Targeting the Intrinsic Pathway of Coagulation with RNA Aptamers

by

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Richard Becker

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in Genetics and Genomics
in the Graduate School
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2013

<u>ABSTRACT</u>

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Abstract

Thrombosis is associated with the occlusion of a blood vessel and can be triggered by a number of types of injury, such as the rupture of an atherosclerotic plaque on the artery wall, changes in blood composition, or blood stasis. The resulting thrombosis can cause major diseases such as myocardial infarction, stroke, and venous thromboembolic disorders that, collectively, account for the most common cause of death in the developed world. Anticoagulants are used to treat and prevent these thrombotic diseases in a number of clinical and surgical settings. Although commonly prescribed, currently approved anticoagulants have a major limitation of severe drug-induced bleeding, which contributes to the high levels of morbidity and mortality associated with use. The "holy grail" for antithrombotic therapy is to identify a drug that inhibits thrombus formation without promoting bleeding. Understanding the differences between thrombosis and hemostasis in the vascular system is critical to developing these safe and effective anticoagulants, as this depends on striking the correct balance between inhibiting thrombus formation (efficacy) and reducing the risk of severe bleeding (safety). While it is commonly thought that the same factors play a similar role in hemostasis and thrombosis, recent evidence points to differing functions for FXI and FXII in each of these settings. Importantly, these factors seem to contribute to pathological thrombus formation without being involved in normal hemostasis.

The overall goal of this project was to evaluate the inhibition of the intrinsic pathway of coagulation as a potential anticoagulant strategy utilizing the aptamer platform. Aptamers are short, highly structured nucleic acids that act as antagonists by binding to large surface areas on their target protein and thus tend to inhibit protein-protein interactions. High affinity binding aptamers have been isolated that specifically target a diverse range of proteins, including transcription factors, proteases, viral proteins, and growth factors, as well as other coagulation factors. As synthetic molecules, aptamers have a small molecular weight, are highly amenable to modifications that can control their bioavailability, and have not been found to elicit an immune response, thus making them ideal drug candidates. Importantly, aptamers can be rapidly and effectively reversed with either a sequence specific antidote that recognizes the primary sequence of the aptamer or a universal antidote that binds to their backbone and reverses all aptamer activity independent of sequence. This ability lends itself well to their therapeutic application in coagulation, as rapid reversal of a drug upon the onset of bleeding is a key property for increasing the safety of this class of drugs.

Aptamers targeting FXI/FXIa and FXII/FXIIa were isolated in two separate SELEX (systematic evolution of ligands by exponential enrichment) procedures: the FXII aptamer was isolated in a convergent SELEX approach and the FXIa aptamer was isolated from a purified protein selection. In both processes, 2'fluoropyrimidine

modified RNA with a 40-nucleotide random region was incubated with either the

plasma proteome (in initial rounds of the convergent SELEX) or the purified protein

target (FXII or FXIa). The nucleic acids that did not bind to the target were separated

from those that bound, and these molecules were then amplified to generate an enriched

pool with increased binding affinity for the target. This process was repeated under

increasingly stringent conditions to isolate the aptamer that bound with the highest

affinity to the purified target protein. Utilizing biochemical and *in vitro* coagulation

assays, specific, high-affinity binding and functional anticoagulant aptamers were

identified for both protein targets, and the mechanism of anticoagulation was

ascertained for each aptamer.

Overall, both aptamers bound to an exosite on their target protein that was able

to inhibit downstream activation of the next protein in the coagulation cascade. In order

to specifically examine aptamer effects on several parameters of thrombin generation, a

new assay was developed and fully characterized using aptamer anticoagulants

targeting other coagulation factors. Aptamer inhibition of both FXI and FXII was able to

decrease thrombin generation in human plasma. However, limited cross-reactivity in

other animal species by both aptamers hindered our ability to assess aptamer inhibition

in an *in vivo* setting. The further characterization and use of these aptamers in plasma

and blood based settings will allow us to study the diverging functions of the intrinsic

pathway in thrombosis and hemostasis.

A critical need exists for safe and effective anticoagulants to treat and prevent numerous thrombotic procedures and diseases. An ideal anticoagulant is one that strikes the correct balance between inhibiting thrombus formation and reducing drug-induced bleeding. Inhibition or depletion of factors XI and XII of the intrinsic pathway of coagulation have shown reduced thrombus formation without interruption of normal hemostasis in several models of thrombosis. By developing novel RNA aptamer anticoagulants to these factors, we have set the stage for evaluating the net therapeutic benefit of intrinsic pathway inhibition to effectively control coagulation, manage thrombosis, and improve patient outcome. As well as developing a safe anticoagulant, these agents can lead to important biological discoveries concerning the fundamental difference between hemostasis and thrombosis.

Dedication

I would like to first dedicate this work to my parents, Leonard Alfred Smock, PhD., and Ellen Gilinsky, PhD., who have taught me to have an inquisitive mind and have unquestionably supported me in everything I pursue to do in life. In addition, I would also like to dedicate this work to my grandfather, Stanley Ellis Gilinsky, who, during retirement, would read calculus and genetic textbooks for fun and taught me to never, ever stop learning.

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List of Abbreviations

16.3	FVIIa binding RNA aptamer
11F7t	FX binding RNA aptamer
2′F	Fluoro modification on the 2′ position of ribose
2′OMe	2′ O-methyl modification on the 2′ position of ribose
ACS	acute coronary syndrome
AF	atrial fibrillation
APC	activated protein C
aPTT	activated partial thromboplastin time
ARC1779	VWF binding RNA/DNA aptamer
AT	antithrombin
BK	bradykinin
BSA	bovine serum albumin
CAD	coronary artery disease
CTI	corn trypsin inhibitor
CVD	cardiovascular disease
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
EPCR	endothelial protein C receptor
ETP	endogenous thrombin potential

FIX   factor IX

FIXa   activated factor IX

FVa   activated factor V

FVII   factor VII

FVIIa   activated factor VII

FVIII   factor VIII

FX   factor X

FXa   activated factor X

FXI   factor XI

FXII   factor XII

FXIIa   activated factor XII

GP1bα   glycoprotein 1b α-polypeptide

GPVI   glycoprotein VI

HAE   hereditary angioedema

HD1   thrombin binding DNA aptamer

HD22   thrombin binding DNA aptamer

HGFA   human growth factor activator

HIT   heparin-induced thrombocytopenia

HITT   heparin-induced thrombocytopenia with thrombosis

HK   high molecular weight kininogen

MI	myocardial infarction
mTGA	modified thrombin generation assay
NETs	neutrophil extracellular traps
NU172	thrombin binding DNA aptamer
PAD	peripheral artery disease
PAI-1	plasminogen activator 1
PCI	percutaneous coronary intervention
PCK	D-Pro-Phe-Arg chloromethyl ketone
PE	pulmonary embolism
PEG	polyethylene glycol
PK	prekallikrein
PSMA	prostate-specific membrane antigen
PT	partial thromboplastin time
R4cXII-1	FXII binding RNA aptamer
R9.14	VWF binding RNA aptamer
R9d14t	thrombin binding RNA aptamer
RB006	FIXa binding RNA aptamer
RB007	FIXa aptamer antidote
REG1	FIXa aptamer and antidote system
RFLP	restriction fragment length polymorphism

SELEX	systematic evolution of ligands by exponential enrichment
TAFI	thrombin activated fibrinolysis inhibitor
TEG	thromboelastogram
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGA	thrombin generation assay
TM	thrombomodulin
tMCAO	transient middle cerebral artery stroke model
Tog25	thrombin binding RNA aptamer
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VKA	vitamin K antagonists
VTE	venous thromboembolism
VWF	von willebrand factor

Acknowledgements

First and foremost, I would like to thank my husband, Patrick Marshall Woodruff. During my graduate school career, we were engaged, got married, bought our first house, got our first dog, travelled to new continents, and had many, many wonderful memories. I could have never gotten through this process without you. Thank you for always being supportive of me, pretending to listen as I prattled on about sciency things, and never letting me take the easy way out. Everyday, I am more and more thankful of the love and relationship that we have.

A close second to thank would be my dog Bailey, who was by my side as I wrote every word of this dissertation, mainly sleeping on the couch. Loyal doesn't even begin to describe her.

I would also like to thank my family, especially my parents to whom this work is dedicated. They met while in their respective PhD program's at UNC, so I didn't really have a chance of being anything else but a scientist. Although David and I hated stopping at every stream on our hikes to count the bugs underneath the rocks, I consider both of you my role models in how a scientist should balance work and life. You both were also models in your respective scientific career paths – with Dad's respected and longtime position as chairman of the Biology Department at VCU and Mom's path first in environmental consulting and then into government, culminating in her senior advisory position in Washington with the EPA. These different career paths gave me a

great perspective on the different paths that a scientist can take, even if our subject matters are completely opposite. I would also like to thank my brother David, who went as far away from science as possible, for giving the rest of our family some balance and someone to call if we ever have computer issues.

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To Jon Kotula – you get your own paragraph! Thank you for getting me through graduate school. I am still convinced that one day we will start a crazy venture that may or may not be a home run (pretty sure that 99% of our ideas would be on the fail pile – but that 1% is going to be legendary). I am so grateful for all of our scientific and life discussions – those have really helped to shape what I want out of my scientific career. Patrick and I are so lucky to have found such awesome friends in you and Michelle – from the Traveling Nachos Doughman Team to making Bloomin' Onions to Aspen vacations. It will be hard to find a group that will get along better than the six of us – Bailey and Meatball included. Our PhD Europe trip is going to be fantastic, and I can't wait to take many more trips with you and Michelle in the future.

To Bruce: I can't begin to tell you how much I admire you – both as a family man and as a scientist. In coming to graduate school, I wanted to find a mentor that mirrored the type of person that I wanted to be – and I have never once wavered in my decision to join your lab. Your commitment to family is unparalleled, especially in such a competitive academic environment. I will always appreciate how you allowed me to take time away from the lab to help my Dad during his cancer treatments, and I have also enjoyed getting to know your daughters and helping them with swimming! In the lab, you have always treated me as an equal, given me enough independence to find my own way as a scientist, and have supported me every step of the way. Thank you for teaching me the importance of collaboration, and also to think very far out of the box. I

greatly appreciate all of the opportunities that you have encouraged me to do during my

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competitive sport. I am not only a much smarter scientist, but also a better person after

six years in your lab.

To the Duke Cycling Team: What an amazing, smart, talented, motivated and

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serious sport, I came to the team in January 2012 looking for a way to try out a sport that

I didn't know much about, but looked fairly fun. Four months later, I find myself flying

across the country to compete in the Collegiate National Championships. I would not

have made it there, and still be racing today, if it wasn't for the help several patient and

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bike etiquette. Thank you for all the crazy conversations that happen when you get an

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with everyone about their respective research has put mine in perspective, and also aided my broader thinking of my project.

A special thanks is in order to my Carolina family. Thanks for letting me go to Duke, and not giving me too much lip about it. I still maintain to this day, that I never bought a shirt or other article of clothing that said Duke on it. Exceptions of course to Duke Cycling, as I proudly wear my Duke kit (absolutely no relation what so ever to Duke basketball).

And to all of the many, many amazing North Carolina friends that we have made… Thank you for you support! Patrick and I are so lucky to have such a wonderful group of friends.

1. Introduction

1.1 Coagulation

1.1.1 Thrombosis and Hemostasis

Hemostasis is the process that occurs to maintain the integrity of the vascular system after injury by keeping blood contained within the circulatory system and to sustain patency of a blood vessel. This process is crucial for life and is highly conserved throughout organisms, from worms to zebrafish to humans (1). Thrombosis, or the formation of a blood clot which can grow to occlude or partially occlude a blood vessel, is thought to result from pathological processes that ultimately overwhelm the normal regulatory systems of hemostasis (2). Thrombosis is responsible for the three most common vascular diseases: myocardial infarction (MI), ischemic stroke, and venous thromboembolism (VTE), which collectively include deep venous thrombosis (DVT) and pulmonary embolism (PE). Including other abnormalities in blood coagulation (such as inherited bleeding disorders) and other coronary diseases, cardiovascular disease (CVD) is the leading cause of death worldwide (3).

The process of thrombosis is often described using Virchow's triad, which states that changes in blood flow, blood composition, or the endothelial cell lining of the vessel walls all contribute to thrombosis (4). In the development of a thrombus, reduced blood flow, stasis, and the resulting decreased oxygen tension can allow for the buildup of procoagulant proteases and mechanisms that can overcome the natural anticoagulant

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processes present at that site. A number of disease states and environmental and genetic

risk factors, such as age, cancer, pregnancy, obesity, and the use of hormone

replacement therapy or contraceptives, which can all result in thrombophilia, can alter

the composition of blood. Thrombophilia is a blood disorder in which blood has an

increased tendency to clot, generally caused by either an increase in clotting proteins,

procoagulant mutations in clotting proteins, decreases in natural anticoagulant proteins,

or decreased fibrinolysis.   The endothelial cells lining the vessel walls are normally

found in an anticoagulant state, with the expression of protein anticoagulants and a

dampened ability to attach to proteins and cells present in flowing blood. Changes in

the endothelial cells, upon reduced oxygen tension or mechanical injury, can drastically

alter their ability in adhering to and activating inflammatory cells, platelets, and

coagulation proteases and thus initiate clotting. While a defect in one of these elements

can go unnoticed, the addition of a second abnormality could be additive or even

synergistic in causing thrombosis, and effective clinical diagnosis and treatment relies on

the simultaneous consideration of all soluble, cellular, and physiological markers (5).

1.1.2 Coagulation Cascade

The ability of blood to coagulate, or form clots, has been documented from the time

of Hippocrates, where it was noted that blood removed from the body congealed upon

cooling (6). This view was widely accepted until the mid 1850's, when scientists showed

that blood clotting could be accelerated in the body with the addition of foreign

substances, therefore, the ability of blood to clot was "intrinsic" to itself. Throughout the

next century, the various components of the clotting cascade were discovered, beginning

with the four basic factors: fibrinogen, prothrombin, thromboplastin (now called tissue

factor), and calcium. At the turn of the century, Paul Morawitz first described the

"classic theory" of coagulation, whereby, in the presence of tissue factor (TF) and

calcium, prothrombin was converted to thrombin, which would then convert fibrinogen

to fibrin to achieve clotting (6). Throughout the next century, the other clotting factors

were discovered, mainly through the examination of coagulopathic patients deficient in

one of these factors.

By the mid-1960s, a "waterfall"(7) or "cascade"(8) schematic of coagulation was

organized, whereby an activated serine protease sequentially activates the next zymogen

in the overall sequence, culminating in thrombin production and fibrin clot formation.

These processes were known to proceed in the presence of a lipid and calcium (Factor

IV). This cascade has become slightly more defined since the publication of those

seminal papers, as the discovery of the action of cofactors, naturally occurring inhibitors,

feedback loops, and multiple ways to activate the cascade have slightly altered the

original format.
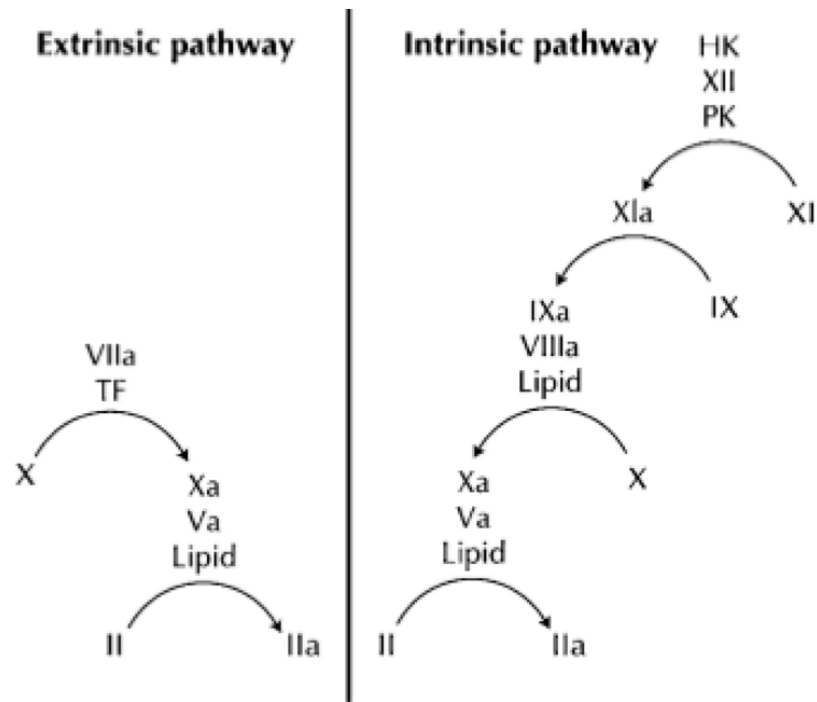
Figure 1. The cascade model of coagulation
Adapted from Hoffman and Monroe, Hematol Oncol Clin North Am, 2007 (9)

Today, this model describes two converging pathways initiated by either

exposure of TF on the vessel wall at the site of injury (extrinsic pathway) or by activation

of blood-borne components in the vasculature (intrinsic, or contact activation, pathway)

(Figure 1). The extrinsic pathway is essential for localized fibrin clot formation at the site

of injury, and is triggered by the binding and subsequent activation of factor VII (FVII)

to exposed endothelial TF, thus forming the extrinsic tenase complex. The intrinsic, or

'contact' pathway, is activated when factor XII (FXII) is exposed to negatively charged

surfaces, causing autoactivation of FXII to FXIIa. FXIIa then activates factor XI (FXI),

which can then activate factor IX (FIX). FIXa can then complex with its cofactor,

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activated factor VIII (FVIIIa), leading to the formation of the intrinsic tenase complex.

The extrinsic and intrinsic pathways converge with the activation of factor X (FX) by

either the extrinsic tenase or the intrinsic tenase. FXa then binds to its cofactor factor Va

(FVa), forming the prothrombinase complex, which can then activate prothrombin to

thrombin, leading to the conversion of fibrinogen to fibrin (1).

While this cascade models elegantly depicts the series of reactions necessary to

form a clot, it does not explain several clinical observations. First, this model does not

depict why patients with hemophilia A or B (FVIII or FIX deficiency) have severe

bleeding phenotypes, even though the extrinsic pathway in these patients is intact. In

addition, patients with deficiencies of the two initiating members of the intrinsic

pathway, FXII and FXI (hemophilia C), either have no bleeding tendencies or an injury-

related variable bleeding tendency, respectively. Thus, the extrinsic pathway could

seemingly compensate for the loss of FXII or FXI, but not FIX or FVIII, an observation

that the current cascade model did not explain (9).

To address these and other observations regarding platelet and endothelial cell

interactions with coagulation factors, a revised, cell-based model of coagulation was

presented in which TF-bearing cells and activated platelets serve as a scaffold for

procoagulant proteins to interact and ultimately form a localized fibrin clot to repair the

vessel wall (10). In this system, coagulation occurs in three overlapping stages:

initiation, amplification, and propagation (Figure 2).

Figure 2. The cell-based model of coagulation
Adapted from Hoffman and Monroe, Hematol Oncol Clin North Am, 2007 (9)

Initiation occurs primarily through the exposed TF/VIIa extrinsic complex on the

surface of an endothelial cell at the site of injury. This complex can then activate FIX,

thus linking the extrinsic and intrinsic pathways, which can then diffuse from the

surface of the cell and localize on the platelet surface. The extrinsic tenase can also

activate FXa on the surface of the TF-bearing cell. This activation is localized by TF

pathway inhibitor (TFPI) or antithrombin (AT), which rapidly inhibits any FXa that

diffuses away from the site of injury. FXa then combines with FVa on the surface of the

TF-bearing cell to form the prothrombinase complex. In this stage, FV can become activated by two ways: 1) by FXa or non-coagulation proteases and 2) the release of FVa from α-granules of partially activated platelets. The resulting prothrombinase complex on the surface of exposed, TF-bearing cells then activate low levels of thrombin (9).

In the amplification stage, this small amount of thrombin can feedback and prime the coagulation cascade for rapid thrombin generation. Thrombin has several roles in this stage. First, thrombin can bind to receptors on the platelet surface, thus accelerating platelet activation. In addition, thrombin activates FVIII, FXI, and additional FV in feedback loops that result in high levels of activated coagulation proteases on the platelet surface. It is this augmentation and activation of various cofactors (FVIII, FV) that leads into the propagation stage, where activated FIX, formed from both FXIa and TF/VIIa, can complex with FVIIIa to activate FX on the surface of the platelet. The resulting prothrombinase complex can then produce the burst of thrombin necessary to form a stable fibrin clot. This generated thrombin can also activate other mechanisms (platelet receptors, FXIII, inhibitors of fibrinolysis) to stabilize the clot (10).

With this model, it is clear that the extrinsic and intrinsic pathways are not redundant; in fact, the extrinsic pathway initiates thrombin generation on exposed TF bearing cells, whereas the intrinsic pathway amplifies thrombin generation on the surface of the platelet to form and stabilize the clot. A notably absent coagulation factor in this scheme is FXII, as thrombin feedback of FXI on the surface of platelet provides

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sufficient amounts of FXIa to boost the production of FIX (11). These observations fit

with clinical data, as FXII does not seem to be essential for hemostasis. However, recent

observations, discussed in more detail later in this chapter, suggest that FXII might have

a mechanism in thrombus formation, as continued FXIa and subsequent thrombin

generation on the surface of a clot may contribute to continued thrombus stability.

While the numerous coagulation proteases work primarily to generate thrombin

and form a fibrin clot, platelets, which are anuclear cells circulating at high

concentration in the blood, also become activated at the site of vascular injury and form

an integral part of the clot that seals the vasculature. At the site of vessel wall injury,

there is numerous crosstalk between coagulation proteases, platelets, and endothelial

cells. While platelet activation is a complex topic reviewed extensively (1, 12), platelets

offer a procoagulant surface for coagulation proteases to generate thrombin, as well as

providing stable adhesion to the vessel wall and mediating signaling activities to

enhance thrombus formation. That platelets are essential for coagulation is evident by

the severe bleeding phenotypes in patients with thrombocytopenia (decreased amount

of platelets in the blood) or genetic disorders affecting platelet pathways.

