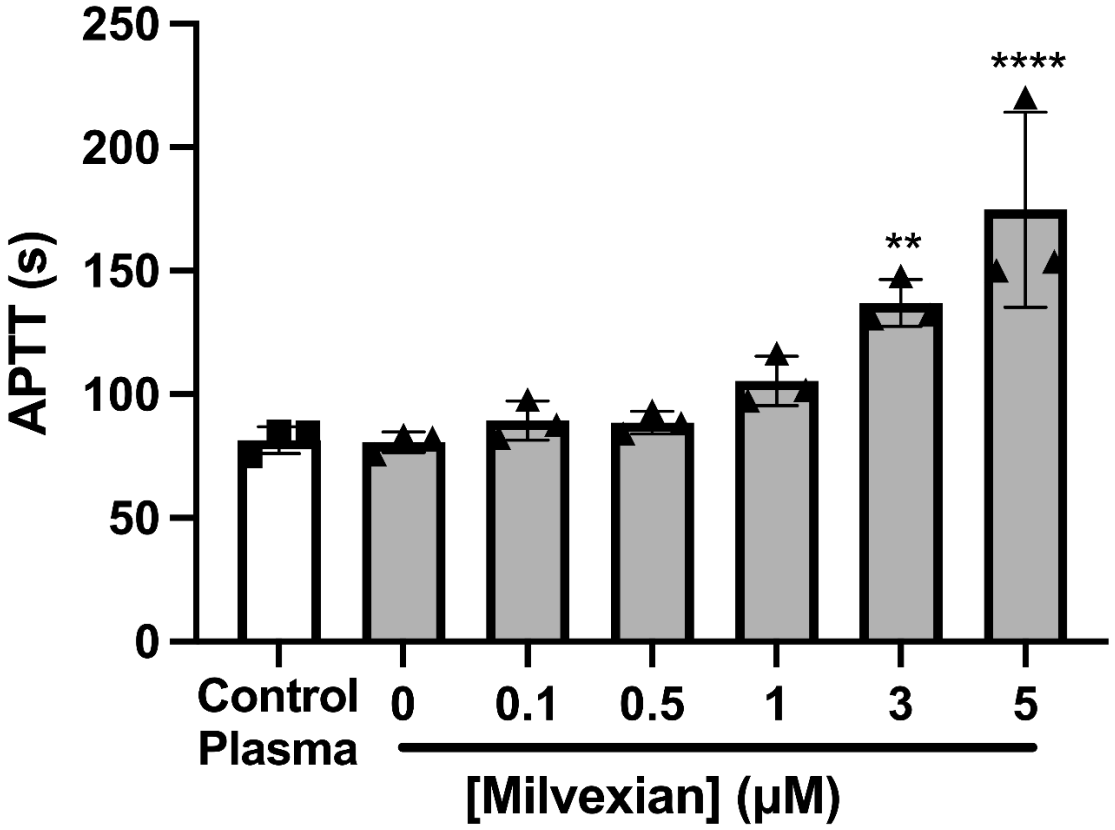

Figure 4.2: DS-L adsorbs only up to 1 μM milvexian.

The APTT was measured in plasma treated with 20 μL of DS-L (grey bars, triangles) or in control plasma (white bar, squares) containing milvexian at the indicated concentrations. Bars represent the mean, whereas the lines above the bars indicate the standard deviation of 3 determinations performed in triplicate. **P < 0.01, ****P < 0.0001. Statistical significance annotations indicate comparison with control plasma without milvexian and DS-L treatment. There were no significant differences between samples treated with 0-1 μM milvexian and the control (one-way analysis of variance, Tukey method).

CHAPTER 5 – GENERAL DISCUSSION

5.1 COAGULATION ASSAYS AND INTERFERENCE

Coagulation tests, such as the PT and APTT, are used by clinicians for routine anticoagulant treatment management (86). A major unresolved challenge occurs when coagulation testing is required for patients on DOAC treatment, such as during preoperative assessment or in the event of bleeding (129). DOACs are effective inhibitors of thrombin and FXa and, as a result, interfere with test results. Notably, the presence of DOACs significantly interferes with lupus anticoagulant testing as DOACs and the presence of lupus anticoagulant both present as a prolonged APTT (90,130,131). As such, this overlap may lead to misdiagnosis of APS. To address DOAC interference, it has been proposed to cease anticoagulant therapy prior to testing, use DOAC-specific reversal agents, or alternatively use DOAC-insensitive assays (90,130,131). However, none of the proposed methods are standard practice due to their shortcomings. Thus, there is still a critical need to establish a standard procedure to overcome the roadblock that DOAC therapy poses on coagulation tests.