While the process described above outlines how coagulation occurs to repair

injury to the vascular system, an important physiological concept includes the process of

anticoagulation, which continually works to regulate and localize coagulation to the site

of injury, and also fibrinolysis, which breaks down the clot once it is no longer needed in

order to keep blood vessels patent. While these two mechanisms will not be outlined in detailed, a brief mention of these systems is important in understanding the entire hemostatic system.

Natural anticoagulant mechanisms are critical in preventing widespread clot formation. This is evident by the fact that mice lacking one of the three major anticoagulant pathways, protein C (inhibiting FVIIa and FVa), TFPI (inhibiting FXa), and AT (inhibiting FIXa, FXa and thrombin), are not compatible with life (13). Protein C becomes activated by increasing amounts of thrombin binding to thrombomodulin (TM) on the surface of endothelial cells, which can then cleave protein C held in complex with the endothelial protein C receptor (EPCR). Activated protein C (APC) can then complex with Protein S to effectively inhibit the action of the important cofactors FVIIIa and FVa. Dysfunction of this pathway is implicated in disseminated intravascular coagulation (DIC), which results in widespread clotting and subsequent bleeding from consumption of coagulation factors. TFPI limits the initiation of coagulation by directly inhibiting free FXa, and also interacting with the TF/FVIIa/FX complex. TFPI function seems to be partially dependent on Protein S. AT inhibits three key coagulation proteases, FIXa, FXa, and thrombin, and is as such is one of the more important inhibitors of thrombin generation. True AT deficiency has never been observed in humans, while homozygous deficient mice die in utero, suggesting that AT deficiency is lethal (1).

In order to maintain blood flow, the fibrinolytic pathway degrades fibrin clots once they are no longer needed to seal a site of injury. Plasminogen, the protease that degrades fibrin into soluble fibrin degradation products, is activated by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). While tPA actually binds to fibrin and aids in the lysis of clots, uPA acts on the cellular level. There are several inhibitors of fibrinolysis, including plasminogen activator 1 (PAI-1), α2-antiplasmin, and thrombin activated fibrinolysis inhibitor (TAFI). The importance of these proteins are evident, as patients deficient in plasminogen have an increased likelihood of clotting, and deficiencies of PAI-1 result in an increased tendency to bleed (14). A more complete view of the coagulation cascade, including anticoagulant and fibrinolytic mechanisms, are detailed in Figure 3.
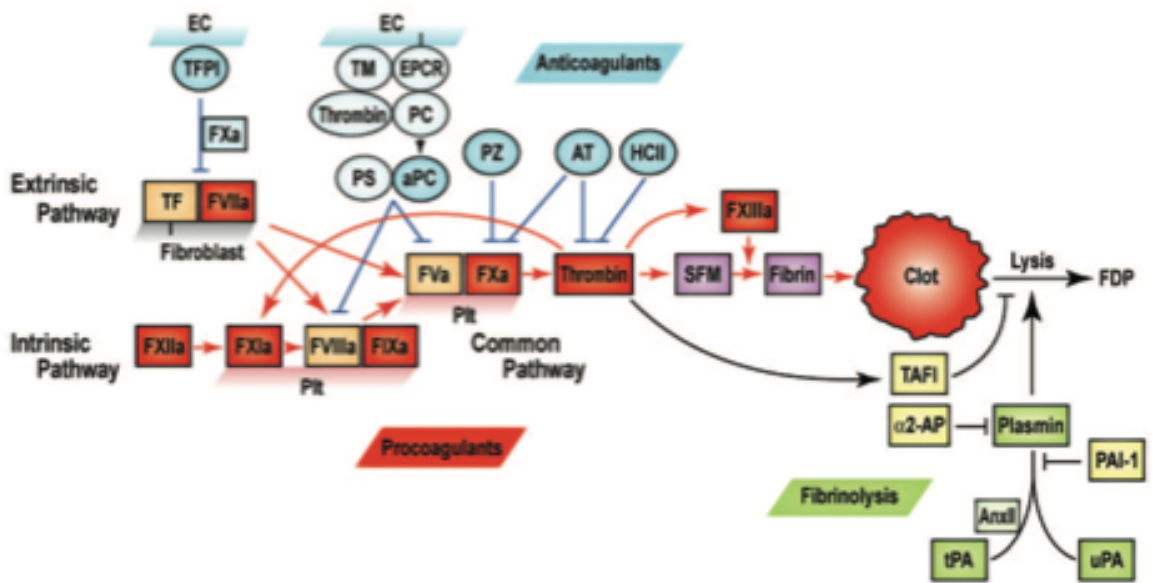
Figure 3. The coagulation cascade, including the anticoagulant and fibrinolytic systems
Adapted from Mackman, Arterioscler Thromb Vasc Biol. 2005. (13)

1.2 Contact Pathway/Intrinsic pathway

Portions of this section have been adapted from the manuscript entitled "The many faces of the

contact pathway and their role in thrombosis" published in the Journal of Thrombosis and

Thrombolysis on July 2011, 32(1): 9-20 and authored by Rebecca Woodruff, Bruce Sullenger, and

Richard Becker, as well as a second manuscript entitled "Antithrombotic therapy in acute

coronary syndrome: how far up the coagulation cascade will we go?" published in Current

Cardiology Reports on July 2010, 12(4): 315-20 and authored by Rebecca Woodruff, Bruce

Sullenger, and Richard Becker.

1.2.1 FXII

Human FXII is an 80kDa glycoprotein that, upon activation, forms α-FXIIa comprised of a 50kDa amino acid heavy chain and a 30kDa light chain linked by a disulfide bond (15). While the light chain contains the catalytic domain capable of activating FXI and prekallikrein (PK), the heavy chain consists of several conserved domains that mediate binding to other proteins and surfaces, including fibronectin type I and II domains, two EGF-like domains, a kringle domain, and a proline rich region (16). The gene for FXII (F12) is closely related to human growth factor activator (HGFA) (17), and contains considerable homologous structural regions, including epidermal growth factor domains, with the fibrinolytic proteins tPA and uPA (18-20).

Binding by the heavy chain to a negatively charged surface, such as dextran sulfate or kaolin, induces a conformational change that allows FXII to be more susceptible to cleavage (21, 22). This cleavage can occur slowly by FXII autoactivation or more efficiently by kallikrein in a feedback loop (23, 24). Biologically, a number of negatively charged substances, including polyphosphates, nucleic acids, mast cell heparin, and collagen, among others, have been found to activate FXII *in vivo* (25-28).

Once activated, surface bound α-FXIIa can cleave FXI and PK, and plasma kallikrein can then reciprocally activate FXII in a positive feedback loop (29, 30). Further cleavage of FXII leads to a smaller 30kDa fragment, β-FXII, which retains catalytic activity for PK but not FXI, and can diffuse away from the activating surface (22, 31).

FXII has also been shown to bind to the surface of endothelial cells (mediated through

the urokinase plasminogen activator receptor (uPAR) (32), as well as platelets (32, 33),

and can thus affect other systems aside from coagulation. Binding of zymogen FXII to

uPAR on endothelial cells can stimulate angiogenesis (34). FXII-mediated activation of

PK leads to activation of the bradykinin (BK) proinflammatory signaling pathway that

can induce vasodilation, chemotaxis of neutrophils, and increase vascular permeability.

In addition, FXII can activate the complement pathway through activation of the C1

esterase (35), as well as trigger the fibrinolytic system through urokinase activation. The

main physiologic inhibitor for FXII is the C1 esterase inhibitor (36), although FXII

activation by platelets seems to be regulated by antithrombin (37). While FXII can

influence a number of systems *in vitro*, FXII's contribution to these systems *in vivo* is

unclear.

Recognition that thrombin can directly activate FXI and bypass the need for FXII-

mediated activation may explain the absence of a bleeding phenotype in FXII-deficient

patients and also supports an emerging theory that FXII-mediated thrombin generation

and fibrin formation is not an absolute prerequisite for normal hemostasis (38, 39).

However, FXII-deficient mice are protected against thrombus formation in several

models of arterial and venous thrombosis, but maintain normal hemostatic capacity.

Intravital microscopy and blood flow monitoring suggest that while thrombus initiation

proceeds following vascular injury, the thrombi themselves are unstable and disperse

rather developing to the point of occlusion (40). Infusion of human FXII into these mice

recovers the wild type phenotype, suggesting that the observed effects are highly

dependent on plasma levels of FXII (40). Protection from thrombosis is not limited to

arterial injury, as FXII-deficient mice have reduced brain infarct volume following

transient middle cerebral artery stroke model (tMCAO) model without an increase in

hemorrhage (41). Therefore, in mice, it is evident that FXII plays a role in pathological

thrombus formation. A search for the primary (or dominant) activator *in vivo* has been

challenging, as a number of negatively charged substances commonly found at the site

of a thrombus, such as polyphosphates, nucleic acids, sulfatides, fatty acids, protein

aggregates, and activated platelets have each been found to activate FXII *in vitro* (27, 42-

44). It is well known that activated platelets can accelerate FXII-mediated fibrin

formation, and the discovery that platelet released polyphosphate can directly activate

FXII may serve as this link (45).

While FXII deficiency in mice confers thrombotic protection, full deficiency of

FXII is rare, and thus not well described. However, there is a history of anecdotal

reports suggesting that complete deficiency of FXII is prothrombotic, including the first

recorded FXII deficient patient, John Hageman, who died from a pulmonary embolism

(46). Other studies have argued against FXII as an independent risk factor for

thromboembolism, but cite other risk factors in a FXII deficient background (47).

Interestingly, a large Viennese patient study (n=8936) showed that all-cause mortality of

patients severely deficient in FXII (<10%) was similar to patients with 100% FXII plasma

levels, suggesting a dual role of FXII in thrombosis (48). Because FXII deficiency causes

unstable thrombi in mice, it may interfere with thrombosis in areas with high shear

stress that can break apart the thrombus before it grows to a larger size. However,

venous thrombi can still develop in areas with low shear stress, and perhaps have a

larger tendency to embolize during FXII deficiency. In addition, other functions of FXII,

such as its growth factor activity or role in inflammation, could also affect mortality

rates in severely deficient patients. Future epidemiologic studies will need to be carried

out to further elucidate FXII's role in humans.

1.2.2 FXI

Human FXI(a) is a 160-kDa serine protease glycoprotein that circulates as a

disulfide linked homodimer of two identical 607 amino acid subunits. Each polypeptide

consists of a 35-kDa C-terminal light chain containing the trypsin-like catalytic domain,

and an N-terminal 45-kDa heavy chain with four ~90 amino acid tandem repeats termed

apple domains (49). These apple domains confer binding to other proteins: A1 contains

binding sites to high molecular weight kininogen (HK) and thrombin (50), A2 & A3 to

FIX (51), A3 to the platelet receptor GP1bα and heparin (52), and A4 to FXII and the

other subunit of FXI (53, 54). FXI circulates in complex with HK, and is structurally

related to PK, as both are hallmarked by the PAN-related apple domains forming a disc-

like structure around the base of the catalytic domain (55) (Figure 4).
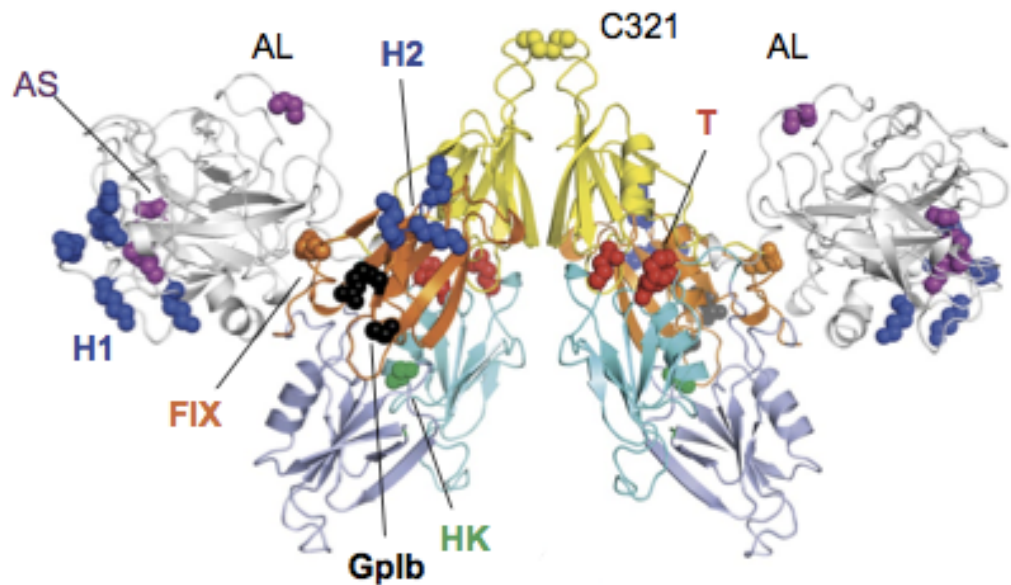
Figure 4. A crystal structure of dimerized zymogen FXI
In this topology diagram, the catalytic domain is in white, and sites of interactions are highlighted. H1 and H2 denote heparin binding sites, T is thrombin, HK is high molecular weight kininogen, Gp1b is the platelet receptor GP1bα, FIX is factor IX, AL is the activation loop cleavage site, and AS is the active site residues. This figure is adapted from Emsley, Blood, 2010 (55).

FXI can be activated by FXIIa and thrombin, both of which cleave at the same site on FXI, and activation proceeds through an intermediate structure with one activated subunit (1/2FXIa) which can be detected in plasma and may represent the predominant active species (56). FXIa subsequently activates FIX to FIXαβ by the sequential cleavage of two activation sites on FIX (56-59). Dimerization seems to be required for FXI activation, as it is thought that the proteases bind to one subunit while activating the other (60). In addition, dimerization might be important in tethering FXI to the site of injury, as the zymogen subunit can bind to the platelet receptor GP1bα, and leave the

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active subunit free to bind to and activate FIX. Zymogen FXI binds to platelets better than FXIa, with approximately 1500 binding sites per platelet (61). Whereas optimal binding requires HK, zinc ions, residues in the apple 3 domain of FXI, and the N-terminal domain of GP1bα, recent work has suggested that FXI binds to the platelet receptor ApoER2, which colocalizes with GP1bα (55). FXIa also binds to platelets, but with a much lower frequency of 250 binding sites per platelet (62). FXI and FXIa bind to platelets through a separate binding site and mechanism, as FXIa binding to platelets seemingly requires the catalytic domain, but not the apple 3 domain, whereas the apple 3 domain is required for FXI binding (55).

Early observations revealed that FXI could be activated by thrombin as well as FXIIa and suggested that this route of bioamplification was favored *in vivo* over FXII-mediated activation (38). Subsequent experiments revealed that FXI-mediated activation of thrombin was essential for continued thrombin generation in the presence of low levels of TF, but not when higher levels of TF were present, such as that found at the endothelial cell sites of injury (63). This continued thrombin generation is important for the activation of TAFI, which can protect the clot from fibrinolysis degradation (63). In addition, the GP1bα receptor on the surface of platelets contains binding sites for FXII, thrombin and FXI, bringing these proteins into close proximity on the platelet surface and allowing FXI to be activated and subsequently activate FIX in the presence of calcium ions, thus amplifying coagulation and thrombin generation (56-59).

The effect of FXI concentration on thrombin kinetics varies widely among individuals (64), likely reflecting the several unique functional characteristics of the protease, as well as the dependency on the levels of other procoagulant proteases, and mirroring the variable bleeding phenotype seen in FXI deficient patients. In a cell based model system, as little as 5% of normal plasma levels of FXI led to some thrombin generation, with a maximal amount of thrombin generated at 50% of normal FXI levels(59). These observations lend to the fact that FXI deficiency, or hemophilia C, has historically been difficult to predict bleeding tendency as there has been a poor correlation between bleeding phenotype and plasma FXI levels. Hemophilia C is a rare bleeding disorder that affects approximately one in 10,000 people worldwide, but is more common in distinct populations, such as Ashkenazi Jews (65). While hemophilia C does not normally result in spontaneous bleeding, it is a mild to moderate bleeding disorder with increased bleeding tendencies in tissues with high fibrinolytic activity. With over 190 reported mutations in the F11 gene (www.factorxi.com), there are two main types of mutation: CRM- (Type I) are mutations causing low FXI plasma levels while CRM+ (Type II) are mostly functional mutations correlating with normal FXI levels but reduced coagulant activity.

Studies on human patients with FXI deficiency have been similarly muddled. While severe deficiency of FXI is rare, there is some evidence that these patients may be protected against VTE and even ischemic stroke (66, 67). The Leiden Thrombophilia

study, a large, population based study, identified high FXI activity levels as a risk factor for DVT (68). In addition, a retrospective analysis of patients reported that FXI activity above 95% was associated with a heightened risk of ischemic stroke (68, 69). Patients with acute coronary syndrome (ACS), compared to patients with stable angina pectoris, have higher levels of FXIa-C1 inhibitor complexes, reflecting acute activation of FXI (70). Overall, while FXI is considered a "procoagulant" and elevated levels have been associated with prothrombotic phenotypes, FXI plasma activity in humans correlates poorly with bleeding risk (71).

The potential uncoupling of FXI's role in thrombosis and hemostasis is supported by observations from several models of arterial thrombosis, venous thrombosis and stroke in FXI- deficient mice and higher species. FXI deficient mice exhibit prolonged aPTT clotting times and reduced FXI plasma activity levels, but had no evidence of a bleeding phenotype. Several studies using a ferric chloride model of arterial injury with these mice demonstrated that FXI deficiency protected from thrombus occlusion at a level comparable to FIX deficiency or high concentrations of unfractionated heparin, but without uncontrolled bleeding as measure by a tail vein bleeding assay (40, 72, 73). The thrombi that did form were unstable and failed to occlude the vessel (40). FXI deficiency also conferred a thromboprotective effect in a tMCAO, with a lower volume of infarcted brain tissue and less fibrin deposition in distal microvessels compared to wild type mice without any detectable hemorrhage (41). The

potential antithrombotic effect of FXI depletion or inhibition has also been observed in

species other than mice. Antibody-mediated FXI inhibition in rabbits increased clot lysis

in jugular veins and reduced thrombus propagation on injured neointima of the iliac

artery (74, 75), while infusion of FXI antibodies in baboons prevented growth of

occlusive thrombi in arterio-venous shunt and vascular graft occlusion models(76, 77).

Administration of a FXI specific antisense oligonucleotide in mice reduced levels of FXI

while conferring an antithrombotic effect similar to warfarin without abnormal bleeding

times (78).

1.2.3 The roles of the contact pathway

The relevance of the contact activation pathway *in vivo* for maintaining

hemostatic capacity has been debated as patients deficient in FXII, PK, or HK do not

typically present with a bleeding phenotype, and FXI deficiency (hemophilia C), is a

mild to moderate, tissue-specific bleeding disorder. In contrast, patients deficient in the

downstream intrinsic pathway factors, such as FIX or FVIII, demonstrate more severe

bleeding tendencies, including spontaneous bleeding, while patients completely

deficient in TF, FX or FII have not been described. Upholding these observations, it is

widely accepted that activation of the extrinsic pathway is the main pathway

responsible for hemostasis. In addition, the actions of FXII can be bypassed by other

constituents of the coagulation pathway, including thrombin, which can directly activate

FXI, leading to continued thrombin generation (38, 79). This mechanism is thought to be

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increasingly important in settings where extrinsic activation, or the amount of TF

present, is low, such as on the surface of a growing clot.

In the past decade, *in vitro* and animal model studies have suggested a larger role

in FXII-mediated activation of FXI in contributing to stable thrombus formation from

continued thrombin generation on the surface of a growing thrombus. In thrombosis,

substances that are present at a developing thrombus, such as aggregated proteins,

extracellular RNA or other nucleotides, collagen, and polyphosphate, could contribute

to the continued activation of FXII and FXI, leading to excessive thrombin generation

and platelet activation to attenuate the growth of the thrombus. The role of FXII and FXI

in thrombosis could vary depending on the event, and while the contact pathway might

play a large role in thrombus formation where or when the extrinsic pathway has been

inhibited, such as on the extended surface of a platelet rich thrombus, FXII and FXI

might not be required for thrombosis at a ruptured plaque, where a large amount of TF

can trigger thrombin generation independently (80).

The procoagulant environment contributing to thrombus formation is not solely

influenced by activation of the coagulation cascade and platelets, but is also affected by

the activity of other systems, including inflammatory or fibrinolytic systems, that FXI

and FXII can also influence. Full understanding of the systems that the contact pathway

affects is essential as many vascular disorders, such as atherosclerosis, are influenced by

a number of these processes. In fact, activated FXII is found at the site of an early

atherosclerotic lesion, and could contribute to the inflammatory and angiogenic

processes that help to develop an atherosclerotic plaque (81). Additional fibrinolytic

and/or inflammatory properties of FXII and FXI could also play a role in pathological

thrombus formation. Therefore, a detailed exploration into all of the contributions of the

contact pathway can provide insight into the mechanisms of thrombosis and guide

future drug development.

1.2.3.1 The Evolution of the Contact Pathway

It is widely thought that a series of gene duplications throughout evolution led to

the complete clotting cascade as it is seen in humans. Thus, all clotting factors contain

many of the same conserved domains and are closely related to one another (19). A

simple version of the clotting cascade appeared in the earliest vertebrates, and as

vertebrates themselves have become more complex with more intricate and contained

cardiovascular systems, the clotting cascade has evolved similarly. While thrombin was

the first coagulation factor to emerge before the appearance of the first vertebrates, the

contact pathway was the last to evolve, as genes for HK, FXII and a single paralog of

PK/XI are found later in vertebrate development (19). A duplication event of the FXI/PK

predecessor gene led to the separation of these factors during the development of early

mammals, with all placental mammals carrying two distinct genes for PK and FXI (18).

Because of this late emergence, these proteins are 58% similar with the same overall

domain structure (82, 83).   Interestingly, the gene for FXII has been lost in some species

during evolution as some marine mammals, such as whales and dolphins, as well as

birds, lack a gene for FXII, supporting the view that this protein is not needed for

hemostasis (19, 84). Similarly, the gene for FXI is absent in birds, such as the chicken,

duck, and vulture (85).