Several studies have since investigated a novel strategy to eliminate the impact that DOACs elicit on coagulation assays by treating plasma with activated charcoal-based compounds, such as DS (108,115,116). Studies with dabigatran (115,116), apixaban (108,115,116,132), rivaroxaban (108,115,116,132), and edoxaban (116) demonstrate that the APTT values are returned to baseline with DS treatment, therefore suggesting that DS neutralizes the effect of DOACs on the APTT. Thus, the DS procedure should be applicable to future DOACs. Furthermore, activated charcoal compounds adsorb medications such as aspirin and paracetamol, and oral gavage with activated charcoal is standard practice for emergency overdose situations (113,114). Despite the success presented in these studies and the use of activated charcoal for adsorption of medications, DS use

is still not standard practice. Due to the unmet need for a standardized method to reverse or eliminate the effect of anticoagulants on coagulation assays, there is ongoing interest for more research on the use of activated charcoal compounds for reversal (119,133).

5.2 FACTOR XI(a) AS A TARGET FOR ANTICOAGULANT THERAPY

FXI(a) inhibitor development is of major importance because of the minimal impact FXI has on hemostasis, but critical role it plays in thrombus formation (134). To date, the FXI(a) inhibitors, like the small molecules, asundexian and milvexian, and the monoclonal antibody, abelacimab, are under evaluation in several Phase 3 investigations. Despite their potential as therapeutic agents for thrombosis due to their superior safety and pharmaco-dynamic and -kinetic profiles, FXI(a) inhibitors interfere with specialized coagulation tests much like traditional anticoagulants (65,105). The prolongation of APTT values caused by FXI(a) inhibitors therefore complicates investigative coagulation tests on patients prescribed these anticoagulants as the expected impact of FXI(a) inhibitors on the APTT may be misinterpreted as a positive test result for a coagulopathy (90,130). Furthermore, these FXI(a) inhibitors pose a challenge of distinguishing between the effects of heparin and these inhibitors on the APTT during clinical trials. To our knowledge, whether DS could reverse the effects of FXI(a) inhibitors is not yet established.

5.3 PROJECT OVERVIEW

The primary aim of this thesis was to investigate the efficacy of DS on its ability to reverse the effects of FXI(a) inhibitors on the APTT. To address this aim, the specific objectives were to (a) evaluate the ability of DS to reverse the effects of asundexian, milvexian, and abelacimab on the APTT; (b) evaluate whether DS can distinguish between the effects of milvexian and UFH on

the APTT; (c) determine the capacity of DS for milvexian reversal; and (d) evaluate the ability of DS-L to reverse the effect of milvexian on the APTT.

In this thesis, we report that: (a) DS reverses the effect of milvexian or asundexian on the APTT, but not the effect of abelacimab (Chapter 3); (b) DS distinguishes between the effects of milvexian and UFH on the APTT (Chapter 3); (c) one DS tablet reverses up to 10 μM milvexian (Chapter 3); (d) the recommended volume of DS-L does not reach the potency of one DS tablet (Chapter 4). Altogether, these results suggest that DS works on the small molecule inhibitors of FXIa, but not FXI(a) monoclonal antibodies. Furthermore, the suggested volume of DS-L may be insufficient for the reversal of the milvexian concentrations expected to be achieved in Phase 3 trials. Therefore, re-evaluation of the indicated volumes for DS-L may be required to properly address clinically relevant concentrations of FXI(a) inhibitors.

5.4 EVALUATING DS TABLETS AND DS-L

In Chapter 3, we investigated the ability of DS to neutralize traditional anticoagulants and FXI(a) inhibitors. We first treated plasma samples containing dabigatran and UFH with DS. Treatment with DS worked as expected where it eliminated the dose-dependent response of dabigatran, but not UFH. Our results are consistent with previous reports that confirm the ability of DS to eliminate the effect of dabigatran on the APTT from both patient-derived and spiked samples (115,116). Previous studies demonstrate the dose-dependent prolongation that asundexian, milvexian, and abelacimab elicit on the APTT (65,105). We first confirmed this prolongation and then explored the ability of DS to abolish such effect. To evaluate DS with the FXI(a) inhibitors, we treated plasma samples containing asundexian, milvexian, and abelacimab with DS. DS eliminated the dose-dependent response of asundexian and milvexian, but not abelacimab. The ability of DS to reverse only the effects of dabigatran, asundexian, and milvexian