1.2.3.2 Hemostasis and Thrombosis

In the coagulation cascade, FXI can be activated either by FXIIa or in a feedback

loop by thrombin, and these two routes may have diverging functions. FXI-mediated

activation of thrombin is essential for continued thrombin generation in the presence of

low levels of TF, but, when higher levels of TF are present, thrombin generation is FXI,

and also FXII, independent (63, 86). To this end, preliminary studies show that low

TF/FXI deficient mice die *in utero*, while FXI deficient nice have no bleeding phenotype,

suggesting that FXI is essential for hemostasis while the extrinsic pathway is impaired

(87). Because the thrombin-mediated FXI activation pathway seems to be favored *in*

vivo over FXII-mediated activation it is widely considered that FXII-mediated thrombin

formation did not have a significant role in maintaining normal hemostasis (38, 88).

The site at a growing thrombus, however, is quite different from a hematological

challenge, and could differ from thrombus to thrombus. A number of negatively

charged substances found in the body and at sites of vascular injury, such as

polyphosphates (26, 45), extracellular oligonucleotides (27, 89), collagen (25), laminin

(90), and aggregated proteins (44), have been found to activate FXII. While all of these

substances could contribute the FXII activation, the main *in vivo* physiological activator

of FXII, if there is just one, has not been fully elucidated. Polyphosphate released from

activated platelets is a logical FXII activator contained to the site of thrombus formation.

However, polyphosphate can act as a cofactor to augment thrombin's ability to activate

FXI itself and therefore bypass the requirement for FXII. However, there is mounting *in

vivo* evidence that FXII-mediated FXI activation contributes to thrombus formation.

A growing clot at the site of a ruptured plaque could be exposed to larger

amounts of inflammatory proteins or the contents of dead and dying cells, all which

could contribute the activation of FXII. To this end, while mice deficient in FXI or FXII

do not exhibit spontaneous or abnormal bleeding, these mice are protected against

thrombus formation in several models of arterial and venous thrombosis, as well as

stroke. In several models of thrombotic injury, intravital microscopy work and blood

flow monitoring suggest that, while thrombus initiation is not interrupted on the vessel

wall in both FXI and FXII deficient mice, thrombi that are formed are unstable and break

up before occlusion of a blood vessel can occur (40, 72, 73, 91).   Protection from

thrombosis is not limited to arterial injury, as FXII and FXI deficiency also confer a

thromboprotective effect in a tMCAO, with a lower volume of infarcted brain tissue and

less fibrin deposition in distal microvessels without any hemorrhaging detected (41).

MRI's performed in FXII deficient mice undergoing tMCAO found that, while both

groups show severe hypoperfusion in the first two hours after removing the occluding

thread, the FXII deficient mice show an increase in cerebral blood flow from 2 –24 hours

compared to wild type mice (92). The antithrombotic effect of FXI deficiency is also seen

across species, as inhibition of FXI with antibodies in rabbits resulted in increased clot

lysis in jugular veins and reduced thrombus propagation on injured neointima of the

iliac artery (74, 75). In addition, infusion of FXI antibodies in baboons prevented the

growth of occlusive thrombi in arterio-venous shunts and vascular graft occlusion (76,

77). Even at low shear rates in an endothelial denudation rabbit jugular vein model, FXI

was found to contribute to platelet aggregation and fibrin formation (93).

Activation of FXII can lead to activation of FXI, and thus coagulation, but FXIIa

can also activate PK. PK circulates in plasma bound to HK, and once activated, kallikrein

cleaves the nonapeptide BK from HK. BK is a vasoactive peptide that mediates several

inflammatory responses, including vasodilation and vascular permeability. In order to

elucidate which pathway, FXII-mediated FXI activation or FXII-mediated PK activation,

plays a larger role in thrombosis, thrombosis models with animals deficient in these

contact pathway proteins were undertaken. In murine studies with deletions of other

constituents of the contact pathway, BK B2 receptor (the main receptor for BK) and

kininogen gene 1 (encodes for HK) knockout mice caused a delayed time to vessel

occlusion in a Rose Bengal/laser injury carotid artery thrombosis model (94, 95). This

model causes less endothelial damage and collagen exposure than a applying the free

radical FeCl₃. While FXI and FXII deficient mice were able to prolong the time to vessel

occlusion in this model (96), pharmacologic inhibition of plasma kallikrein was shown to be prothrombotic, as shorter occlusion times in the carotid artery after laser injury were observed (97). Conversely, BK2 knockout mice in different strain backgrounds demonstrated opposing phenotypes in an experimental stroke model, with one having reduced infarct volumes and improved motor skills (98), whereas another study showed an increase in infarct volumes and mortality rates (99). While the role of some of the contact pathway proteins in thrombosis has not completely been elucidated, the *in vivo* evidence suggests that FXII and FXI, though not involved in initiating fibrin formation, are involved in stabilizing occlusive thrombi in several animal models.

If FXI and FXII deficiency protect from thrombosis in an animal model, than human should follow suite. In fact, either FXI or FXII deficient humans present with a more complex phenotypic picture. Elevated levels of FXI have been found to be prothrombotic, as FXI levels are considered to be a risk factor for deep venous thrombosis and ischemic stroke (68, 69), and FXI deficiency reduced the incidence of ischemic stroke (67). FXI deficiency does not seem to offer protection against acute myocardial infarction (100), but high FXI levels were found to increase the risk of myocardial infarction. Interestingly, in this same study, low levels of FXII increased the risk of myocardial infarction. FXII plasma activity has been found to exhibit a U-shaped curve with respect to mortality, with the lowest 10% of plasma levels associated with mortality rates comparable to 100% FXII levels (48). However, FXII has not been found

to be an independent prothrombotic risk factor, and no studies have been conducted to determine if FXII deficiency offers thromboprotection (101). These findings highlight the difference between mice and human, and also point to the probable importance of other functions of the contact pathway in modulating thrombosis.

1.3.2.3 Fibrinolysis

In addition to its role in the coagulation cascade, the contact pathway has also been shown to influence fibrinolysis. *In vitro*, it was recognized that the presence of FXI was able to inhibit clot lysis in plasma (63). Further studies found that thrombin mediated feedback activation of FXI contributes to sustained thrombin generation and TAFI production, thus stabilizing and protecting the clot from fibrinolysis (102, 103). TAFI inhibits fibrinolysis of the formed clot by removing carboxy-terminal lysine residues from fibrin, thereby altering the ability of plasminogen to bind to fibrin, and thus preventing the breakdown of the fibrin clot (104). The importance of FXI-mediated continued TAFI production is evident from the increased risk of bleeding in FXI deficient patients undergoing surgeries in tissues with high local fibrinolytic activity. Additionally, inhibition of FXI in a rabbit jugular vein model caused a two-fold increase in endogenous thrombolysis and clot lysis, presumably through reduced activation of TAFI (74).

While FXI acts indirectly as an antifibrinolytic by attenuating thrombin mediated TAFI activation, FXII has been shown to directly and indirectly promote fibrinolysis.

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FXII structurally resembles the fibrinolytic proteins tPa and uPa, and FXIIa and kallikrein can activate plasminogen directly in plasma to contribute to wound healing and tissue remodeling (105, 106). Kallikrein can also directly activate uPa and tPa, and enhance the activation of tPa on endothelial cells by releasing BK, which stimulates tPa release (107). In addition, a recent study showed that FXII could bind to and inactivate PAI-1 to enhance fibrinolysis in an *in vitro* assay (108). Overall, the contact pathway seems to be involved in both pro and antifibrinolytic mechanisms, although the relative efficiency and importance of each system *in vivo* has yet to be determined.

1.3.2.4 Platelets

Activated platelets are known to promote FXII activation and FXII- mediated thrombin generation, although the mechanism of activation *in vivo* has been debated (43, 109). Polyphosphates released from the dense granules of activated platelets can directly activate FXII, trigger coagulation and inflammation, and delay clot lysis, possibly through FXI-mediated TAFI activation (26, 43, 45). Mice deficient in FXII were protected from a lethal pulmonary embolism model triggered by infusion of polyphosphates and exhibited reduced fibrin formation in the lungs, while infusion of human FXII protein corrected thrombosis susceptibility in this model (45). Collagen, a strong platelet activator itself, is also able to independently bind to and activate FXII to induce thrombin generation and clotting in a FXI-dependent manner (25, 40). Under flow conditions, collagen-initiated thrombin formation in reconstituted platelet rich

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plasma was highly dependent on the presence of FXII and FXI, and this dependence was abolished by adding tissue factor, suggesting that FXII- and FXI- mediated thrombin generation is particularly important in conditions where the extrinsic pathway has been impaired (25). The relative importance of polyphosphate-mediated activation of FXII versus collagen activation, as well as the possibility of FXII activation from other materials present in a developing thrombus such as RNA and aggregated proteins, will require detailed investigation, although the unstable thrombi in FXII deficient mice suggest that one or possibly all of these factors effect FXII- mediated coagulation (26, 27, 40).

Binding to platelets serves as an important mechanism for FXI-mediated FIX activation, as thrombin can activate enough FXI on the platelet surface without any FXII contribution to sustain thrombin generation (59). A naturally occurring FXI mutant that cannot bind platelets has a severely reduced rate of activation by thrombin (110), whereas thrombin activates FXI at a 5-10,000 fold enhancement when bound to the platelet surface (61). This enhancement could be due to the recent finding that polyphosphates release from activated platelets serve a cofactor to greatly increase thrombin activation of FXI (111). While there are ~1500 binding sites for FXI on the surface of the platelet (61), FXIa binds to only ~250 sites (112), suggesting that FXI and FXIa bind in a different mechanism. In fact, FXI and FXIa do not compete for binding on the platelet surface (62), and biochemical studies suggest that FXI binds to GP1bα in

complex with either HK or prothrombin through its exposed A3 domain, but, once activated, residues in the catalytic domain are required for binding (112). Dimeric FXI seems to be required for binding, as monomeric FXI has a reduced activity in activated platelet dependent clotting assays, suggesting that FXI binds to GP1bα with one subunit and to FIX with the other (58). In addition, FXI has recently been shown to bind to ApoER2, a receptor that co-localizes with GP1bα on the platelet surface, and FXI or FXIa can induce platelet aggregation and intracellular signaling events on wild type, but not ApoER2-deficient platelets (113). Binding of FXI to the platelet surface can help to localize hemostasis to only the site of injury, as the FXI inhibitor protein nexin 2 released from activated platelets inhibits only free FXIa (112, 114). Thus, platelets can accelerate the activation of both FXII and FXI in several respects, and may serve as important mediator of intrinsic pathway activation at the site of a growing thrombus.

1.3.2.5 Inflammation

Activation of the contact system can induce inflammatory reactions through activation of the classical cascade of the complement system, leading to anaphylatoxin production causing smooth muscle cell contraction, enhanced vascular permeability and recruitment of inflammatory cells to produce a local immune response. Mast cells are commonly found at sites of infection, and are associated with increased vascular permeability in host defense of allergic reactions. Heparin released by these mast cells has been shown to activate FXII, thus leading to release of BK, a small peptide that is

cleaved from HK by kallikrein which induces vascular permeability (28, 35). BK can also

bind to its receptor on endothelial cells to promote nitric oxide and prostacyclin release,

inducing smooth muscle cell relaxation and vasodilation (115). FXII seems to play an

integral part of contact-stimulated BK generation, as plasma levels of BK are reduced in

FXII deficient mice, even though kallikrein can be activated independent of FXII by

other proteins (heat shock protein 90, prolylcarboxypeptidase) (116). Activation of both

of these systems causes the signs and symptoms seen in septic shock and hereditary

angioedema. In septic shock, FXII can be activated by bacterial cell walls, endotoxin,

polyphosphate, and various microbial proteases present from bacteria in the blood,

leading to a rapid decrease in blood pressure and possibly disseminated intravascular

coagulation, a thrombotic and hemorrhagic disorder resulting from widespread

misregulation of the clotting and fibrinolytic systems (117-120). Unregulated contact

pathway and complement activation is also implicated in hereditary angioedema (HAE),

a condition caused by a deficiency or defect in the main inhibitor of these two pathways,

C1-inhibitor (121). HAE is marked by the recurrent episodes of acute, localized increase

in vascular permeability in the skin and various mucosal tissues, mediated through the

action of BK and complement anaphylatoxin. HAE-TypeIII is caused by a mutation in

the FXII gene, however, plasma concentrations and aPTT clotting times are both normal

in these patients (101). Kallikrein and BK may also be involved in cross-talk with the

renin-angiotensin system involved in regulating blood pressure and fluid balance.

Constituents of the contact system can also bind to and activate immune cells directly, as kallikrein can induce neutrophil homing, aggregation, and degranulation (115, 122, 123), and FXII mobilizes leukocytes to the site of injury by activating the chemoattractant chemerin (124). FXII also stimulates monocytes to produce the cytokine interleukin-1 (125). In addition, activated HK can bind to receptors on monocytes and neutrophils, stimulating the release of cytokines and inhibiting their migration, keeping them at the site of injury, and contributing to a chronic inflammatory response (115). Overall, these studies indicate that all of the contact factors, except FXI, may play an important role in modulating the inflammatory response pathway. Thus, inhibition of FXI is touted to have less potential off-target effects, and therefore be a more attractive therapeutic target.

1.3.2.6 Angiogenesis

Binding of contact pathway proteins to endothelial cells can also induce angiogenesis, and this generation of new blood vessels can be important in tumor progression and wound healing. HK and PK bind to endothelial cells through a multiprotein receptor complex, including complement receptor gC1qR, uPAR, and cytokeratin 1 (126). While activation of PK can occur on the surface of endothelial cells without FXII by the enzyme prolylcarboxypeptidase (95), FXII can bind to gC1qR and increase the rate of kallikrein formation (127). Assembly of contact factors in the presence of Zn^{2+} ions on endothelial cells results in the release of BK, which, through the

action of its receptors, stimulates cell migration and proliferation by increasing the

concentration of angiogenic mediators (32, 128, 129). In addition, BK was recently

shown to enhance the homing of circulating endothelial progenitor cells, a mechanism

also important in forming new blood vessels (130).

Zymogen FXII is also directly implicated in angiogenesis, as FXII can bind to

uPAR and induce cell proliferative intracellular signaling events at levels just 12.5% of

its normal plasma concentration (34, 128). In addition, FXII induced aortic sprouting in

mice at concentrations as low as 3.2% of normal levels, and FXII deficient mice had

fewer vessels in skin biopsies compared to wild type (34). While FXII is not essential for

developmental angiogenesis as FXII deficiency is not lethal, it could contribute to the

repair process after injury or inflammation. HK and FXII compete for the same binding

region on the gC1qR-uPAR-cytokeratin 1 complex, and plasma concentrations of HK

inhibit FXII binding and thus inhibit FXII- mediated signaling. Activated HK itself

inhibits angiogenesis by preventing endothelial cell migration and adhesion through

disruption of the uPA-uPAR signaling complex that regulates these processes (32, 34,

131).

1.3.2.7 Inhibitors of the contact pathway

Inhibition strategies aimed at FXIa include antisense oligonucleotides,

neutralizing antibodies, and peptide and small molecule inhibitors. Several antibodies

have been tested in higher animals, including rabbits and baboons, and all were

efficacious in preventing thrombus occlusion without any reported effects on bleeding times (76, 77, 96). Administration of a FXI specific antisense oligonucleotide in mice reduced levels of FXI while conferring an antithrombotic effect similar to warfarin without abnormal bleeding times (78). A selective small molecule inhibitor has recently been described that prevents activation of FIX and prolongs clotting times (132).

Novel 3-carboxamide-coumarin inhibitors were developed to be potent and selective inhibitors of FXIIa (133); however, they did not protect mice from ischemic stroke in a tMCAO at the given concentration (134). D-Pro-Phe-Arg chloromethyl ketone (PCK) inhibits the amidolytic activity of FXIIa, was able to increase clotting time in a dose-dependent manner, and protect mice against ischemic brain stroke in a tMCAO (41). However, PCK is not completely selective for FXIIa, as it is a more potent inhibitor of kallikrein (135), and PCK treatment was prothrombotic in a Rose Bengal murine thrombosis model (97). The fourth domain of a specific thrombin inhibitor, infestin, that was isolated from the insect *Triatoma infestans*, can specifically inhibit FXIIa (136). This protein, termed infestin-4, was fused to recombinant human albumin, and was highly specific for FXIIa. This compound protected mice from ischemic stroke and protected rats and mice from arterial thrombosis without affecting hemostasis (136). Several inhibition strategies against both proteins mirror the data seen with factor deficient mice and encouragingly protect against thrombosis without affecting bleeding.

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1.3 Antithrombotics

1.3.1 Cardiovascular Disease

CVD includes any disease of the heart or blood vessels and is the leading cause

of morbidity and mortality, accounting for one in three deaths in the United States.

While mortality trends have declined since 2000, CVD has accounted for more deaths

that any other major cause of death since the early 1900s. Currently one in three adults

have one or more types of CVD, with the prevalence higher amongst men and

increasing with age. Thus, CVD costs more than any other major diagnostic group and

currently constitutes over 17% of overall national health expenditures. Unfortunately,

by 2030, 40.5% of the population is predicted to have a CVD, with direct medical costs

expected to triple to an estimated $818 billion (3, 137).

The three most common CVDs include acute coronary syndromes (ACS), stroke,

and VTE. ACS includes an array of symptoms relating to an obstruction of the coronary

arteries, including MI and unstable angina. In coronary artery disease (CAD),

insufficient amounts of oxygen are reaching the myocardium, most commonly due to

arteriosclerosis, or the buildup of a lipid-filled plaque in the endothelium of one or up to

all three coronary arteries. This plaque can slowly grow over the course of years to

occlude the blood vessel, or rupture, thus immediately forming a thrombus that can

occlude blood flow, and thus oxygenation of the myocardial tissue. Prolonged occlusion

can cause death of the myocardium, resulting in an MI. Angina pectoris is chest pain

associated with temporary myocardial ischemia that does not occur long enough for

death of the heart tissue to occur. While stable angina is associated with chest pain due

to increased oxygen requirements of the myocardium when coronary blood flow is

reduced, most likely due to an atherosclerotic plaque, unstable angina is associated with

a partially occlusive thrombus and can lead to MI.

Atrial Fibrillation (AF) is the most common heart arrhythmia and can be caused

by a number of different risk factors, including hypertension and CAD. In AF, the left

atrium is not contracting properly, leading to blood stasis and an increased probability

for thrombus formation. AF greatly increases the risk of stroke, as thrombi formed in

the atrium can travel to the cerebral artery to result in an ischemic event. Ischemic

strokes account for 85% of all strokes, and stroke, as a whole, is the leading cause of

severe disability (137).

VTE occurs with blood clots forming in the large veins, oftentimes in the legs, a

process termed deep vein thrombosis (DVT). While there are approximately 1-3 cases

per 1000 people per year, the prevalence of VTE increases with age, with approximately

1 in every 100 people per year over the age of 50 presenting with a VTE (4). Long

periods of immobilization, such as that which occurs on long plane flights or leading a

sedentary lifestyle, results in blood stasis, which can increase the risk for VTE. In

addition, orthopedic surgery, such as hip or knee replacements, can increase the risk for

VTE up to two months. A pulmonary embolism (PE) is an embolized thrombus,

oftentimes from a DVT, that travels to and blocks the pulmonary artery. PE is the third

most common cause of hospital-related death, and one-quarter of PE's result in sudden

death (137).

While arterial thrombosis is largely responsible for ACS, including MI, stroke,

and peripheral artery disease (PAD), which occlude obstructions in other large arteries

(excluding coronary, aortic or brain), venous thrombosis is primarily responsible for

VTE, including DVT and PE.   These two types of thrombosis differ in mechanism, as

outlined below, and thus in the types of agents used to treat them (Figure 5).
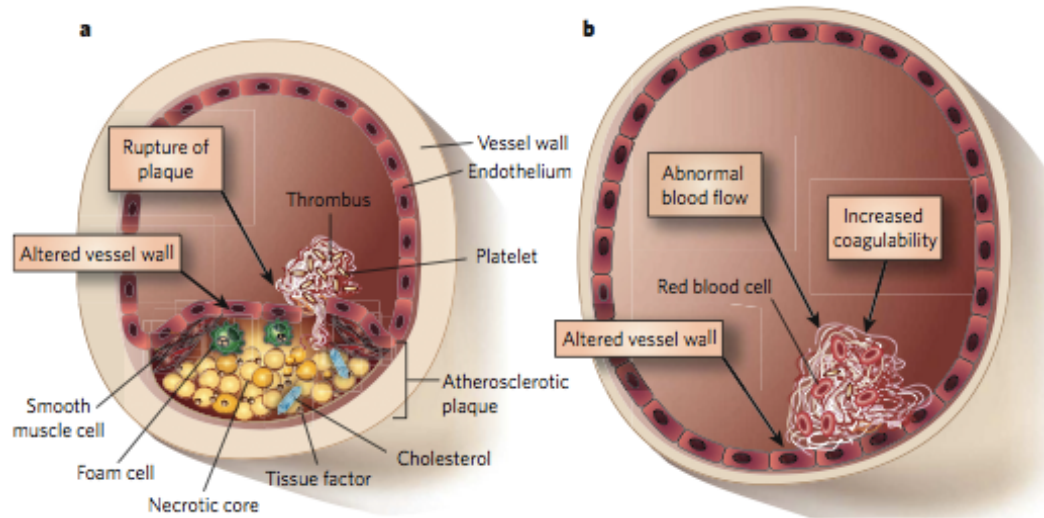
Figure 5. A depiction of two types of thrombosis
The differences between (A) arterial and (B) venous thrombosis. Adapted from
Mackman, Nature 2008 (138).

Arterial thrombosis is associated with atherosclerosis, and often occurs on the

surface of a ruptured atherosclerotic plaque. Exposure of procoagulant material, such as

collagen, von willebrand factor (VWF), lipid-rich macrophages, TF and subendothelial

cells in the high shear arterial environment rapidly recruits platelets to the site of injury

and activates procoagulant proteases, thus forming platelet-rich "white" clots. These

clots can grow to occlude an artery, where they can cause disrupted blood flow to the

tissue supplied by the artery. Arterial thrombi are the most common cause of MI and

stroke, collectively the most common cause of death in the world. Risk factors

associated with arterial thrombi include hypertension, diabetes, smoking, and

hypercholesterolemia. Interestingly, the risk factors for arterial thrombosis only

modestly increase VTE risk, and vice versa, thus supporting that these thrombotic

disorders have different mechanisms (5).

Venous thrombosis usually occurs with "red" clots in the large veins in the legs,

where part or all of this thrombus can break away and travel to the lungs, possibly

forming a PE and disrupting the flow of blood in the pulmonary artery. Prolonged

immobilization, such as that which occurs during bed rest after surgery or prolonged

periods of sitting, can cause blood stasis and decreased oxygen tension, especially in

sinus valve pockets in the large leg vein. Decreased oxygen tension can induce

endothelial cell stress response systems, resulting in activation of cell adhesion

molecules on the surface of the cell that recruit platelets, microparticles, and

inflammatory cells, all of which carry low levels of TF. This local increase in TF, in

addition to a higher number of procoagulant proteases present due to blood stasis, leads

to activation of the coagulation cascade, thrombin generation and fibrin clot formation.

In addition, inflammatory cells release neutrophil extracellular traps (NETs), DNA,

RNA, and platelet-released polyphosphate, thus activating the intrinsic pathway

through FXII, and contributing to more thrombin generation (139). While

immobilization alone might not cause DVT, genetic factors including deficiencies in

anticoagulant proteases or prothrombotic mutations, and other acquired risk factors

such as age, surgery, hormone replacements or contraceptives, and obesity, can increase

the risk for developing a DVT.

In general, arterial thrombosis is treated with a class of drugs called antiplatelets, which prevent platelet activation and aggregation, while venous thrombosis is treated with anticoagulants, which prevent fibrin clot formation. Collectively, these two classes of drugs are called antithrombotics, and are divided into classes based on their mechanism of action. Antithrombotics are also used to prevent blood coagulation in surgical settings, where catheter insertions, stent placements, extracorporeal circuits or orthopedic surgery can present a highly thrombogenic environment.

1.3.2 Anticoagulant agents

Anticoagulants are generally used to prevent the generation, growth and embolization of a thrombus, although they do not dissolve an already present clot. There are several classes of anticoagulants, including the vitamin K antagonists (VKA), FXa inhibitors, and direct and indirect thrombin inhibitors. A partial list of the currently approved anticoagulants, including their targets, mechanism of action, and indication, are outlined in Table 1.

Table 1. Current anticoagulant agents

All information was obtained from www.fda.gov

Drug Class	Generic Name	Trade Name	Type of Drug	Route	Use
Indirect Thrombin Inhibitors	Heparin	Heparin	Glycosamino-glycan	Parental	Prophylaxis & Treatment of DVT; PCI; CABG
	Enoxaparin	Lovenox	Glycosamino-glycan	Parental	Prophylaxis & Treatment of DVT
	Fondaparinux	Arixtra	Pentasaccharide	Parental	Prophylaxis & Treatment of DVT
VKA	Warfarin	Coumadin	Small Molecule	Oral	Prophylaxis & Treatment of DVT; Prophylaxis & Treatment of VTE in valve replacement & AF; Prophylaxis of recurrent MI or stroke
Direct Thrombin Inhibitors	Argatroban	Acova	Small Molecule	Parental	Prophylaxis & Treatment of DVT; PCI
	Bivalirudin	Angiomax	Peptide	Parental	Prophylaxis & Treatment of DVT; PCI
	Dabigatran	Pradaxa	Small Molecule	Oral	Prophylaxis of VTE & stroke in nonvalvular AF
Direct FXa Inhibitors	Apixaban	Eliquis	Small Molecule	Oral	Prophylaxis of VTE & stroke in nonvalvular AF
	Rivaroxaban	Xarelto	Small Molecule	Oral	Prophylaxis & Treatment of DVT; Prophylaxis of stroke in nonvalvular AF

There are two routes of administration of anticoagulants: parental or oral.

Parental anticoagulants are traditionally used in acute care settings when a rapid onset

is necessary, such as a patient presenting with unstable angina or MI, during cardiac

operations, or for short-term prophylaxis of VTE after orthopedic surgery (140). The

indirect thrombin inhibitor heparin is a glycosaminoglycan that enhances antithrombin's

inhibition of thrombin and FXa. Heparin has been a mainstay of anticoagulation since

the 1940s. Because of its rapid and predictable mode of action, heparin is oftentimes

used to initiate anticoagulant therapy. In addition, heparin is commonly used in

surgical settings, such as cardiopulmonary bypass (CPB), because of its strong

anticoagulant effect and its ability to be reversed by a small, positively charged protein,

protamine. Heparin, while affective, is associated with several complications, including

heparin-induced thrombocytopenia (HIT) or heparin-induced thrombocytopenia with

thrombosis (HITT), which results when heparin interacts with other extracellular

proteins, such as platelet factor 4, to cause immune reactions (138). Lower molecular

weight heparins, such as enoxaparin (Lovenox) or fondaparinux (Arixtra), are safer and

easier to administer and monitor, and have thus replaced heparin for prophylactic

treatment of VTE. However, these heparin derivatives cannot be reversed by protamine,

and are thus not used for surgical procedures (141). The parental direct thrombin

inhibitors, such as argatroban (Acova) and bivalirudin (Angiomax), have recently been

introduced to replace the indirect thrombin inhibitors in patients with or at risk of

developing HIT or HITT, who cannot be given heparin, during percutaneous coronary

intervention (PCI, or "angioplasty") surgery or as a prophylactic treatment of VTE.

While these parental anticoagulants are effective, they all confer a high risk of bleeding,

with no direct antidote available to reverse their action.

For long-term anticoagulation therapy, an oral anticoagulant is administered

after initial treatment with a rapidly acting parental anticoagulant. In general, oral

anticoagulants are easier to administer and have higher patient compliance for chronic

therapy, as parental anticoagulants require repeated injections. The oral VKA, warfarin

(Coumadin), has been the mainstay of oral anticoagulant therapy for the past 65 years. While effective, warfarin has several drawbacks that have led for a search for new oral anticoagulants. Namely, warfarin has numerous food and drug interactions, a narrow therapeutic window, and is difficult to dose and monitor, with over half of patients failing to maintain an acceptable level of anticoagulation (140). This puts a high number of patients at risk for bleeding events (over-anticoagulation) or thrombotic events (under-anticoagulation).

Recent advances have led to emergence of new oral anticoagulants directly targeting either thrombin or FXa that will likely begin to replace warfarin.   These compounds include rivaroxaban (Xarelto), apixaban (Eliquis) and dabigatran (Pradaxa), which all show efficacy in preventing short-term VTE for surgical indications such as hip or knee replacement. These oral anticoagulants have also shown efficacy in long-term anticoagulant therapy for the life-long prevention of stroke in patients with AF, which represent a large and growing population (142). While dabigatran has already been licensed for this indication, other direct FXa inhibitors are in advanced stages of development. These new anticoagulants show comparable efficacy to warfarin with less bleeding incidents, indicating that the field has made a large step in replacing warfarin in this at-risk population (142).

1.3.3 Antiplatelet agents

Chronic antiplatelet therapy is used to treat a range of cardiovascular diseases associated with arterial thrombosis, including treating patients with MI, stroke, or angina, as well as prevention in patients at-risk for these events and others, such as PAD or AF. In addition, antiplatelet agents are also used as a rapid onset anticoagulant in cardiac surgeries, especially PCI, where insertion of foreign bodies, such as a catheter or a stent, can cause thrombosis. Several of the currently used antiplatelet agents are summarized in Table 2.

Table 2. Current antiplatelet agents
All information was obtained from www.fda.gov

Target	Generic Name	Trade Name	Type of Drug	Route	Use
PDE	Cilostazol	Pletal	Small Molecule	Oral	Treatment of PAD
PDE	Dipyridamole	Persantine	Small Molecule	Oral	Prophylaxis of thrombosis in valve replacement
P2Y12	Prasugrel	Effient	Small Molecule	Oral	Prophylaxis of thrombotic CV events in ACS; Prophylaxis of stent thrombosis
P2Y12	Clopidogrel	Plavix	Small Molecule	Oral	Prophylaxis of thrombotic CV events in ACS; Prophylaxis of stroke, MI, or PAD
P2Y12	Ticagrelor	Brilinta	Small Molecule	Oral	Prophylaxis of thrombotic CV events in ACS
P2Y12	Ticlopidine	Ticlid	Small Molecule	Oral	Prophylaxis of stroke; Prophylaxis of stent thrombosis
GPIIb-IIIa	Abciximab	ReoPro	Monoclonal Antibody	Parental	PCI
GPIIb-IIIa	Eptifibatide	Integrilin	Peptide	Parental	Prophylaxis of thrombotic CV events in ACS; PCI
GPIIb-IIIa	Tirofiban	Aggrastat	Small Molecule	Oral	Prophylaxis of thrombotic CV events in ACS
COX	Aspirin	Aspirin	Small Molecule	Oral	Low Dose: Long term prophylaxis of MI & stroke

Aspirin has been the gold standard for chronic oral antiplatelet agent use, as it is effective in reducing the incidence of MI and stroke without increasing the risk of bleeding (142). Clopidogrel (Plavix) is more recent antiplatelet agent that has shown widespread tolerance across patient populations with ACS, as well as slightly more efficacy than aspirin in reducing serious vascular events. However, both of these agents were only able to reduce the incidence of these events by approximately 20%, leaving a wide proportion of the population at risk, and a high probability for breakthrough thrombotic events (12, 142).

The limited efficacy of aspirin and clopidogrel, which only target one receptor on the platelet surface, respectively, suggests that the redundancy of the platelet activation pathway, where any number of pathways can be activated by different stimuli and lead to platelet aggregation, is important, and that only inhibiting one pathway or receptor might not be efficacious (142). To this end, combination therapy, either combining an antiplatelet and an anticoagulant agent, or two antiplatelet agents (such as aspirin and clopidogrel), is hampered by an increase in major bleeding, thus not leading to a clinical benefit for chronic use. This type of therapy is reserved for use in high-risk populations, as well as inhibitors against the platelet receptor GPIIb-IIIa, which is the key receptor in mediating platelet aggregation. These inhibitors exhibit high bleeding risk, but are still used in certain settings, such as in PCI to inhibit catheter and stent-mediated thrombosis, where their use can be highly monitored and controlled (12).

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Newer antiplatelet agents have been developed that have higher potency than clopidogrel, but target the same pathway. Prasugrel (Effient) is P2Y12 receptor antagonist that exhibits more potent and consistent inhibition of platelet aggregation (142). Unlike clopidogrel, ticagrelor (Brilinta) does not have to be metabolized, and thus has a fast onset of action, a characteristic that lends itself well to surgery, where anticoagulation needs to occur quickly. The challenge of both of these agents, however, is their increased risk of bleeding compared to clopidogrel (142).

Overall, antiplatelet therapy has suffered due to a narrow therapeutic window, where too much inhibition can cause bleeding and too little inhibition can cause breakthrough thrombotic events. Another problem with antiplatelet therapy is that irreversible inhibitors are present for the life of a platelet, so a patient on antiplatelet drugs ideally should stop therapy for several days before surgery to decrease the risk of bleeding. Oftentimes, this is not possible for this subset of the population, as urgent surgery in ACS patients is often necessary (141). The combination of the correct dose and target is important, and further studies into the inhibition of alternative platelet receptors has been underway to attempt to identify new, safer antiplatelet agents.

1.3.4 Ideal Antithrombotic Agents

While cardiovascular disease is already the leading cause of death in many westernized countries, the global incidence of cardiovascular disease is expected to

continue to increase, as more cultures are changing their lifestyle to lead more sedentary

lives and eat higher fat diets. As such, the antithrombotic market is expected to increase

from approximately 4 billion in 2010 to 12 billion in 2016 in the United States alone, from

a combination of increased patient population for long term therapy due to rising

incidence of obesity and atherosclerosis, and an increase of cardiac procedures

performed in the aging population (143). While a number of new agents have been

introduced into the market that have more favorable profiles than the standard of care,

there is still a large unmet need for convenient and effective antithrombotics that do not

incur a bleeding risk. This risk of bleeding associated with antithrombotic drug therapy

can be caused by many factors, including narrow therapeutic windows (the balance

between inhibiting thrombosis and causing bleeding), patient drug metabolism

variability, an undefined threshold of anticoagulation needed for clinical benefit,

complex interactions between the drug and multiple hemostatic factors, and limited

control of the drug's pharmacodynamics (144). As in the example of antiplatelet

therapy, increased potency does not always mean increased efficacy, and increased risks

of bleeding can actually decrease the clinical benefit of a more potent therapeutic.

There are several ways to design an antithrombotic with a more favorable safety

profile. The first strategy is to formulate a drug that has a rapid onset and offset of

action. After drug administration is halted, the compound is rapidly cleared from the

body and anticoagulation values quickly return to baseline in the absence of drug

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administration. This strategy requires a large amount of compound, and is not viable

for a chronic therapy setting. An alternative drug design strategy is to develop a stable

compound that can be rapidly controlled with an antidote. Antidote reversal of

stabilized drugs reduces the amount of compound required (and therefore the cost),

increases the number of indications that the drug can be used for, and enhances the

safety profile (145). In addition, an active antidote can be appropriately dosed to reverse

some, but not all, of the circulating drug, allowing for a higher level of anticoagulant

control. This ability of antidotes to modulate, rather than completely neutralize the

therapeutic compound offers clinicians the ability to fine-tune the drug's titratability and

the level of clinical anticoagulation in real time (145). Currently, the only antidote-

reversible antithrombotic available is the heparin/protamine pair, which is the standard

for clinical anticoagulation despite dangerous side effects and complex and

unpredictable pharmacodynamics that make dosing a challenge. Other, newer

anticoagulant agents that have potentially less bleeding risk than heparin, such as

enoxaparin or argatroban, do not have an antidote, making their clinical use limited

(146).

Because of the wide range of cardiovascular events and the different mechanism

contributing to each, a "magic bullet" therapy that is universally effective is not likely

(12). Instead, therapeutics designed to treat closely related cardiovascular events and

settings are needed. For example, cardiologists in a hospital setting need an effective

antithrombotic for highly thrombogenic settings, including cardiology procedures and critical care settings, where the risk of bleeding might not offset the clinical benefit. This antithrombotic ideally has a rapid onset of action, is most often parentally administered, is highly effective at anticoagulation, and can be instantly reversed after surgery or if bleeding complications arise. In contrast, a parental antithrombotic for a long term chronic setting might not be feasible (141). A long-term antithrombotic must be conveniently administered, is most often orally administered, be highly tolerable in a wide range of patient populations, have a predictable pharmacokinetic and pharmacodynamics response, have compatibility with other reagents, and be effective without causing bleeding or have an antidote readily available if bleeding complications arise. While there are similarities between these two strategies, in general, the most important characteristics involved in designing a successful antithrombotic is reliability, convenience, and safety (143).

Another strategy in striking the balance between inhibiting thrombus formation (efficacy) and reducing the risk of severe bleeding (safety) of an antithrombotic includes inhibiting the correct target. While most of the factors involved in hemostasis and thrombosis are conserved, determining the differences between these two states, and subsequently only inhibiting those factors involved in thrombosis, can lead to an effective antithrombotic with a reduced bleeding risk. In general, targeting of the downstream, essential coagulation factors interrupts both thrombosis and hemostasis to

ensure that blood clotting cannot occur. In these settings, an antidote is needed in order

to control potential bleeding for this strategy to be safely administered. While most of

the currently approved antithrombotics target these downstream factors, their use is

limited due to the high risk of bleeding associated with therapy, and no antidote to

reverse it. However, recent studies have suggested that, at least in animal models,

inhibition of the upstream, intrinsic coagulation factors XI and XII can hinder

thrombosis without affecting normal hemostatic events. While these factors are

associated with the stability of a pathological thrombus, they are not essential for

hemostasis in humans, as deficiencies of FXI and FXII do not confer a severe bleeding

phenotype. Therefore, pharmacological inhibition of FXI and FXII, or other factors not

involved in hemostasis, could produce an effective anticoagulant to treat and prevent

thromboembolic disorders without a high risk of bleeding.

1.4 Aptamers and SELEX

Portions of this section have been adapted from the manuscript entitled "Antidote-control

of aptamer therapeutics: The road to a safer class of drug agents" published in Current

Pharmaceutical Biotechnology on August 2012, 13(10):1924-34 and co-authored by Kristin

Bompiani and Rebecca Woodruff, as well as Richard Becker, Shahid Nimjee, and Bruce Sullenger.

Other portions of this section were adapted from the manuscript entitled "Application of

aptamers for targeted therapeutics" published in Arch Immunol Ther Exp (Warsz) on April

2013, and co-authored by Partha Ray, Kristi Viles, Erin Soule and Rebecca Woodruff.

1.4.1 Emergence of Aptamers & SELEX

The central dogma of molecular biology states: "DNA makes RNA makes

protein." While the oligonucleotides in this equation are responsible for information

storage and transfer, proteins actually carry out all the functions of the cell. A clue that

this might not precisely be the case was discovered when the structure of tRNA was

solved, and it was determined that highly structured RNA's serve a function other than

information storage in the translation process. The discovery of catalytic RNAs was so

groundbreaking that it was later awarded the Nobel Prize. Out of this oligonucleotide

revolution came the idea of an RNA world, where more diverse oligonucleotides than

what is present today could have been able to both encode and carry out complex

functions until amino acids took over part of their job. To do this, oligonucleotides

would have needed to evolve diverse shapes and affinities towards different substrates,

and this information inherently would have been encoded by their linear sequence. By

screening large libraries of single stranded oligonucleotides, scientists can identify the

exact sequences responsible for binding to molecules, including nucleic-acid binding

proteins (147).

The process of SELEX (systematic evolution of ligands by exponential

enrichment) was first developed to quickly discover the specific nucleotide sequence(s)

essential for binding to a DNA or a single stranded RNA binding protein. In their

groundbreaking paper, Tuerk and Gold demonstrated that large libraries of randomized

oligonucleotide (RNA or DNA) sequences could be screened *in vitro* for ligands that

bind with the highest affinity to T4 DNA polymerase (148). Using this process, their

group discovered that T4 DNA polymerase recognizes eight specific nucleotides. Two

aptamers, or specific sequences of DNA, dominated the finished pool and bound with

equal affinity: one with the exact nucleotide sequence of its natural DNA binding site

and one with four differences. These binding sequences were termed "aptamer" after

the Greek *aptus,* meaning "to fit" (149). Early on, it was determined that SELEX could

actually select sequences that bind with higher affinity than those that are present in

nature (150).   Because the *in vitro* SELEX process is not constrained by biological fitness,

these additional, and sometimes truncated, sequences that bind with higher affinity can

be isolated. This process was therefore exploited, and during the last twenty years, has

been used to isolate aptamers to a diverse number of proteins, including transcription

factors, proteases, viral proteins, growth factors and cell adhesion molecules, among

others.

　　　The starting pool of a selection is a combinatorial nucleic acid library containing

a certain number of randomized nucleotides, flanked on either end with constant

regions to aid with molecular biology techniques. The randomized region is essential for

library diversity, and can range from a few nucleotides up to hundreds of nucleotides,

and can be continuous or found in blocks. Importantly, enough randomization of the library needs to occur in order to form enough unique stable aptamer structures (or oligonucleotide sequences) to perform the selection. A library with approximately 25 randomized nucleotides yields $\sim10^{15}$ different aptamer sequences, which allows for almost all sequences present in the starting pool to be examined (147).

Traditionally, the SELEX process begins by incubating this nucleic acid library with a purified protein target or molecule. Oligonucleotides that bind to the protein or molecule are separated from the unbound aptamers through various techniques (nitrocellulose filtration, column chromatography, immunoprecipitation, *etc.*) and are subsequently amplified to generate an enriched pool with increased binding affinity for the target. The entire process of incubation, partitioning, elution, and amplification completes one round of SELEX. This process is repeated under increasingly stringent conditions to isolate a pool of oligonucleotide sequences that bind with the highest affinity for the target.   Increased stringency is generated by creating greater competition for binding to the target in a round; this is facilitated by either increasing the RNA to protein ratio, increasing salt concentrations, or decreasing the overall amount of protein used. The number of rounds required to drive a selection to completion depends on both the innate ability of the target to bind to the library oligonucleotide and how much stringency is increased in each round. Importantly, stringency is often kept minimal during the beginning rounds to reduce the risk of loosing a high affinity binder when

there are many aptamer sequences present and limited number of areas on the protein to bind. After significant increases in binding affinity by the pool are made, and weak or non-binders have been selected out, stringency can be increased to quickly drive the selection to completion (147).

Several variations on the traditional SELEX procedure have since been derived from the original method described by Tuerk and Gold. For example, "toggle" SELEX was developed to isolate an aptamer that can bind to conserved regions on two homologous proteins, primarily between a protein from two different species (151). This method was developed to facilitate aptamer cross-reactivity for pre-clinical studies. "Complex" selection is used to create a pool of aptamers that can bind to multiple targets in a complex mixture, such as the plasma proteome or red blood cell membranes, to create a focused library (152, 153). This focused pool can be amplified and used to isolate aptamers from nearly any protein found in the complex mixture in approximately half the number of rounds compared to traditional SELEX (152). Other types of SELEX have been developed with cells or a whole organism as the target, not just soluble proteins or molecules. Cell-based SELEX can be employed in the cancer biomarker setting to discover aptamers that selectively bind to cancer cells, but not normal cells (154). Similarly, aptamers have been isolated that can detect differences between pancreatic cancerous cell secretomes versus non-cancerous secretomes (155). In

addition, SELEX has been performed *in vivo* in a mouse to identify aptamers that bound specifically to cancer metastases (156).

The SELEX process generates an enriched pool of aptamer sequences that demonstrate high affinity binding toward the target. After completion of SELEX, the oligonucleotide pool is cloned and sequenced, yielding individual aptamer sequences. Enriched pools at the end of a selection often have "structural families", or sequences that share common stretches of nucleotide composition or common structural motifs. If SELEX has been driven to completion, the final pool is comprised of a single aptamer sequence. While SELEX completion produces an obvious candidate compound for further testing, generation of sequence families can yield valuable information as conserved structural domains across structural families may indicate a region of sequence that is important for target binding and/or functionality.

Once several aptamer sequences have been identified, *in vitro* studies are used to compare the individual aptamer sequences for their binding affinity and intended functionality toward the therapeutic target (enzyme inhibition, receptor-mediated signaling, *etc.*). The aptamer with a combination of the tightest binding and most potent functionality is selected for optimization and further studies.

1.4.2 Aptamers as Therapeutics

In essence, aptamers are designer ligands that can be made toward theoretically any target molecule of interest. As a novel class of compounds that has rapidly gained clinical interest over the past twenty years, aptamers combine the best characteristics of small molecules and antibodies and have some additional unique characteristics of their own.