is consistent with its mechanism of action (109,110). The ability of DS to interact with dabigatran suggests that adsorption may depend primarily on overall size and hydrophobicity of the compound. This hypothesis is explained by DS adsorbing small compounds composed of many hydrophobic cyclic rings like dabigatran (32), asundexian (75), and milvexian (77), but not large anticoagulants like UFH, which is a highly acidic polysaccharide (27), or abelacimab, which is a large protein (65). We then demonstrated the selectivity of DS for small molecule inhibitors by treating plasma containing both milvexian and UFH, where DS eliminated only the effect of milvexian on the APTT. This is important for situations where patients on a milvexian regimen may also be prescribed UFH, such as during percutaneous coronary interventions (135). As such, the use of DS may be useful as a perioperative procedure to distinguish the between the effects of the two anticoagulants. Lastly, we evaluated the capacity of DS to reverse the effect of milvexian on the APTT, where DS is able to reverse up to 10 μM milvexian. This finding is of importance as DS is able to reverse milvexian concentrations more than three times greater than those expected to be achieved in clinical evaluation (136). Our results confirm that DS works regardless of molecular target and depends mostly on molecular size and hydrophobicity. Therefore, the use of DS may be a prospective tool for diagnostic laboratory testing as it reverses the effect of small molecule FXIa inhibitors on the APTT where these inhibitors are prescribed alone or in conjunction with UFH.

In Chapter 4, we evaluated a liquid formulation of DS called DS-L. DS-L was introduced as a potential advance to DS due to a more user-friendly formulation (137). The potential for its superiority above DS is attributed to its ability to treat smaller volumes of plasma, readily disperse in plasma samples, and omit the necessity for sedimentation in assays that do not involve optical detection methods (137). Thus, DS-L allows for treatment of limited plasma volumes and less

preparation time required prior to testing. Similar to DS, DS-L reversed the effect of milvexian on the APTT. However, treatment with more than double the recommended volume of DS-L was required to reach the potency of one DS tablet for milvexian reversal. This observation is likely explained by the formulation of activated charcoal in DS compared with DS-L. Therefore, re-evaluation of the recommended procedure of DS-L is required. Despite this, DS-L may still be a useful alternative to DS tablets where patient plasma is limited.

5.5 THE EFFECT OF DS IN PLASMA

A potential area of concern is the effect that activated charcoal compounds, like DS, elicit in plasma. While most studies showed no such effect, one study observed decreases in TFPI with DS treatment (138). Another study observed a prolongation in the APTT with the addition of raw activated charcoal to plasma lacking anticoagulant (139). As a preliminary evaluation of the effect of DS on plasma, we treated plasma without anticoagulant with DS. Our findings are consistent with previous reports using samples without drug where no significant difference in the APTT was observed between control and DS-treated samples (115,116). These findings suggest that DS does not elicit a procoagulant effect in plasma as seen in some previous reports (138,139). However, we did not explore the potential for DS or DS-L to adsorb coagulation proteins from plasma samples. Therefore, further experiments are required to properly address this concern given the potential use of DS in diagnostic laboratory testing for APS.

5.6 IMPACT OF DS

DS, both in tablet and liquid formulation, reverses the effects of the FXIa inhibitors asundexian and milvexian on the APTT. In addition to DS, DOAC-Remove (5-Diagnostics AG) and DOAC Filter (Diagostica Stago, France) are also under evaluation to address the need for a standardized way to eliminate DOAC interference on coagulation assays. DOAC-Remove applies

the same principles as DS and involves treating plasma with an activated charcoal tablet (140,141). One study reported that treatment with DOAC-Remove reverses the effect of apixaban, rivaroxaban, and dabigatran on two screening tests for lupus anticoagulant (140). Furthermore, with samples from patients on dabigatran, rivaroxaban, or apixaban, treatment with DOAC-Remove decreased the concentrations of these anticoagulants; however, it was less effective for apixaban removal (141). In contrast, the DOAC Filter procedure involves passing plasma over a filtration cartridge where DOACs are adsorbed using noncovalent binding mechanisms and solid phase extraction (142,143). DOAC Filter is a newer product, and its efficacy has yet to be extensively evaluated. The first report of DOAC Filter utilized plasma spiked with dabigatran, rivaroxaban, apixaban, edoxaban, or betrixaban, where DOAC Filter treatment significantly reduces the DOAC concentrations (142). In a study which compared DS with DOAC Filter, both treatments result in significant reductions in the concentrations of apixaban, rivaroxaban, edoxaban, and dabigatran (143). In conclusion, the use of activated charcoal products like DS, DOAC-Remove, and DOAC Filter as a standard practice to remove DOACs from plasma samples serves as a potential solution to coagulation test interference but requires further evaluation.