As small, single-stranded oligonucleotides, aptamers form specific three-dimensional structures that allow them to bind to their targets with high affinity and specificity. Crystal structures of aptamer-protein binding have shown that aptamers fold into a tertiary structure and present an extended conformational surface that is complementary to the target protein, thus binding and concealing a large surface area on the protein (157). There are numerous electrostatic, hydrophilic, and van der Waals interactions within the extended aptamer/protein binding interface, including hydrogen and pi-bond stacking with the amino side chains of the proteins. Because of these specific interactions, aptamers often bind their targets with dissociation constants (K_ds) that are typically in the low nanomolar (10^{-9}) to high picomolar range (10^{-12}). The unique surface, shape, and charge of the aptamer-binding region on the target profoundly influences the affinity of aptamer-target binding and thus dictates specificity. A single substitution of an amino acid or nucleic acid base in the binding region can completely disrupt aptamer binding and thus function (158). Since a large part of the target protein

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is concealed by aptamer binding, aptamers tend to act as antagonists by blocking protein-protein interactions.

Unlike small molecules or antibodies, aptamers can easily be pharmacokinetically manipulated, thus controlling their duration of action from a few hours to days. Unmodified oligonucleotides, especially RNA, are rapidly degraded in biological fluids by endogenous endonucleases. Therefore, internal modification strategies with chemically synthesized bases are oftentimes utilized to increase resistance to enzymatic hydrolysis. Modified bases are typically introduced at the beginning of the SELEX process, since a change in chemical structure can influence aptamer folding and target binding. Commonly, the 2' ribose position of the pyrimidine nucleotides is modified to include functional groups such as an amine (2'N), O-methyl (2'OMe) or fluoro (2'F), as substitutions at this position are effective at increasing nuclease resistance (159). These modifications can easily be incorporated during in vitro transcription by using a mutant form of RNA polymerase (RNAP) that can recognize its respective modified base. While all of these modifications greatly increase the half-life of an aptamer in biological fluids, there are subtle unique advantages and disadvantages to each type of modification. Overall, many aptamers have been generated with 2'fluropyrimidines, as higher transcription yields incorporating this modification makes the development process easier. However, 2'OMe modifications have traditionally been less expensive than any of the other common modifications, and are thus attractive in

clinical development of aptamers (159). Although internal modification of the aptamer

sequence protects against endonuclease cleavage, a 3′end cap of deoxythimidine is often

incorporated to protect the end of the aptamer from exonuclease cleavage.

Because aptamers are relatively small (8-15 kDa) and thus are rapidly cleared by

the kidneys, bulky, inert moieties, such as cholesterol or polyethylene glycol (PEG), are

typically conjugated to the 5′ end of an aptamer to increase the molecular weight,

thereby decreasing the clearance rate and increasing the half-life of an aptamer *in vivo*.

Cholesterol conjugation of an aptamer can increase the half-life to several hours (160),

while PEG conjugation can extend the half-life to days or even weeks, depending on the

mode of administration (161). Therefore, the bioavailability of a specific aptamer can be

easily manipulated.

Similar to small molecule therapeutics, aptamers can be chemically synthesized

by solid-phase oligonucleotide synthesis to generate large, homogenous and highly

purified quantities of compound. Shorter aptamers, like other custom oligonucleotides,

have higher yields and are most cost effective to produce; thus, aptamers are typically

truncated to less than 50 nucleotides or the smallest functional sequence possible.

Additionally, as synthetic molecules, the chemical composition of individual bases in the

aptamer sequence can be altered to increase the yield or decrease the cost of

manufacturing, as long as the changed modification does not disrupt the aptamer's

structure or function. Previously, 2′F modified nucleotides could often be substituted

with the less expensive 2'OMe nucleotides, resulting in a functional aptamer that is more cost effective to produce in high quantities. However, the cost of 2'F modified nucleotides has recently been reduced to a similar cost as 2'OMe nucleotides, allowing for the feasibility of developing a therapeutic 2'F modified aptamer.

In addition to being chemically synthesized, aptamers have a stable shelf life and can recover their folded structure after wide temperature fluctuations. Moreover, aptamers are well tolerated in the body, and can thus be repeatedly administered (162). Overall, ribonucleic acids are non-immunogenic, with extensive analysis in clinical trials. The incidence of allergic reactions have been low, and thought to be caused by antibody generation to the PEG group appended to the aptamer (163).

While the above properties of aptamers highlight their flexibility and utility as potential therapeutics, the most unique characteristic of aptamers over antibodies or small molecules is their ability to be functionality controlled with antidotes. Two types of antidote control have been developed: a sequence specific antidote that recognizes the primary sequence of the aptamer and disrupts the aptamer's structure, and a universal antidote that nonspecifically binds to an oligonucleotide and can reverse the activity of any aptamer, independent of sequence or shape (164, 165).

A matched oligonucleotide aptamer antidote takes advantage of the fact that aptamers form stable secondary conformations, such as hairpins and pseudoknots, which fold into a stable tertiary structure to bind to a complementary surface on their

target (157). Theoretically, a molecule that binds to and alters the tertiary structure of an aptamer should disrupt the ability of an aptamer to bind to its target protein. Because oligonucleotides inherently bind to each other through simple Watson-Crick base pairing, a short, complementary "antisense" oligonucleotide disrupts the structural elements essential for binding and abolishes aptamer activity (164).

Although matched oligonucleotide antidotes can successfully modulate the function of aptamer anticoagulants, there are a few drawbacks of this strategy. First, this method of antidote design is labor intensive and costly, because custom antidotes must be designed and tested for each new aptamer. Moreover, it can be challenging to develop oligonucleotide antidotes to highly structured aptamers (unpublished data). Finally, because aptamer-oligonucleotide antidote complexes are double-stranded RNA, they may trigger an innate immune response through activation of toll-like receptors, although there has been no direct evidence of this to date.

Because of these potential limitations of oligonucleotide antidotes, a universal antidote strategy was developed. In theory, a universal antidote is a molecule that can bind to and control the activity of any aptamer, regardless of sequence or conformational shape. In healthy individuals, no endogenous oligonucleotides circulate in the blood; therefore, an antidote that can recognize and bind to any oligonucleotides will be able to neutralize the administered aptamers, regardless of sequence. Because all aptamers are oligonucleotides and thus negatively charged, the most obvious choice for

a universal antidote is a positively charged molecule that can bind an aptamer through electrostatic interactions. Several compounds were shown to be successful universal antidotes, including protamine and a number of nucleic acid binding polymers, both *in vitro* and *in vivo*. In contrast to the specific base pairing mechanism required for oligonucleotide antidotes, binding of a universal antidote to the oligonucleotide backbone is thought to envelope the aptamer, thus providing a barrier around the folded aptamer to prevent binding to the therapeutic target. In essence, a universal antidote functions like a "molecular sponge" by binding and sequestering any systemically administered oligonucleotides.

Both types of antidotes have several benefits that led themselves well to therapeutic administration. Because of their specificity and ability to modulate aptamer functionality, matched oligonucleotide antidotes can allow clinicians to fine-tune therapy to the degree required, rather than turn it on or off. This level of drug control can allow clinicians to respond immediately to urgent clinical needs and is a valuable advance in drug therapy. In addition, if two aptamers are administered simultaneously, one could use an oligonucleotide antidote to specifically turn off a single aptamer, thus reinstating the functionality of a single pathway while leaving the other pathway(s) inhibited. However, the clear advantage of a universal antidote lies in it its simplicity and cost-effectiveness - one antidote compound can be used for any aptamer

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therapeutic. Additionally, a universal antidote could simultaneously reverse the activity of any aptamer combinations.

The high binding affinity and specificity of aptamers allow for isolation of an artificial ligand for theoretically any therapeutic target of interest. Chemical manipulations of aptamers also allow for fine-tuning of their bioavailability, and antidote control greatly expands their clinical use. Thus the aptamer platform offers a promising therapeutic.

1.4.3 Current aptamer therapeutics

The concept of using nucleic acids to modify protein function originated from the study of viruses, where short, structured RNAs encoded by the virus bound to proteins to either assist with virus replication or inhibit antiviral responses. In the first study testing the idea of a therapeutic aptamer, Sullenger et al. demonstrated that the single stranded RNA TAR aptamer could successfully inhibit HIV virus replication by binding to the natural nucleic acid binding site of the viral protein Tat, and thus act as a decoy to block normal Tat function (166, 167). Coupled with the emergence of SELEX, it was recognized that this process could be used to rapidly generate oligonucleotide designer ligands to essentially any therapeutic target, including soluble proteins and enzymes, prions (pathogenic misfolded proteins), cellular surface receptors, and transcription factors (168).

While a number of therapeutic aptamers have been developed, only one aptamer has gained FDA approval: an anti-VEGF aptamer, Macugen, for treatment of age-related macular degeneration (169). Since its approval in 2004, a number of novel aptamers have been introduced into clinical trials, as summarized in Table 3.

Table 3. Aptamers in clinical trials

Only the most recent trial for each aptamer was included. Data from
www.clinicaltrials.gov.

Aptamer (company)	Target	Condition	Phase (status)	Identifier (www.Clinical Trials.gov)
ARC1905 (Ophthotech Corporation)	Factor C5 of the complement cascade	Age-Related Macular Degeneration	Phase I (unknown)	NCT00950638
E10030 (Ophthotech)	Platelet-derived growth factor-B (PDGF-B)	Neovascular Age-Related Macular Degeneration	Phase 2 (completed)	NCT01089517
NOX-A12 (Noxxon Pharma AG)	Stromal cell-derived factor-1 (SDF-1)	Multiple Myeloma	Phase 2 (recruiting)	NCT01521533
NOX-E36 (Noxxon Pharma AG)	Monocyte chemotactic protein-1 (MCP-1)	Type 2 Diabetes Mellitus, Albuminuria	Phase 2 (recruiting)	NCT01547897
Pegaptanib sodium (Valeant Pharmaceuticals International, Inc.)	Vascular endothelial growth factor (VEGF-165)	Age-Related Macular Degeneration, Retinal Vein Occlusion, Diabetic Macular Edema	Phase 4 (active, recruiting)	NCT01573572
REG1 (Regado Biosciences)	Factor IX	Acute Coronary Syndrome	Phase 2 (completed)	NCT00932100
AS1411 (Antisoma Research)	Nucleolin	Acute Myeloid Leukemia	Phase 2 (terminated)	NCT01034410
ARC1779 (Archemix Corp.)	Von Willebrand Factor	Von Willebrand Disease	Phase 2 (withdrawn)	NCT00694785
ARC19499 (Archemix Corp.)	Tissue Factor Pathway Inhibitor (TFPI)	Hemophilia	Phase 1/2 (unknown)	NCT01191372

While aptamers function well as antagonists due to their propensity to inhibit protein-protein interactions, they can also be used to stimulate protein function. A multimerized aptamer targeting the immune surface costimulatory receptor 4-1BB was generated that mimicked natural ligand binding to active 4-1BB and promoted T-cell proliferation, survival, and expansion *in vitro* and led to tumor rejection when administered *in vivo* (170).

In addition to functioning as antagonists or agonists, it was quickly realized that the ability to easily manipulate aptamers offers a platform for targeted delivery. Aptamers can be made as chimeras, with a small molecule, nanoparticle, toxin, splice-switching oligonucleotide, or siRNA, to name a few, appended to the end of the aptamer sequence, to act as a delivery molecule for targeted therapy (171). While the aptamer targets a certain protein and offers specificity, the cargo attached to the end of the aptamer imparts a function. While there are several examples in the literature of this, one of the first displaying this concept was for targeting prostate-specific membrane antigen (PSMA) positive prostate cancer cells *in vitro* (172). An aptamer targeting PSMA was hybridized with an anti-PLK1 or anti-BCL2 siRNA and the complex was shown to specifically bind to PSMA receptor on the surface of the cancer, be internalized, and silence PLK1 or BCL2 function, resulting in apoptosis and decreased cell proliferation. This technology has wide application in the cancer field, offering a tumor-specific targeting device for other therapeutic agents, hopefully leading to targeted cancer

therapy. While there are many aptamers targeting tumor markers available, each aptamer has to be validated individually, as internalization rates and methods differ from receptor to receptor. Identifying a single aptamer that can internalize into many cancerous cell types would be advantageous in further developing this therapeutic (173).

Aside from direct applications as therapeutics, aptamers have been designed to have clinical diagnostic functions ranging from the detection of tumor cells or cancerous secretomes to uses in imaging, flow cytometry, and microarray detection. The multiple functions of therapeutic aptamers have been extensively reviewed (168, 171, 174)

1.4.4 Aptamers as antithrombotics

While the use of antithrombotics will only increase as more of the population is expected to have a cardiovascular disease, the field itself has been hampered by nonselective agents with narrow therapeutic windows and no dosing flexibility or reversal agent. As a therapeutic, aptamers lend themselves well as anticoagulants, as aptamers targeting coagulation factors can be systemically delivered with rapid onset in a highly reproducible and readily measurable manner, as well as be rapidly modulated or completely reversed within minutes after the administration of an antidote. Thus, aptamers targeting several members of the coagulation pathway have been developed, and a few of those compounds have progressed into clinical trials.

1.4.4.1 Inhibiting the common pathway

Prothrombin/Thrombin

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Thrombin is the final step in the blood coagulation pathway, and mediates many

important features of blood coagulation, from cleaving fibrinogen into fibrin to

activating platelets. Thrombin has three main binding sites: the catalytic active site, and

two anionic bindings sites: exosite I and II, which are located on opposite sides of the

protein. Exosite I is present on both prothrombin and thrombin, and binds to

fibrinogen, FV, FVIII, thrombomodulin, and platelet PAR receptors. In this way, exosite

I mediates fibrinogen cleavage, feedback activation of clotting cofactors, and platelet

activation. The other anion binding site, exosite II, binds to heparin and also to a platelet

surface receptor. Therefore, exosite II mediates platelet activation, as well as inhibition

by heparin. Thus, inhibitors targeting different sites on thrombin have different modes

of action, and combining inhibitors to both exosites can result in a very potent inhibition

of thrombin function (175).

Because of thrombin's central role in coagulation, this well-characterized protein

has been the target for some of the first *in vitro* aptamer selections. The first aptamer to

be isolated to a non-nucleic acid binding protein was termed HD1, or is also known as

ARC183. This aptamer is a unmodified 15 nucleotide single-stranded DNA aptamer

that forms a stable G-quadraplex and binds to exosite I, thus inhibiting exosite I

functions, including fibrinogen cleavage (176, 177). Due to its anticoagulant activity, this

aptamer was explored in several animal models as a cardiopulmonary bypass agent. In

cynomolgous monkeys, HD1 has a very short half-life (108 seconds) and rapid clearance

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by the body (2-4 minutes); therefore, a continuous infusion was needed to achieve

efficacious anticoagulation (178). Once administration was halted, coagulation values

returned to baseline in approximately 10 minutes, abolishing the need for a reversal

antidote. While preliminary human studies were commenced, the large quantities of

aptamer required for continual administration for anticoagulation resulted in

suboptimal dosing profile (179).

HD22 is another unmodified DNA aptamer, 29 nucleotides long, that also forms

a G-quadraplex. This aptamer, however, binds to exosite II on thrombin to inhibit

thrombin-mediated activation of platelets, but has minimal effects on fibrinogen

cleavage (180). A very potent DNA aptamer was made by designing a hybrid aptamer

joining both HD1 and HD22 with a linker. HD1-22 thus binds to both exosites,

effectively shutting down thrombin function (70). A third DNA aptamer, Nu172, is a

26nt single stranded unmodified DNA aptamer, which produced a dose dependent

increase of clinical coagulation assays during phase I testing in healthy male volunteers.

Since this aptamer has a short half-life, after terminating infusion of Nu172, coagulation

values returned to baseline. This aptamer is currently being evaluated in phase II

clinical trials for acute cardiovascular surgical procedures (NCT00808964).

While many of the first thrombin aptamers were DNA, several RNA aptamers

targeting thrombin have since been developed. Whereas unmodified DNA has a very

short half-life in the body, RNA can be modified to be resistant to endonuclease

cleavage, thus greatly increasing an aptamer's plasma stability. A 25nt 2'F pyrimidine

RNA aptamer, Tog25, was selected against thrombin using a "toggle" SELEX method to

promote species cross-reactivity. In order to isolate an aptamer that was efficacious in

both human and porcine, the selection was toggled back and forth each round with

human and porcine thrombin (151). Tog25 binds to exosite II of thrombin, thus

inhibiting platelet activation but having a minimal effect on fibrinogen cleavage (158).

In contrast to the G-quadruplex architecture of the DNA aptamers, Tog25 has a

traditional stem-loop structure with an internal bulge. A crystal structure of Tog25 in

complex with human thrombin shows that the RNA forms an elaborate three-

dimensional structure to present an extended molecular surface complementary to the

protein. A number of key interactions, including an "A-Arg zipper", which involves a

number of adenine-arginine stacking interactions, as well as hydrogen bonds and van

der Walls interactions, contribute to the intricate folding that allows Tog25 to tightly

interact with thrombin (Figure 6) (157). While Tog25 is not itself a potent anticoagulant,

dual administration with the exosite I binding DNA aptamer, HD-1, results in

synergistic anticoagulation, similar to the bivalent DNA aptamer described above (175).
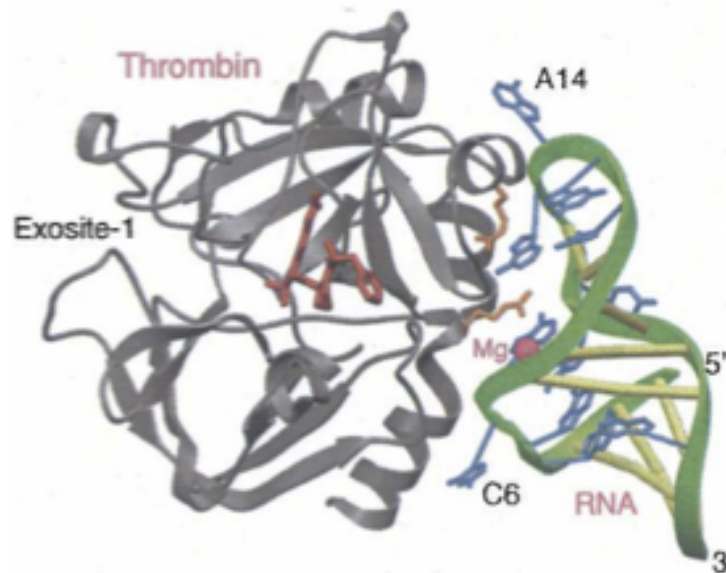
Figure 6. Crystal structure of the RNA aptamer Tog25 to thrombin
Adapted from Long, et al., RNA, 2008 (157).

Recently, another 2'F pyrimidine modified RNA aptamer was described. R9d14t

is 58 nucleotides and binds to both prothrombin and thrombin at exosite I, thus

inhibiting fibrin clot formation, FV feedback activity, and platelet activation mediated by

the PAR receptors. In addition, because the aptamer binds to the pro-exosite I on

prothrombin, it inhibits thrombin generation by the extrinsic tenase complex. This

aptamer is a potent inhibitor of clinical clotting assays. In addition, an oligonucleotide

antidote was generated towards R9d14t that can rapidly and stably reverse

anticoagulation. While this aptamer binds to the same exosite as the DNA aptamer HD-

1, R9d14t is a more potent inhibitor of thrombin due to its increased stability and higher

binding affinity (181). Aside from NU172, the other thrombin aptamers described above

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have not advanced clinically due to limited anticoagulant ability, rapid clearance, or chemical instability. R9d14t is a promising thrombin aptamer therapeutic with an elegant reversal strategy; however, prothrombin is present at a high concentration of approximately 1.4μM and will therefore require large amounts of compound to fully saturate the target. Further optimization of Rd914t will be warranted before pre-clinical studies can proceed.

FX

FX combines with its cofactor, FVa, on the surface of a platelet to cleave prothrombin to thrombin. While FX has some protease activity itself, the formation of the prothrombinase complex yields a ~10^5 fold increase in thrombin generation. Since prothrombinase cleavage of thrombin is the only reaction not duplicated in the coagulation pathway, inhibition of this complex would yield a potent anticoagulant. An aptamer, 11F7t, was isolated that binds to FX and FXa. This aptamer works as a potent anticoagulant in clinical coagulation assays by blocking the assembly of the prothrombinase complex (182). Biochemically, 11F7t binds to FX and competes for binding with FVa. Therefore, in the presence of aptamer, the prothrombinase complex cannot form, resulting in a decreased amount of thrombin able to be formed. Interestingly, the aptamer does not affect membrane binding, catalytic site activity, or influence substrate binding. In addition, 11F7t blocks the ability of FXa to activate FVIII in a feedback loop, as well as blocking TFPI from inhibiting FXa (182). As a potent

anticoagulant, this aptamer unfortunately does not exhibit a high amount of cross-reactivity to other species (unpublished data). Optimization of the aptamer, or the isolation of a separate FX aptamer, would be needed in order continue developing this aptamer as a therapeutic.

1.4.4.2 Inhibiting the extrinsic pathway

<u>FVII</u>

The principle activator of the extrinsic pathway, and also the major activator *in vivo*, is the TF/FVIIa complex. Upon TF exposure, FVIIa binds to TF, allowing FVIIa to then cleave FIX and FX to their active proteases, and thus initiate thrombin generation at the site of injury. An inhibitor against FVIIa would therefore shut down the initiation of thrombin generation. A 2'aminopyrimidine RNA aptamer, termed 16.3, was generated that binds to FVIIa and potently inhibits a prothrombin time clotting assay. This aptamer inhibits the TF/FVIIa mediated activation of FX, at least in part by preventing this complex to form, to function as an anticoagulant (183). Unfortunately, the folding of this aptamer is temperature sensitive, and at 37°C, has decreased binding affinity for FVIIa. Therefore, this aptamer was not further developed.

1.4.4.3 Inhibiting the intrinsic pathway

<u>FIX aptamer</u>

The serine protease FIX is a critical component in coagulation, where it plays a fundamental role in both the initiation and propagation phases leading to thrombin

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generation and stable fibrin clot formation. Although a member of the intrinsic

pathway, FIX can be activated by both FXI and the TF/FVIIa complex. It then forms a

complex with its cofactor, FVIIIa, to activate a large amount of FX on the platelet surface.

Inhibiting FIX is thought to be a "safer" target, since current FIX-targeting agents show

reduced bleeding risks at their effective doses. However, at higher doses, these agents

still have bleeding risks, making an antidote strategy attractive.

A 35 nucleotide, 2'F pyrimidine modified RNA aptamer was isolated that

inhibits FIXa activity and prolongs plasma clotting in a dose-dependent manner.

Optimal doses of this aptamer induce a transient, Hemophilia A-like state where >99%

FIXa activity is inhibited. This aptamer can also be rapidly reversed by a number of

short 2'OMe RNA antidote oligonucleotides (164). Biochemical studies indicate that this

aptamer blocks an extended substrate binding site so as to inhibit FIX-mediated

activation of FX, but it does not inhibit FVIII binding to FIX (184).

This FIXa aptamer-antidote pair was next tested in a porcine cardiopulmonary

bypass surgery model, and it was found that this strategy could limit thrombin

generation and inflammation more effectively than heparin while exhibiting none of the

dehabilitating side effects, such as reduced cardiac function, that are associated with

protamine administration (185).   Because of its low immunogenicity, this regimen could

lend itself well to applications in patients who cannot tolerate heparin/protamine (HIT,

HITT, or sensitization to protamine) and currently have no other option for reversible anticoagulation.

Because of the success in the preclinical development of establishing the FIX aptamer-antidote pair as a nontoxic, predictable, and reversible system in animals, the aptamer-antidote pair was next optimized for use in humans and moved into clinical development as the REG1 Anticoagulation System (Regado Biosciences, Durham, NC). The REG1 system consists of two compounds: RB006 is the FIXa aptamer further modified with active 2'F and 2'OMe nucleotides and a 3' inverted deoxythymidine cap conjugated to a 40kDa PEG carrier to increase its half life and RB007 is a 2'OMe modified 15 nucleotide RNA oligonucleotide antidote complementary to a portion of RB006 (162) (Figure 7).
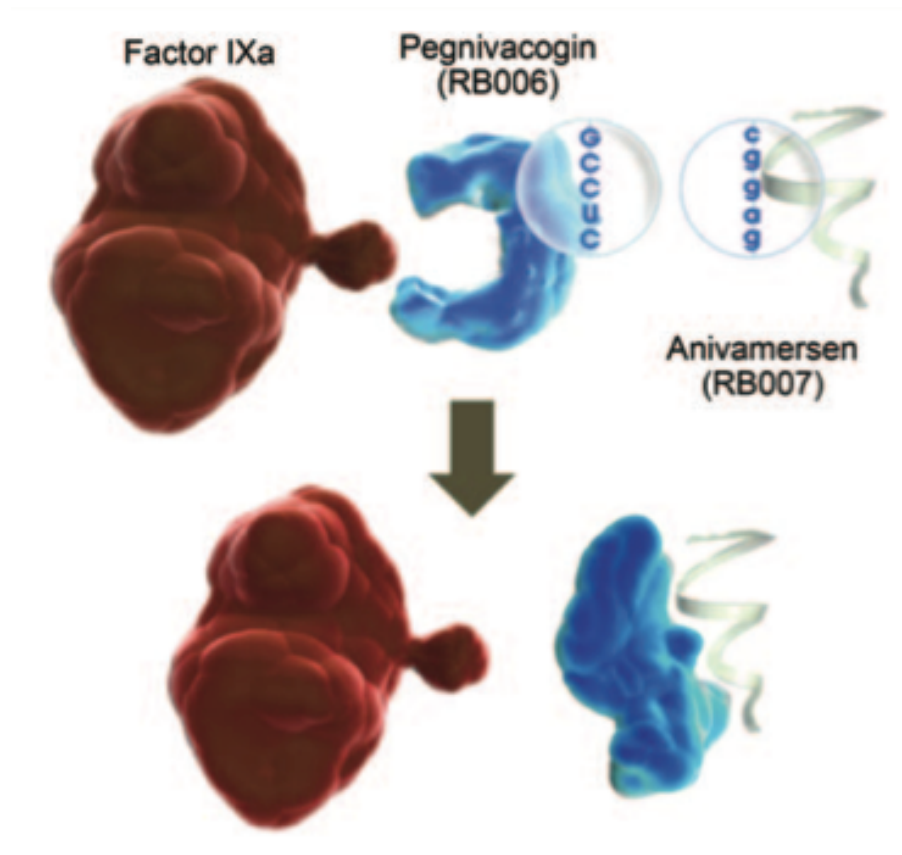
Figure 7. The REG1 anticoagulation system
Adapted from Povsic, et al., *Euro Hrt Jrl.*, 2012 (163)

Three phase one clinical studies were performed to determine if the REG1

Anticoagulation System would be well tolerated in healthy subjects and patients with

stable CAD currently on antiplatelet agents, as well as to determine the relationship

between drug dose and clinical monitoring tests (161, 186). Overall, The RB006/RB007

complex was found to be both stable and biologically inactive, with irreversible binding

of aptamer to antidote, thus exhibiting no rebound anticoagulation effect due to

dissociation of aptamer and antidote. Anticoagulation, either bolus or weight-adjusted,

was rapid and stable for several hours, whereas antidote administration rapidly reversed the aptamer's activity within minutes (161). While clearance of free RB006 involves mainly intravascular and some renal mechanisms, clearance of RB007 and the RB006/RB007 complex is believed to occur rapidly through endogenous endonuclease degradation, allowing for the re-administration of aptamer with immediate restoration of anticoagulant effects (162, 186). The REG1 system was also shown to be well tolerated and predictable in patients with stable CAD that commonly undergo procedures requiring anticoagulation. These patients tend to be older with reduced drug clearance and are currently on other medications, such as oral antiplatelets, that affect hemostatic factors and can increase the patient's propensity for bleeding (186). The success of these studies highlight the versatility of the aptamer-antidote platform for use in a wide number of clinical indications, as the pair can be repeatedly administered with highly predictive results and no evidence of toxicity, immunogenicity, or adverse bleeding events. Importantly, drug reversal can be closely controlled by titrating in antidote as needed to neutralize the anticoagulant effects of the aptamer to the necessary degree (187).

Reversal-PCI (NCT00113997) was a phase 2a pilot study that was undertaken to assess the REG1 system as the sole anticoagulant for use in elective PCI in low risk patients with stable CAD. This study represented the first clinical use of the REG1 system and exhibited partial or complete reversal of REG1 to allow for a safe sheath

removal. Overall, the REG1 system exhibited low inter-patient variability, with a rapid

onset and stable anticoagulation throughout the procedure. RB006 was either fully or

partially reversed upon administration of the appropriate dose of RB007 in a predictable

manner. This ability to slowly and partially reverse is useful in clinical settings where

incomplete or slow reversal of anticoagulation is desired, such as PCI, where

anticoagulation can be slowly reversed in conjunction with sheath removal. This study

proved that REG1 could replace heparin/protamine in PCI with stable and predictable

anticoagulation, and laid the groundwork for a larger clinical trial in higher risk patients

undergoing PCI (188).

A Phase 2b study termed RADAR (A Randomized, Partially Blinded,

Multicenter, Active-Controlled, Dose-Ranging Study Assessing the Safety, Efficacy, and

Pharmacodynamics of the REG1 Anticoagulation System in Patients with ACS)

(NCT00932100), was designed to asses the degree of reversal required for early sheath

removal and mitigate bleeding following cardiac catheterization (163). A total of 640

non-ST elevated ACS patients from multiple international centers were randomly

assigned to one of four REG1 treatment arms, all receiving a weight adjusted dose of

RB006 (now termed pegnivacogin) with four different RB007 (now termed anivamersen)

reversal strategies. Overall, it was determined that at least 50% reversal of pegnivacogin

is required for safe sheath removal. In addition, full FIX inhibition with pegnivacogin,

followed by full reversal with anivamersen, did not increase bleeding or subsequent

thrombotic events despite early sheath removal, suggesting that this strategy is efficacious for ACS patients undergoing invasive surgery. While the incidence of bleeding rates and adverse events were similar with REG1 and heparin, enrollment in RADAR was halted after three allergic events thought to be due to exposure to PEG and not the aptamer itself. In addition, all three patients had a history of allergic reactions (163). Overall, the study goals were met, and larger clinical trials determining the efficacy of the REG1 system compared to other anticoagulation strategies are warranted.

While the REG1 system was administered intravenously in these previous studies for rapid onset anticoagulation, a subcutaneous route of injection could be valuable in settings that require chronic anticoagulation, such as VTE. In a phase 1a clinical trial in healthy volunteers, subcutaneous administration of pegnivacogin resulted in anticoagulation beginning at 8-12 hours after injection, with peak anticoagulation at ~48 hours. This effect was durable with a half-life of 6 days. Anivamersin administration successfully neutralized RB006 plasma activity, but did not inhibit further absorption of RB006 in the bloodstream, resulting in only transient reversal (189). A phase 2b trial with multiple dose escalation is planned.

Overall, the REG1 anticoagulation system is the first aptamer-antidote pair to be tested in humans, and it has shown to be safe and effective in the clinical setting of PCI with a number of advantages over the standard of care heparin/protamine. Importantly, REG1 was able to predictably and stably anticoagulate patients and was easily

monitored with routine point of care assays. Antidote reversal allowed for immediate

restoration of FIX function, allowing for repeat administration with stable and

predictable anticoagulation. The hallmark of this system allows for unprecedented

control over drug titratability, with the ability to fully or partially neutralize the aptamer

as needed.

1.4.4.4 Inhibiting platelet pathways

<u>VWF Aptamer</u>

VWF is a large, multimeric glycoprotein (ranging from 600 to 20,000 kDa)

expressed in platelets and endothelial cells and mediates platelet adhesion, activation,

and aggregation. Once VWF is exposed on the injured vessel wall, it can then interact

with collagen near the site of injury, as well as adhesion platelet receptors to tether

platelets to the site of injury, allowing them to become activated and aggregate, thus

forming a plug at the site of injury. Inhibiting the interaction of platelets with VWF,

especially in a high flow environment, could severely impair coagulation by not

allowing platelets to adhere to the site of injury. This defect is evident in von

Willebrand's disease, where patients with deficiencies of VWF have moderate to severe

bleeding phenotypes.

A mixed modified DNA/RNA aptamer against VWF was isolated that binds to

the A1 domain of VWF. This domain is responsible for platelet binding to the

glycoprotein. As such, this aptamer, termed ARC1779, blocks the ability of VWF to

interact with platelet receptors, thus interrupting VWF-mediated aggregation and activation of platelets in clinical platelet aggregation assays. Given as a continuous infusion, this aptamer was efficacious in preventing occlusive thrombi from forming in a cynomolgous monkey arterial thrombosis model (190). A PEGylated version of ARC1779 was subsequently administered in healthy volunteers in a Phase I clinical trial sponsored by Archemix Corporation. While the drug was well tolerated in this study, with high specificity and no reports of excess bleeding or toxicity, further clinical studies with this aptamer were either terminated or halted (190).

A 2'F pyrimidine RNA VWF aptamer was isolated using a convergent SELEX approach by first selecting against the entire plasma proteome. Then, using this focused library, several rounds of purified protein selection against VWF were performed. The lead candidate, R9.14, was shown to bind within the A1 domain that mediates platelet binding to VWF, and therefore, this aptamer was able to inhibit platelet aggregation and activation in a clinical platelet aggregation assay. An antidote was also generated that could potently reverse the aptamer's action within two minutes (191).

A truncated, cholesterol-modified version of R9.14 was next tested in animal models. These modifications allow for more cost effective synthesis, and increase the half-life of the aptamer in an animal. In a mouse ferric-chloride arterial thrombosis model, administration of R9.14 kept the vessel patent and free of thrombi for up to one hour (192). While there was no spontaneous bleeding in these mice, the aptamer dose-

dependently increased bleeding as measured by a tail transection. However,

administration of either a matched oligonucleotide antidote or a universal antidote

completely reversed the bleeding phenotype (192). As use of antiplatelets has been

limited due to the high risk of hemorrhage, especially from coupling these agents with

other anticoagulants, an aptamer-antidote pair could be rapidly controlled by physicians

in response to adverse bleeding events in a clinical setting, while still protecting the

patient from thrombotic complications. Thus, the VWF aptamer-antidote system is

currently undergoing further optimization and subsequent pre-clinical trials.

GPVI

The platelet receptor Glycoprotein VI (GPVI) binds collagen to activate platelets,

and is a promising target for a thrombosis therapeutic, as inhibition of GPVI impairs

stable platelet aggregation without a bleeding phenotype. Regado Biosciences has

developed a GPVI aptamer-antidote pair that can inhibit collagen-induced platelet

aggregation and activation. While this aptamer is still in preclinical trials, it is expected

to enter safety trials in 2013. In addition, a 2'F pyrimidine RNA aptamer was isolated

against GPVI by the Sullenger lab (unpublished data). While this aptamer exhibited

high affinity binding towards the receptor, the aptamer exhibited agonistic effects by

rapidly activating platelets and stimulating aggregation (unpublished data). It is

thought that at high concentrations, the aptamer multimerizes and thus mimics other

GPVI agonists to activate platelets.

2. Characterization of an anticoagulant aptamer against FXII

Chapter 2 was modified from a manuscript entitled "Inhibiting the intrinsic pathway of coagulation with a FXII-targeting RNA Aptamer" published in the Journal of Thrombosis and Haemostasis in May 2013 (epub ahead of print). The authors were Rebecca Woodruff, Yiming Xu, Juliana Layzer, Weizhen Wu, Martin Ogletree, and Bruce Sullenger.

2.1 Introduction

The hemostatic system maintains the integrity of the vasculature by sequentially activating a series of proteases, culminating in thrombin production and fibrin clot formation. Activation of coagulation can occur by either exposure of TF on the vessel wall at the site of injury (extrinsic pathway) or by activation of blood-borne components (factor XII) in the vasculature (intrinsic, or contact activation, pathway) (10). While the extrinsic pathway is thought to initiate thrombin formation at the site of injury, the intrinsic pathway is thought to mediate continued thrombin generation to stabilize a thrombus (39). Deficiency of FXII in animals is associated with protection from occlusive thrombus formation after arterial injury and stroke models (40, 41, 96). While thrombi can still form in these models, they are unstable and fail to occlude the vessel. Importantly, while offering a protective thrombotic effect, deficiency of FXII did not interrupt normal hemostatic events (40). Thus, pharmacologic inhibition of FXII is an attractive alternative in providing protection from pathologic thrombus formation while minimizing hemorrhagic risk.

Human FXII is an 80kDa glycoprotein consisting of an enzymatic light chain and a heavy chain comprised of several conserved domains that mediate binding to anionic surfaces and other proteins (15, 20). Binding of the FXII heavy chain to an anionic surface induces a conformational change and results in a small amount of activated FXII (FXIIa) formed (21). While a number of negatively charged substances have been shown to autoactivate FXII, several compounds thought to be involved in or associated with thrombosis or inflammation, such as polyphosphates (45), nucleic acids (27, 89), activated platelets (43), protein aggregates (44), collagen (25), and mast cell heparin (28) have been identified as potential autoactivators of FXII *in vivo*. Once activated, FXIIa can then activate FXI and the intrinsic pathway of coagulation to generate thrombin, leading to fibrin clot formation and stabilization and platelet and cellular activation via protease activated receptors. Additionally, FXIIa can activate plasma kallikrein (PK) to cleave the inflammatory mediator bradykinin (BK) from high-molecular weight kininogen (HK) to facilitate inflammatory responses (193).

Aptamers are single stranded, highly structured oligonucleotides that act as protein antagonists by binding to large surface areas on their target protein to directly inhibit protein-protein interactions. A number of aptamers have been developed as specific and high affinity inhibitors of coagulation proteins (FIX, FVII, VWF, FX, FII) that function as potent anticoagulants by interrupting specific complex macromolecular interactions on their target protease (164, 181-183, 191). The aptamer platform offers a

level of control, as each aptamer can be specifically and effectively reversed by either a

sequence specific antidote that recognizes and binds to the primary sequence of the

aptamer to disrupt aptamer-protein binding (160) or a universal antidote that can

reverse the action of any aptamer independent of its sequence (165). While aptamer

technology has been successfully applied to downstream coagulation factors, an

aptamer inhibitor of the upstream contact pathway has not yet been reported. Herein,

we describe the isolation and characterization of a modified RNA aptamer targeting

FXII/FXIIa. This aptamer functions as a potent anticoagulant in a number of clinical

coagulation assays, and mediates its anticoagulant effects by inhibiting not only the

autoactivation of FXII, but also FXI-mediated intrinsic pathway activation. However,

this aptamer does not affect FXIIa-mediated PK activation, demonstrating that aptamers

can selectively interfere with specific protein-protein interactions without inhibiting the

active site of enzymes.

2.2 Materials and Methods

Materials:

The SELEX DNA template sequence was 5'-

TCTCGGATCCTCAGCGAGTCGTCTG-N40-CCGCATCGTCCTCCCTA-3' and was

purchased from Oligos, etc. (Wilsonville, OR). The 5' and 3' SELEX primer sequence are

5'-GGGGGAATTCTAATACGACTCACTATAGGGAGGACGATGCGG and 5'-

TCTCGGATCCTCAGCGAGTCGTC-3', respectively, and were purchased from

Integrated DNA Technologies (IDT, Coralville, IA). The 2'Flouro modified cytidine and uridine were purchased from Trilink BioTechnology (San Diego, CA).

Kallikrein, prekallikrein, α-FXIIa, and β-fXIIa, were purchased from Enzyme Research Laboratories (South Bend, IN), and FXII, Corn Trypsin Inhibitor (CTI), and all other coagulation proteins were purchased from Haematologic Technologies (Essex Junction, VT). Human normal pooled plasma was purchased from George King Biomedical, Inc. (Overland Park, Kansas). Bovine serum albumin (BSA) was purchased from EMD Chemicals (Gibbstown, NJ), PEG-8000 was purchased from Fluka Biochemika (Buchs, Switzerland), dextran sulfate was purchased from Pharmacia Biotech (Piscataway, NJ), and kaolin was purchased from Sigma-Aldrich (St. Louis, MO). AMV-RT enzyme was purchased from Roche Applied Science (Indianapolis, IN). Pacific Hemostasis APTT-XL Reagent (Ellagic Acid) was purchased from Thermo Scientific (Waltham, MA), and TriniCLOT aPTT S (micronized silica) was purchased from Trinity BioTech (Bray, Co Wicklow, Ireland). All kinetic assays were performed in 96-well flat bottom microtiter plates (Corning Incorporated, Corning, NY), the absorbance was read at 405nm on a Power Wave XS2 kinetic microplate spectrophotometer (Biotek, Winooski, VT), and the data were analyzed using the Graphpad Prism Software (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

Convergent SELEX:

Convergent aptamer selection was performed as described previously (191). The sequence of the starting RNA library was 5'-GGGAGGACGAUGCGG-N40-CAGACGACUCGCUGAGGAUCC-3', where N40 is a 40 nucleotide randomized region, and D and U represent 2'F modified ribonucleotides, respectively. Complex selection rounds were performed by incubating the modified starting library with diluted normal human plasma in a low salt, Hepes-based binding buffer (20mM Hepes, 50mM NaCl, 2mM $CaCl_2$, and .01%BSA). To inhibit non-specific binding of the RNA library to the plasma proteome, yeast tRNA was incorporated into the binding mixture. Bound RNA was separated from unbound RNA by passing the mixture through a 25-mm 0.45μm nitrocellulose filter membrane (Protran BA 85, Whatman, Inc., Piscataway, NJ). The bound RNA was extracted and RT-PCRed to amplify the bound RNA species as previously described (152). After five rounds of selection against unfractionated normal human pooled plasma (George King Biomedical, Inc., Overland Park, KA), four rounds of selection were performed against purified FXII in Hepes-based buffer (20mM Hepes, 150mM NaCl, 2mM $CaCl_2$, and .01%BSA). Binding of each round to FXII was monitored by a nitrocellulose filter-binding assay. The final round was cloned and sequenced as previously described to distinguish individual aptamer sequences (152).

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RNA Aptamers

For all aptamer sequences, "C" and "U" represent 2'fluorocytosine and 2'fluorouracil.

The 80-nucleotide full length R4cXII-1 sequence is 5' -

GGGAGGACGAUGCGGCCAAAUCUCGGCUGCCAGCAGGUCACGAGUCGCAAGAUAAC

AGACGACUCGCUGAGGAUCCGAGA-3'. The 51 nucleotide truncated R4cXII-1t sequence is

5'- GGCUCGGCUGCCAGCAGGUCACGAGUCGCAGCGACUCGCUGAGGAUCCGAG-3'.

The 51-nucleotide scrambled control RNA sequence is

5'GGGGCAGCCGUGGACCGACUGCCGCAUGCCAUUGACAGUCCGAUGCCAGGC-3'.

The sequence for the point mutant control R4cXII-1T22M is

GGCUCGGCUGAACGCAGGUCACGAGUCGCAGCGACUCGCUGAGGAUCCGAG. All

aptamers were transcribed *in vitro* as previously described using a modified polymerase (152,

194). Before use, all aptamer preparations were diluted into a Hepes-based buffer with or

without BSA (20mM Hepes, 150mM NaCl, 2mM CaCl$_2$, and with or without 0.01%BSA) as

indicated. Diluted aptamers were refolded by heating to 65°C for 5 minutes, and then cooled

for 3 minutes at ambient room temperature.

Nitrocellulose filter binding assay:

A double-filter nitrocellulose binding assay was employed to determine the

apparent binding affinity constants (K$_d$ values) of aptamer to protein (152). The 5' end of

the RNA was dephosphorylated using bacterial alkaline phosphatase (Gibco BRL,

Gaithersburg, MD), and subsequently end-labeled with [$\gamma^{32}P$] ATP (Perkin Elmer,

Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

Varying concentrations of purified protein were incubated with [$\gamma^{32}P$] RNA in Hepes

binding buffer with BSA (20mM Hepes, 150mM NaCl, 2mM $CaCl_2$, and .01%BSA) at

37°C for 5 minutes. The RNA-protein complexes were then separated from the unbound

RNA by passing the mixture through a nitrocellulose filter (Protran BA 85, Whatman

Inc., Florham Park, NJ). The levels of bound RNA were quantified using a Storm 825

phosphoimager (GE Healthcare, Piscataway, NJ). Non-specific binding of the

radiolabeled RNA to the nitrocellulose filter was subtracted, and the data were fitted

using nonlinear regression to determine the apparent binding affinity for the RNA

aptamer-protein interaction (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

Clotting Assays

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays

were carried out using a model ST4 coagulometer (Diagnostica Stago, Parsippany, NJ).