CHAPTER 6 – LIMITATIONS AND FUTURE DIRECTIONS

6.1 OVERVIEW OF LIMITATIONS AND FUTURE DIRECTIONS

In this thesis, we demonstrated that DS reverses the effects of asundexian and milvexian on the APTT, and that DS-L reverses the effects of milvexian. However, several aspects of this thesis warrant further investigation to improve our understanding of the clinical relevance, efficacy, and overall impact of DS. This chapter outlines key limitations and proposes experiments to address them in the following areas: (i) the use of DS on patient plasma; (ii) evaluation of DS on the reversal of additional FXI(a) inhibitors; (iii) evaluation of DS-L on traditional anticoagulants and FXI(a) inhibitors; and (iv) the effect of DS on plasma.

One overarching limitation to the research presented in this thesis is that the relevance of the work presented here with DS and FXI(a) inhibitors depends on whether these inhibitors ever get approval for widespread use as a therapeutic anticoagulation strategy. This approval relies on the safety and efficacy outcomes of these novel inhibitors compared with DOACs for treatment and prevention of indications such as thrombosis (83,85). Establishing this may be limited by the time it takes to complete such evaluations, funding, and enrolment of volunteers that meet the criteria. At the time of writing, several Phase 3 clinical trials continue, many of which aim to evaluate of the safety, tolerability, and efficacy of FXI(a) inhibitors compared with DOACs (83,85).

6.2 DS IN PATIENT PLASMA

A notable limitation of the experiments outlined in this thesis is that the plasma utilized is from healthy participants to which anticoagulants were added instead of from patients on anticoagulant therapy. While we incubated the plasma with anticoagulants prior to treating with DS and the data are consistent with reports on patient plasma containing dabigatran (115), this

procedure does not replicate what occurs in clinical situations. Specifically, the plasma retrieved from patients undergoing coagulation testing will likely include other medications in addition to anticoagulants that are susceptible to activated charcoal compound adsorption, like paracetamol (114,144). As such, these situations raise the question whether DS will perform as efficaciously in reversing the effect of traditional anticoagulants and FXI(a) inhibitors when in the presence of more than one type of adsorbate molecule. To note, we show in Chapter 3 and 4 that there is a limit to milvexian adsorption by DS and DS-L, respectively. Therefore, if other molecules like acetaminophen are present in the patient plasma, it could impede the ability of DS to adsorb anticoagulants and further complicate the interpretation of coagulation test results used to monitor anticoagulant therapy or for APS investigations (92,94,118,119). In addition, using plasma from healthy individuals does not account for potential confounding factors associated with prothrombotic or coagulopathic plasma as seen in those under investigation for APS (92,94). As a result, these factors may independently prolong the APTT and therefore would impact the interpretation of the efficacy of DS for FXI(a) inhibitor reversal. It should be noted, however, that studies with DS on patient samples taking DOACs such as rivaroxaban, dabigatran, apixaban, and edoxaban show significant reduction of APTT values and anticoagulant concentrations with DS treatment (108,116,132,145–147).

Project #1: Evaluating the Effect of DS in Patient Plasma

To address this, the same experiments will be performed, but with plasma derived from patients on FXI(a) anticoagulant therapy. Specifically, a 1-mL aliquot of patient plasma will be treated with DS and incubated on a rotator for 5 min at room temperature. If patient samples are limited, DS-L will be utilized as it can be used for sample volumes as low as 0.5 mL (127). Following incubation, the DS- or DS-L-treated plasma will be subjected to two centrifugation steps

for 2 min or one centrifugation step for 10 min at 5000 X g, respectively. The supernatant will be used in a dilute APTT as described in Chapter 3. Based on reports with DS and patient samples (108,116,132,145–147), we hypothesize that DS will perform comparably with patient samples as with our results using normal plasma spiked with anticoagulant.

Project #2: Evaluating the Effect of DS in Plasma Containing Multiple Drugs

To address whether the adsorptive capacity of DS is impacted by the presence of multiple drugs, the same experiments will be performed with samples spiked with the FXI(a) inhibitors and acetaminophen. Based on our results that highlight the capacity of DS and DS-L for milvexian adsorption and literature that demonstrates activated charcoal adsorption of paracetamol, we hypothesize that the ability of DS to adsorb anticoagulants may be impacted in samples spiked with multiple drugs compared with samples containing only the FXI(a) inhibitors.