Aptamer was diluted in Hepes buffer without BSA (20mM Hepes, 150mM NaCl, 2mM

$CaCl_2$), refolded by heating for 5 minutes at 65°C and then cooled to ambient

temperature for 3 minutes. For the aPTT, aptamer (5μL) was then added to 50μL normal

human pooled plasma (George King Biomedical, Inc., Overland Park, KA) and

incubated for 5 minutes at 37°C. 50μL APTT-XL reagent was then added to activate

plasma and incubated for 5 minutes at 37°C. Clotting was initiated by adding 50μL of

25mM CaCl$_2$ and the time to clot formation was recorded. Aptamer concentrations

represent the final concentration in the entire reaction.

For the PT, refolded aptamer (5μL) was added to 50μL plasma and incubated for

5 minutes at 37°C. TriniClot PT Excel (Trinity BioTech, Bray, Co Wicklow, Ireland) was

added to initiate clotting.

For FXIIa and FXIa clotting times, refolded aptamer (5μL) was added to 45μL of

either 40nM FXIa or 250nM FXIIa and incubated for 5 minutes at 37°C. Aptamer and

protein were added to either FXII or FXI-deficient human plasma (Haematologic

Technologies, Essex Junction, VT) supplemented with 3μM phospholipid (15%

phosphatidylserine, 41% phosphatidylcholine, 44% phosphatidylethanolamine (Avanti

Polar Lipids (Alabaster, AL)) and incubated for 30 seconds at 37°C. Clotting was

initiated by adding 50μL of 25mM CaCl$_2$ and the time to clot formation was recorded.

Aptamer concentrations represent the final concentration in the entire reaction.

Thrombin Generation Assay

In an Immulon 2HB clear U-bottom 96 well plate (Thermo Labsystems, Franklin,

MA), CTI- treated (50μg/mL), pooled normal human platelet poor plasma from healthy,

consented volunteers (60μL) was mixed with aptamers (3μL) diluted in Hepes-based

buffer without BSA and incubated for 5 min at 37°C. Ellagic acid reagent (final

concentration 0.1μM, diluted 1:200 with 4μM phospholipid vesicles (20%

phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine) in

Hepes-based buffered saline)(15μL) was added to the plasma-aptamer mixture, and

incubated for 5 minutes at 37°C. Flu-Ca (Diagnostica Stago)(15μL) was added to start the

assay and thrombin generation was measured using a Fluroskan Ascent Reader

(Thermo Scientific, Waltham, MA). A thrombin calibrator reagent (Diagnostica Stago,

Parsippany, NJ) was used to quantify the amount of thrombin generated in the samples.

Data analysis was performed using Thrombinoscope software (Thrombinoscope BV, the

Netherlands). Aptamer concentrations represent the final concentration in the entire

reaction. The amount of ellagic acid used was standardized to produce a similar curve to

a TF-activated TGA. In addition, the amount of CTI present was standardized to inhibit

contact activation of the plasma sample, while still allowing for robust FXII-dependent

activation upon administration of ellagic acid.

FXIIa small peptide substrate cleavage

FXIIa (final concentration 10nM) was incubated with varying concentrations of

either aptamer or CTI for 10min at 37°C in a Hepes-based reaction buffer (20mM Hepes

pH 7.4, 150mM NaCl, 2mM $CaCl_2$, .01% BSA). The chromogenic substrate Spectrozyme

FXIIa (American Diagnostica Inc. – now Sekisui Diagnostics, Stamford, CT) was added

to a total volume of 100μL. Substrate cleavage was monitored at an absorbance of

405nm using a kinetic microplate spectrophotometer at 37°C, and rates of substrate

cleavage were determined using linear regression analysis.

FXII autoactivation assays

FXII (final concentration 0.2μM) was incubated with varying concentrations of

aptamer, dextran sulfate, or buffer for 1 hour at 37°C in a Hepes-based buffer with BSA

and with or without 0.1% PEG. To determine if the aptamer could block autoactivation

of FXII, 1μM aptamer or buffer was pre-incubated with FXII for 5 minutes prior to the

addition of an autoactivator, and the mixture was incubated for 1 hour at 37°C. The final

concentrations of the autoactivators were: dextran sulfate (1μg/mL), kaolin (100μg/mL),

micronized silica (30x-diluted), and ellagic acid (30x-diluted). In both experiments, the

FXIIa chromogenic substrate Spectrozyme FXIIa (American Diagnostica Inc. – now

Sekisui Diagnostics, Stamford, CT) was added to a total volume of 100μL (final

concentration 0.2mM). Substrate cleavage was monitored at an absorbance of 405nm

using a kinetic microplate spectrophotometer at 37°C, and rates of substrate cleavage

were determined using linear regression analysis.

FXIIa-mediated FXI activation:

Conversion of zymogen FXI to activated FXIa by FXIIa was measured in a two

stage chromogenic assay performed at 37°C in a Hepes-based buffer with BSA. Aptamer

or buffer was incubated with FXIIa (final concentration 5nM) for 5 minutes at 37℃, and reactions were initiated with the addition of FXI (final concentration 25nM). At timed intervals after the initiation of the reaction, samples of the reaction mixture were removed, FXIIa activity was quenched by addition of 1μM CTI, and FXIa activity was assayed by the addition of 1mM Pefachrome FXIa (Centerchem, Inc., Norwalk, CT). The rate of substrate hydrolysis was recorded at 405nM using a kinetic microplate spectrophotometer at 37℃. FXIa cleavage rates of substrate were then used to determine the amount of FXIa formed by extrapolating from a standard curve prepared using purified FXIa.

To determine the inhibition constant (K_i), FXI activation reactions were performed as described above in the presence of varying concentrations of aptamer. The initial rate of FXI activation by FXIIa was determined by linear regression of the amount of FXIa formed versus time for the first 10 minutes of the reaction. Fractional activity (V1/V) was calculated by dividing the initial rate of FXI activation in the presence of aptamer by the initial rate of FXI activation in the absence of aptamer. The K_i was determined using nonlinear regression analysis.

FXIIa-mediated Prekallikrein activation

Conversion of prekallikrein to kallikrein by FXIIa was measured in a two stage chromogenic assay performed at 37℃ in a Hepes-based buffer with BSA and 0.1% PEG.

Aptamer or buffer was incubated with FXIIa (final concentration 4nM) for 5 minutes at

37°C and reactions were initiated with the addition of prekallikrein (final concentration

20nM). At timed intervals after the initiation of the reaction, samples of the reaction

mixture were removed and FXIIa activity was quenched by addition of 1μM CTI.

Kallikrein activity was assayed by the addition of 0.25mM Pefachrome PK (Centerchem,

Inc., Norwalk, CT), and the rate of substrate hydrolysis was recorded at 405nM using a

kinetic microplate spectrophotometer at 37°C. Kallikrein cleavage rates of substrate

were then used to determine the amount of kallikrein formed by extrapolating from a

standard curve prepared using purified kallikrein.

2.3 Results

2.3.1 SELEX Pool Analysis and Lead Clone Selection

High affinity aptamers targeting human FXII were isolated from a convergent

selection approach, whereby a nucleic-acid-based combinatorial library containing

approximately 10^{14} RNA species was first enriched for binding against the entire human

plasma proteome, and then further enriched for binding against purified FXII. To confer

greater stability in human plasma, the library contained 2'F pyrimidines (159). Five

rounds of complex selection were performed against unfractionated human plasma to

obtain a focused library containing aptamers that theoretically bind to all plasma

components. Using this focused library, four rounds of selection to purified FXII

yielded further enrichment for a pool of aptamers targeting FXII as determined by a

nitrocellulose filter-binding assay (Figure 8).
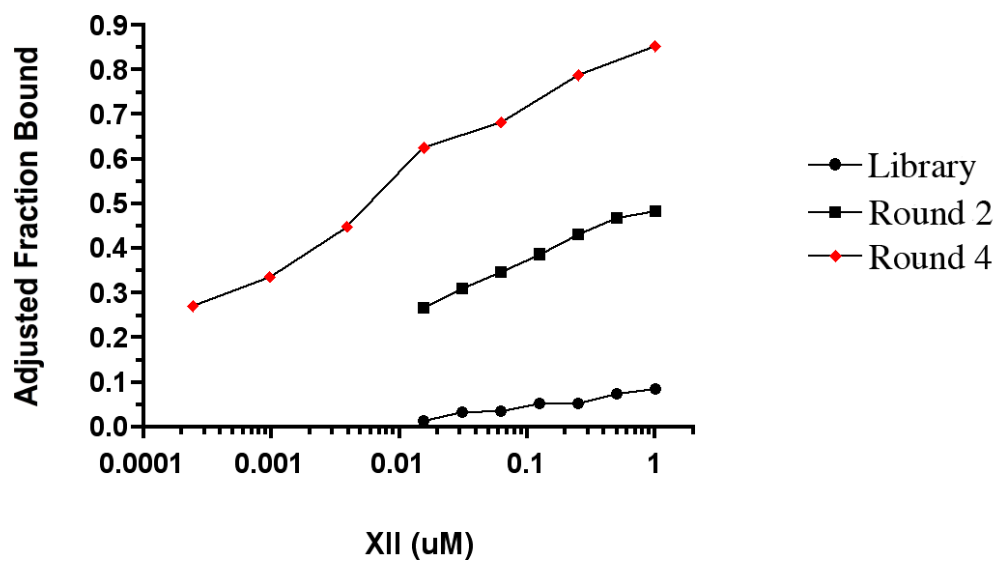
Figure 8. Binding affinity of SELEX rounds against purified FXII
Nitrocellulose filter binding assay of the plasma proteome enriched starting library
(black circles), round 2 of selection against purified FXII (black squares), and round 4 of
selection against purified FXII (red diamonds).   Data courtesy of Juliana Layzer.

The selection process was ended by cloning and sequencing the individual RNA

sequences present in the round 4 pool, and these sequences were then organized into

families based upon their primary sequences (Table 4).

Table 4. RNA aptamer sequences from FXII convergent SELEX

Clone ID	Variable Region Sequence	Frequency
R4cXII-12	ACTCCGCCATGGACCATGCATCTTCACAGCTAACCCCGCA	26.1%
R4cXII-15	TCAAAACTCAACGAGCTCACAT-CTAGCATTGACGGT	17.4%
R4cXII-1	CCAAATCTCGGCTGCCAGCAGGTCACGAGTCGCAAGATAA	13.0%
R4cXII-7	CCCTCCGCACAACCTCGGCTGCCAGCAGAACACGAGTTAC	13.0%
R4cXII-8	CATGAGCTGTCTTCTCGCGGCTGCCAGCAGGAATACGAGT	8.7%
R4cXII-16	TTCGATCCCCTCCGGCTACCAACAGAGTTTCCGAGTCTT	8.7%
R4cXII-11	TCGCTGACGAATAGACGTTCATCCAGGTGATCACATCTAG	4.3%
R4cXII-21	ACNCCGACACGAGATTACNNATTTAGTCACCACNAGAGTGTNGA	4.3%

In order to determine the binding affinity of each family of isolated clones, a nitrocellulose filter-binding assay was performed with a representative clone from each family. A number of aptamers bound to FXII with affinities (measured by their dissociation constants, or K_d's) in the low nanomolar range. Binding to αFXIIa was also tested, and each clone bound with approximately 10-fold increased affinity to the activated form (Table 5). The B_{max}, or the percentage of RNA present that is folded correctly in order to bind to the target protein, was also determined for each individual aptamer.

Table 5. RNA aptamer binding to soluble FXII and FXIIa as determined by a nitrocellulose filter-binding assay

Aptamer	FXII		FXIIa	
	K_d approx (nM)	B_{max} approx (%)	K_d approx (nM)	B_{max} approx (%)
R4cXII-1	8.9	75	0.31	58
R4cXII-7	2.8	53	0.22	28
R4cXII-8	17.9	47	0.52	20
R4cXII-11	20	58	0.24	24
R4cXII-12	9.3	47	0.13	46
R4cXII-15	463	46	0.08	4
R4cXII-16	29	44	0.25	21
R4cXII-21	5.9	45	0.05	8

Although several clones bound with high affinity to FXII and FXIIa, the nitrocellulose filter-binding assay does not examine the functionality of these aptamers as anticoagulants. Therefore, we next tested each aptamer for anticoagulant activity in a common clinical coagulation test, the activated partial thromboplastin time (aPTT). This assay measures the time it takes for a fibrin clot to occur in plasma, and is sensitive to deficiencies or inhibition of the intrinsic and common pathways of coagulation. This assay uses a negatively charged substance, such as kaolin or ellagic acid, to activate FXII and prime the cascade, and, upon addition of Ca^{2+} to initiate clotting, records the time for a fibrin clot to form. Because initial tests using normal human plasma and a micronized silica activator did not show any affect of the FXII aptamers over control RNA at the concentration used on altering the clotting time, the assay was performed in 99% FXII deficient plasma and 1% normal plasma, affectively lowering the amount of

FXII present and allowing for a lower concentration of aptamer to show an effect (Figure

9).
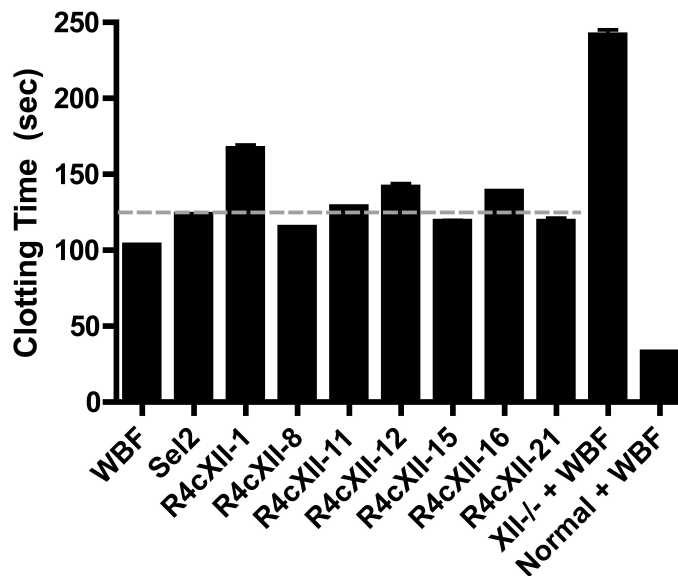
Figure 9. Screen of FXII aptamers in a functional clotting assay, the aPTT
Selected aptamers (0.5μM) were screened for prolongation of clotting time in 1%
Normal/99% FXII deficient human plasma. Aptamer clotting times were compared
against a buffer control (WBF), library RNA (Sel2), 100% FXII deficient plasma (XII-/- +
WBF) and 100% normal human plasma (Normal + WBF). The dotted line represents the
clotting time of library RNA and serves as a baseline clotting time to compare to
aptamer clotting time.

The clone R4cXII-1 was chosen as the lead aptamer due to its greater effect in the

aPTT assay, as well as its superior binding profile compared to all other aptamers.

While R4cXII-1 did not necessarily have the lowest K_d, it did have the highest B_{max},

meaning a larger proportion of the RNA is in a folded state that is able to bind to FXII or

FXIIa compared to the other aptamers. This is reflected in the functional clotting assay,

where R4cXII-1 outperformed all other aptamers.

2.3.2 Lead aptamer binding affinity and specificity

The full length, 80 nucleotide, 2'F pyrimidine modified RNA R4cXII-1 was

chosen as the lead aptamer out of the convergent selection against FXII (Figure 10).
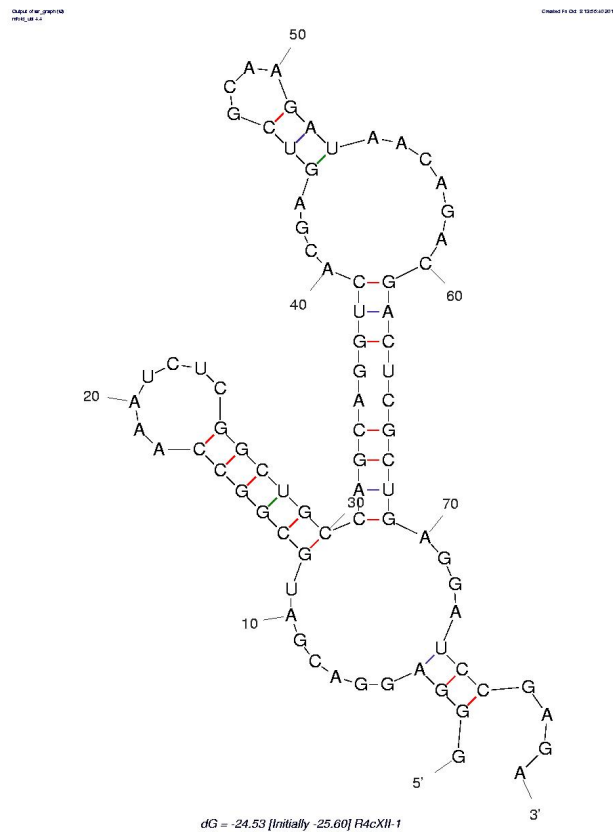
Figure 10. The M-fold predicted secondary structure of the full-length aptamer R4cXII-1

In depth binding of R4cXII-1 was analyzed by a nitrocellulose filter-binding

assay and the aptamer was found to bind to both the zymogen and active form of FXII.

R4cXII-1 bound to FXII with a K_d of 8.9 ± 1.0nM and a B_{max} of 74.8 ± 4.7%and to αFXIIa

with a K_d of 0.5 ± 0.2nM and a B_{max} of 58 ± 3.2% to αFXIIa (Figure 11).
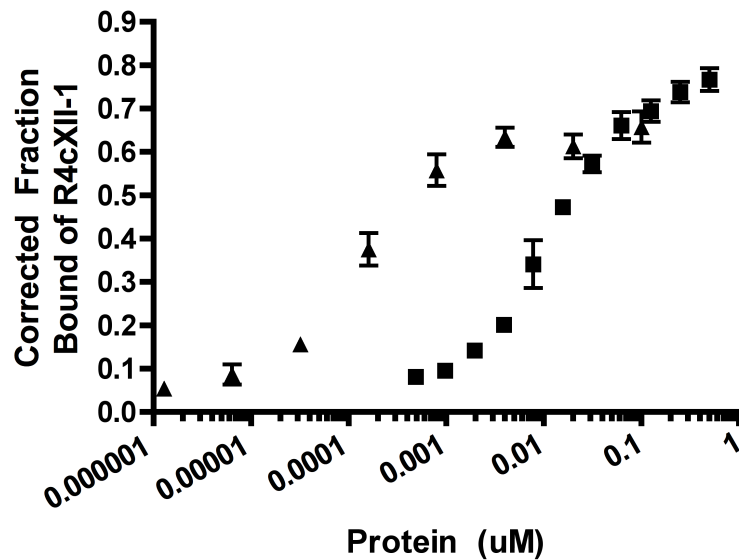
Figure 11. Aptamer R4cXII-1 binds with high affinity to FXII and FXIIa
Nitrocellulose filter binding assays of aptamer to A) FXII (■) and FXIIa (▲) and B)
indicated proteins. C) Linear sequence and dissociation constants of full length R4cXII-1
and truncated R4cXII-1 (R4cXII-1t). In A, the data represent the mean ± SEM of triplicate
measures and in B, the data are representative of at least two independent experiments.

This high affinity binding of R4cXII-1 to FXII and FXIIa was also found to be

specific, as R4cXII-1 binding to a number of other structurally and functionally related

coagulation factors, including thrombin, FXIa, FIXa, FXa, FVIIa, urokinase plasminogen

activator, and tissue plasminogen activator, was shown to be non-existent or very weak,

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with dissociation constants greater than 1000nM and no different from a random RNA

library (data not shown). In addition, R4cXII-1 exhibited greatly reduced binding to β-

fXIIa (dissociation constant K_d of 51.4 ± 9.0nM and a B_{max} of 34.7 ± 2.3%), a cleaved form

of FXIIa which is missing part of the heavy chain (Figure 12).
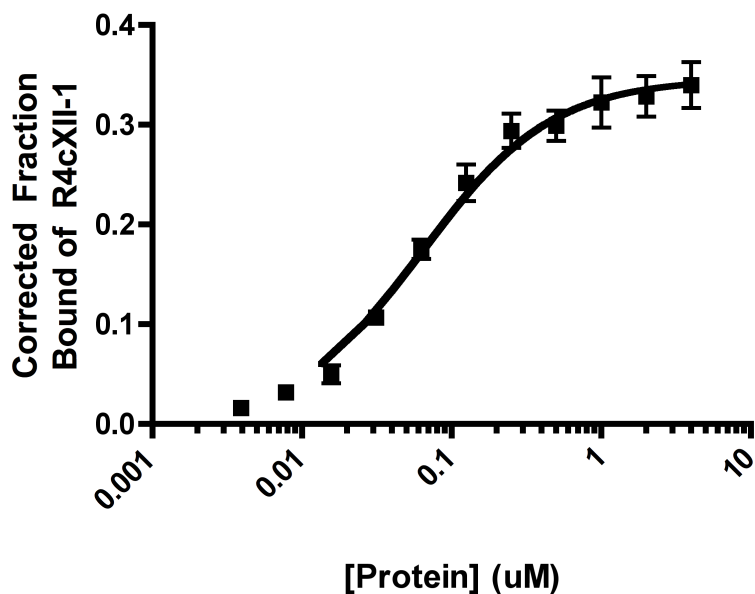
Figure 12. Aptamer R4cXII-1 binds with reduced affinity to β-fXIIa
Nitrocellulose filter binding assay of aptamer to β-fXIIa. The data represent the mean ±
SEM of triplicate measures.

2.3.3 Biochemical Characterization of aptamer inhibition

Assessment of aptamer activity on FXII active site function

A number of purified protein assays were performed to characterize the

mechanism of aptamer binding and inhibition. First, we studied the impact of aptamer

binding on active site function of αFXIIa by monitoring the cleavage of a small peptide

chromogenic substrate in the presence of a range of concentrations of aptamer, size

matched scrambled control RNA, and a known FXIIa active site inhibitor, corn trypsin

inhibitor (CTI). While CTI potently and dose dependently inhibited the ability of FXIIa

to cleave the small peptide chromogenic substrate, both the control RNA and R4cXII-1

did not affect small-substrate cleavage (Figure 13). These data indicate that the

anticoagulant effect of the aptamer is not due to the inhibition of the active site on FXIIa.