6.3 NARROW RANGE OF ANTICOAGULANTS TESTED WITH DS AND DS-L

A further limitation involves the narrow range of monoclonal antibodies and anticoagulants tested with DS and DS-L, respectively (Chapter 3-4). While our findings show that DS does not adsorb the FXI(a) monoclonal antibody, abelacimab (Chapter 3), it would be valuable to confirm this observation with another negative control, osocimab, which is a FXIa monoclonal antibody also undergoing clinical evaluation (58,66). Similarly, the experiments outlined in this thesis with DS-L only evaluate its ability to reverse the effect of milvexian on the APTT (Chapter 4). To improve our understanding of DS-L as another method to reverse anticoagulant interference on coagulation assays, it would be valuable to test the DS-L procedure with dabigatran, as well as the FXI(a) inhibitors, asundexian, abelacimab, and osocimab.

Project #3: Evaluating the Effect of DS Tablets on Osocimab

The experiments outlined in Chapter 3 will be performed with osocimab. Due to the structural similarities between osocimab and abelacimab, we hypothesize that DS will not reverse the effects of osocimab or other FXI antibodies on the APTT (128).

Project #4: Evaluating the Effect of DS-L on Traditional DOACs and FXI(a) Inhibitors

The experiments outlined in Chapter 4 using DS-L will be perform with dabigatran, UFH, asundexian, abelacimab and osocimab. Based on results in Chapter 3 and 4, and structural characteristics, we hypothesize that DS-L will reverse the effects of dabigatran and asundexian on the APTT, but not abelacimab or osocimab.

6.4 DS IN PLASMA

Lastly, this study does not explore the impacts of activated charcoal-based products, like DS, on plasma itself. Notably, Kopatz et al. (2018) highlighted the interactions between DS and TFPI when DS was used in lower volumes than recommended by the manufacturer (138). Further, Buckley et al. (2023) reported that raw activated charcoal elicits a procoagulant effect on the APTT when added to non-anticoagulated plasma, and attribute such effect to either the presence of contaminants in the activated charcoal or interactions with plasma proteins (139). However, neither group pursued further investigation of their findings. In Chapter 3, initial experiments using a more dilute APTT reagent (1:50 final dilution) showed a significant prolongation of the APTT in non-anticoagulated DS-treated plasma compared with control plasma (data not shown). This observation was corrected by instead using a more concentrated APTT reagent (1:10 final dilution), but the experiments outlined did not further investigate this effect (Chapter 3). Thus, this finding raises questions regarding the effect of DS in plasma and whether it interacts with plasma proteins.

Project #5: Assessing the Effect of DS in Plasma

To address this question, nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry will be performed on plasma samples treated with DS (148,149). A 1-mL aliquot of control plasma will be treated with one tablet of DS. After incubation for 10 min on a rotator, the DS-treated sample will be centrifuged for 2 min at 5000 X g. From here, the supernatant will be removed, and the pellet will be resuspended in SDS-PAGE buffer containing Tris-hydrochloride, SDS, glycerol, bromophenol blue, but not β-mercaptoethanol (148). Treatment with SDS allows for separation of proteins solely based on their molecular weight as the proteins are denatured and a uniform negative charge is applied (148). In addition, nonreducing conditions leave disulfide bonds intact, thus leaving multimeric proteins connected by disulfide bonds in their denatured, but complete forms. From here, the produced bands, visualized using a stain like Coomassie Brilliant Blue, will be compared with the known molecular weights of plasma proteins to provide preliminary identification of any proteins adsorbed from the plasma using DS. Mass spectrometry will be utilized as a follow-up experiment to identify any proteins pulled from the plasma (149). Based on the selectivity of activated charcoal adsorption and the known molecular weights of the coagulation proteins, we hypothesize that DS will not interact with plasma proteins.

CHAPTER 7 – CONCLUSION

In conclusion, the results of this study confirm the efficacy of the commercially available activated charcoal-based product, DOAC-Stop™, both in tablet (DS) and liquid form (DS-L). In Chapter 3, we show that DS reverses the anticoagulant effects of two FXIa inhibitors, asundexian and milvexian, on the APTT. We also show that DS distinguishes between the effects of milvexian and UFH on the APTT. Lastly, we show that one DS tablet reverses up to 10 μM milvexian. In Chapter 4, we demonstrate that DS-L reverses the effect of milvexian on the APTT comparable to DS, but only at higher concentrations than recommended. We also show that the recommended volume of DS-L cannot reverse clinically relevant concentrations of milvexian. Collectively, this thesis provides that DOAC-Stop™ is a promising laboratory procedure to address the interference that asundexian and milvexian evoke on the APTT.

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