Because aptamers tend to impede protein-protein interactions without affecting the

active site (157), the ability of the aptamer to inhibit FXII/XIIa-mediated macromolecular

interactions was next evaluated.
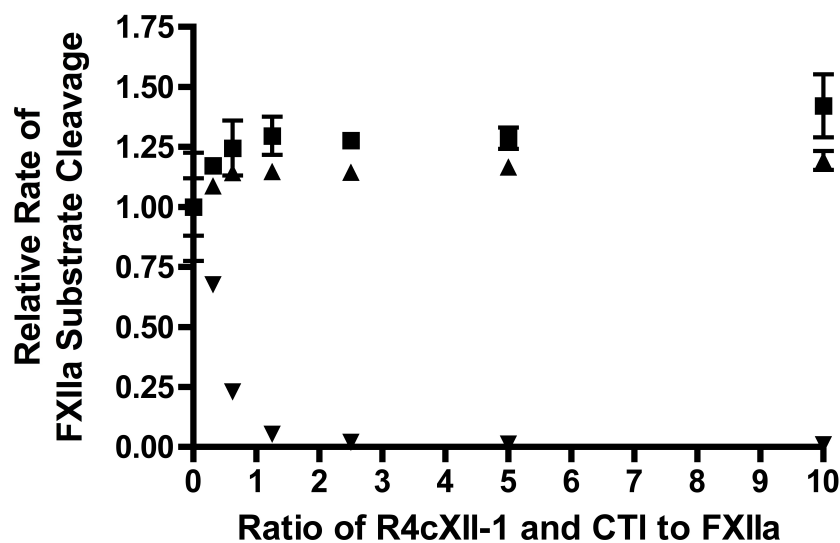
**Figure 13. Aptamer R4cXII-1 does not inhibit FXIIa cleavage of a small peptide
substrate**
αFXIIa (10nM) was incubated with varying amounts of R4cXII-1 (■), size matched
scrambled control RNA (▲), and CTI (▼), and active site activity was measured by an
αFXIIa-specific chromogenic substrate. The data were normalized to the rate in the
absence of compound and represent the mean ± SEM of duplicate measures.

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Assessment of aptamer activity on FXII autoactivation

Since a range of anionic compounds, including unmodified nucleic acids (27, 89), have been found to autoactivate FXII, aptamer and size matched scrambled control RNA were tested to determine if relatively short 2'F pyrimidine modified RNAs could also autoactivate FXII. While a known FXII autoactivator, dextran sulfate, was able to convert FXII to FXIIa with the characteristic bell-shaped concentration curve of a template-related mechanism (195), neither the control RNA nor R4cXII-1 could autoactivate FXII over a wide range of concentrations (Figure 14A).

Next, the ability of R4cXII-1 to block the autoactivation of FXII by various known activators was tested. Dextran sulfate, as well as purified kaolin, and standardized aPTT reagents including micronized silica (TriniClot S) and ellagic acid (APTT-XL) were all tested and found to activate FXII at widely different rates (data not shown). The final concentration of each activator that yielded an optimal rate of autoactivation of FXII was used for further studies. R4cXII-1 (1μM) was able to greatly inhibit the ability of dextran sulfate (1μg/mL) and micronized silica (30x) to autoactivate FXII, and partially inhibit the ability of kaolin (100μg/mL) and ellagic acid (30x-diluted) to autoactivate FXII (Figure 14B). In contrast, a size matched scrambled control RNA was not able to inhibit autoactivation by any activator. Autoactivation assays conducted with physiologic activators, such as polyphosphates, were hampered by the necessity for the aptamer

buffer to contain divalent ions, which assist in proper aptamer folding but inhibit the

ability of these physiologic activators to measurably autoactivate FXII with a cofactor

present (data not shown).
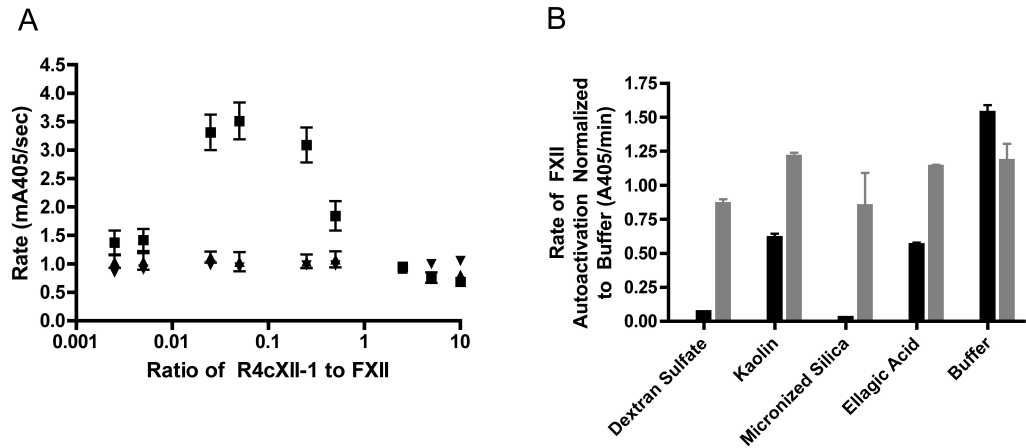
**Figure 14. Aptamer R4cXII-1 does not induce autoactivation of FXII but blocks
the activities of several autoactivators of FXII**
A) FXII (200nM) was incubated for 1 hour with varying amounts of dextran sulfate (■),
R4cXII-1 (▲), or size matched scrambled control RNA (▼), and assayed for the amount
of FXIIa formed by an FXIIa-specific chromogenic substrate. The data represent the
mean ± SEM of triplicate measures. B) FXII (200nM) was incubated for 5 min with
R4cXII-1 (black bars) or size matched scrambled control RNA (grey bars) prior to the
addition of autoactivators, and the amount of FXIIa formed after 1 hour was assayed.
The data were normalized to the rate in the absence of any RNA and represent the mean
± SEM of triplicate measures.

Assessment of aptamer activity on FXIIa-mediated activation of biologic substrates

Once activated, FXIIa can subsequently activate FXI to propagate the intrinsic

pathway of coagulation. The ability of R4cXII-1 to inhibit FXIIa-mediated FXI activation

was assessed by monitoring the amount of FXIa formed in a purified system containing

FXIIa as an activator in the absence of aptamer, or in the presence of R4cXII-1 or control

RNA. R4cXII-1 reduced the initial rate of FXI activation by greater than ~90%, while the

presence of size matched scrambled control RNA did not (Figure 15A). To determine

the potency of inhibition, the initial rate of FXI activation by FXIIa was measured in the

presence of varying concentrations of R4cXII-1. As shown in Figure 15B, R4cXII-1

concentration-dependently decreased the amount of FXIa formed. An apparent

equilibrium inhibition constant (K_i) of 3.0nM ± 0.3nM was obtained by plotting the

fractional rates of FXI activation versus the concentration of R4cXII-1 used (Figure 15C).
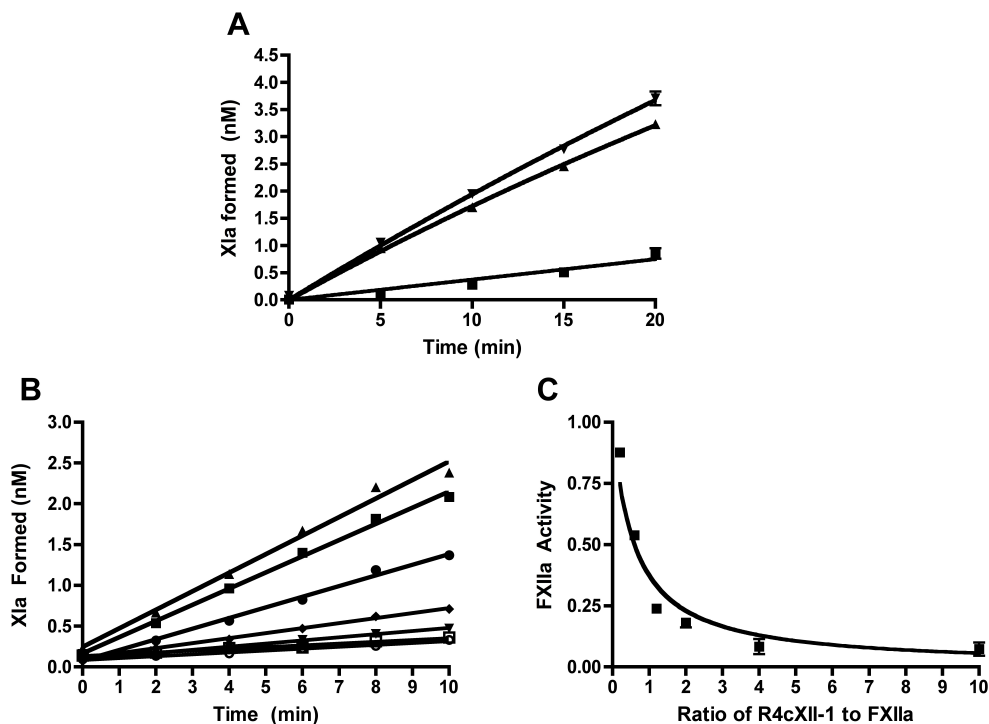
Figure 15. Aptamer R4cXII-1 dose dependently inhibits FXIIa-catalyzed activation of FXI

FXI (25nM) was activated by FXIIa (5nM), the reaction was quenched at various time-points by the addition of 1 μM CTI, and the amount of FXIa was quantified using a chromogenic substrate. A) The amount of FXIa formed at various time points was determined in the presence of 1μM R4cXII-1 (■), 1μM size matched scrambled control aptamer (▼), or buffer (▲). Lines represent linear regression fits of the data. The data represent the mean ± SEM of triplicate measures. B) Dose dependent response on FXIa generation with increasing concentrations of R4cXII-1. The amount of FXIa formed was determined in the presence of R4cXII-1 at final concentrations of 0nM(▲), 1nM (■), 3nM (●), 6nM (◆), 10nM (▼), 20nM (□), and 50nM (○). Lines represent linear regression fits of the data. The data are representative of three independent measures. C) Fractional activity of R4cXII-1 versus the ratio of R4cXII-1 to FXII present in the reaction. Fractional activity of R4cXII-1 was calculated by dividing the initial rate of FXI activation in the presence of R4cXII-1 by the initial rate of FXI activation in the absence of aptamer. The K_i was calculated by nonlinear regression analysis. The data represent the mean ± SEM of triplicate measures.

Aside from activating the intrinsic pathway through FXI activation, FXIIa can also activate plasma kallikrein (PK), which in turn reciprocally activates more FXII in a feedback loop that amplifies the activation of the contact pathway (23). PK is involved in the initiation of the kallikrein-kinin system by cleaving the small peptide bradykinin (BK) from high molecular weight kininogen (HK), leading to activation of various inflammatory processes, including vasodilation and vascular permeability (115). The ability of R4cXII-1 to inhibit prekallikrein activation by FXIIa was examined by monitoring the amount of kallikrein formed in a purified system using FXIIa as an activator. In contrast to the ability of R4cXII-1 to inhibit the generation of activated FXI (Figure 15), the aptamer did not affect prekallikrein activation by FXIIa compared to buffer or size matched scrambled control RNA (Figure 16). This indicates that R4cXII-1 does not bind to FXIIa in a manner that inhibits FXIIa-PK interactions, while it does inhibit FXIIa-FXI interactions. Collectively, these data suggest that R4cXII-1 is a potent inhibitor of both the autoactivation of FXII and FXIIa-mediated activation of FXI, while having no effect on prekallikrein activation.
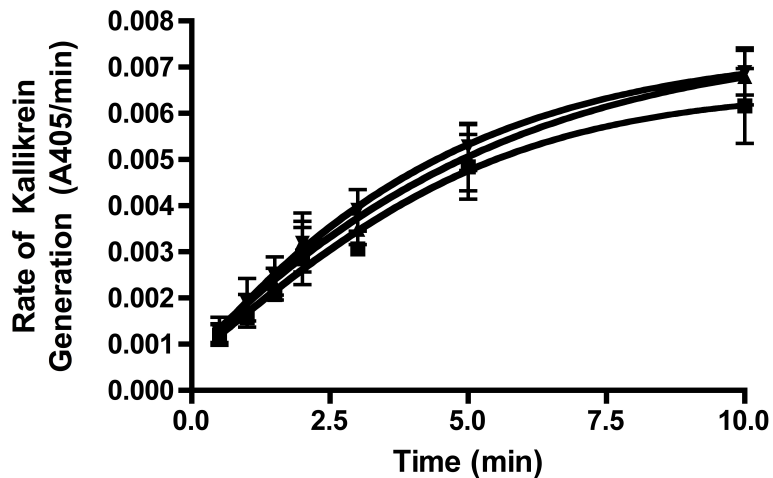
Figure 16. Aptamer R4cXII-1 does not inhibit the ability of FXII to activate prekallikrein

Prekallikrein (20nM) was activated by FXIIa (4nM), the reaction was quenched at various time points by the addition of 1μM CTI, and the amount of kallikrein was quantified using a chromogenic substrate. The amount of kallikrein formed was determined in the presence of 1μM R4cXII-1 (▼), 1μM size matched scrambled control aptamer (▲), or buffer (■). Lines represent linear regression fits of the data. The data represent the mean ± SEM of triplicate measures.

2.3.4 Truncation of aptamer

In order to facilitate optimal transcription yields and reduce background noise due to RNA length, R4cXII-1 was truncated by systematic nucleotide deletion. Three to five constructs were tested at a time, with deletions beginning on each end of the aptamer, and then internally to shorten stems and loops as much as possible without losing binding affinity or functionality. Table 6 depicts the truncation strategy, where each possible truncate was tested for binding affinity and functionality.

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Table 6. Truncation strategy for R4cXII-1

K_d's and B_{max}'s were determined using a nitrocellulose filter-binding assay. FXIa generation inhibition was determined using a chromogenic assay.

Aptamer Name	Length (nt)	K_d approx. (nM)	B_{max} approx (%)	Inhibits FXIa Generation
R4cXII-1	80	4.0	85	Yes
T1	67	8.9	86	Yes
T2	58	109.5	106	Yes
T4	60	5.5	55	Yes
T6	63	9.9	57	Yes
T7	65	120	84	No
T8	60	308	67	No
T9	50	203	70	No
T10	59	38.5	88	Yes
T11	59	60.2	98	Yes
T12	57	4.2	74	Yes
T13	56	28.2	80	Yes
T14	52	5.8	73	Yes
T15	55	277	113	No
T16	55	289	106	No
T17	55	350	123	No
T18	51	50	65	Yes
T19	54	279	104	Yes
T20	50	20.5	72	Yes
T21	48	116	88	No
T22	51	13.2	80	Yes
T23	51	71	101	No

When a truncation lost binding (generally a K_d of over 100nM) and/or

functionality, those deleted portions/nucleotides were considered to be essential in

either facilitating the proper folding and final structure of the aptamer or directly

involved in binding to the target protein. The most optimal truncation that retained

binding affinity to both FXII (apparent K_d of 12.9 ± 2.06nM) and FXIIa (apparent K_d of 0.4

± 0.02 nM), as well as the ability to block FXIIa-mediated FXIa generation, was the 51-nucleotide truncate 22 (R4cXII-1T22). The predicted secondary structure of the aptamer features an "L" shaped aptamer, with several bulge regions and two lengthy stems (Figure 17).
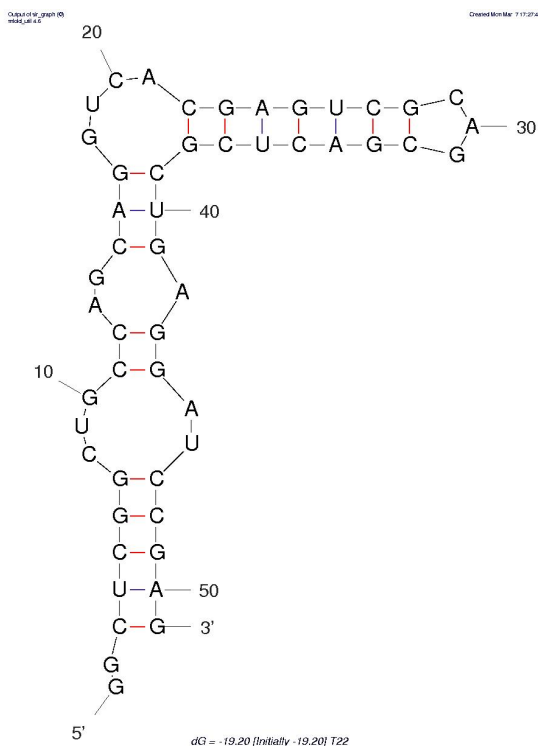
Figure 17. The M-fold predicted secondary structure of the truncated aptamer R4cXII-1T22

For ongoing functional studies, a 51 nucleotide scrambled RNA that had no binding or functional activity against FXII was also employed as an aptamer control.

2.3.5 Functional Characterization of aptamer inhibition

Biochemical characterization of R4cXII-1 suggests that the aptamer acts as an anticoagulant by specifically inhibiting FXII activation and FXIIa-mediated FXI activation. However, the functionality of this aptamer as an anticoagulant in a plasma setting is not certain, as several redundant pathways, including a feedback loop whereby thrombin can directly activate FXI, can contribute to thrombin activation with minimal or no FXII involvement.

We next tested aptamer R4cXII-1 for anticoagulant activity in two common coagulation tests, the activated partial thromboplastin time (aPTT) and the thrombin generation assay (TGA). The aPTT assay uses a negatively charged substance, such as kaolin or ellagic acid, to strongly activate FXII and thus initiate clotting through the intrinsic pathway. Therefore, this test is highly sensitive to deficiencies or inhibition of the intrinsic pathway of coagulation. A 51-nucleotide truncated version of R4cXII-1 (R4cXII-1t) that retained binding affinity to both FXII (apparent K_d of 12.9 ± 2.06nM) and FXIIa (apparent K_d of 0.4 ± 0.02 nM) dose dependently anticoagulated human plasma in an aPTT activated with ellagic acid. This inhibition was specific to the intrinsic pathway, as the aptamer had no effect on PT, which measures the function of the extrinsic pathway of coagulation (Figure 18). A size matched scrambled control RNA that does not retain binding to FXII/FXIIa had minimal impact on clotting time (Figure 18). R4cXII-1t also dose-dependently increased clotting time when a micronized silica

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based reagent was utilized, indicating that inhibition of the intrinsic pathway of

coagulation occurs regardless of the FXII activator (data not shown).
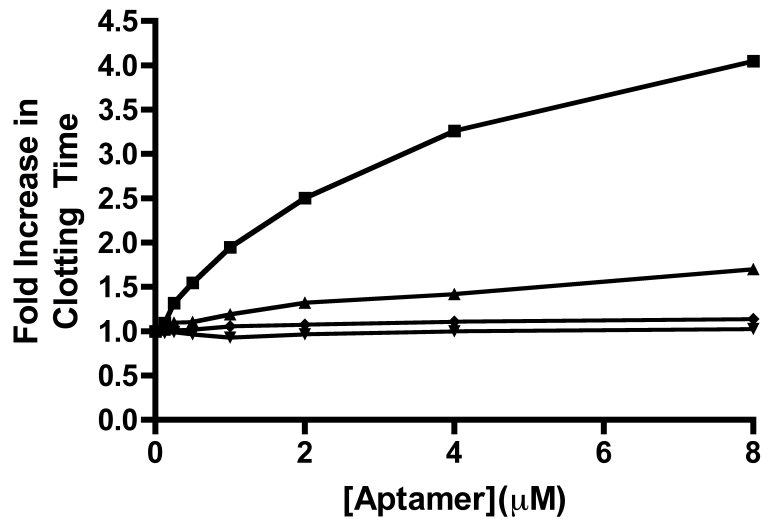
Figure 18. Aptamer R4cXII-1t dose dependently anticoagulates human plasma in an aPTT, but not a PT clotting assay

Effect of the 51-nucleotide R4cXII-1t (■) and size matched scrambled mutant control (▲) in an aPTT clotting assay activated with ellagic acid, and R4cXII-1t (▼) in a PT clotting assay activated with tissue factor. The data were normalized to the baseline clot time without aptamer present and represent the mean ± SEM of duplicate measures.

In addition, this prolongation was specific to FXII/FXIIa inhibition, as the

aptamer dose-dependently prolonged FXIIa clotting times, with no effect on the FXIa

clotting time (Figure 19).
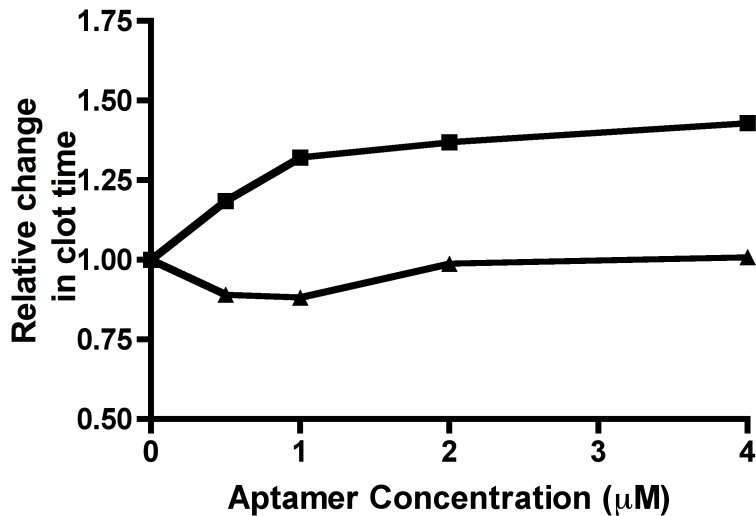
Figure 19. Aptamer R4cXII-1t dose dependently inhibits FXIIa clotting times, but not FXIa clotting times

Effect of the 51-nucleotide R4cXII-1t in a clotting assay activated with 250nM FXIIa (■) or 40nM FXIa (▲). The data were normalized to the baseline clotting time of a size-matched control RNA and represent the mean ± SEM of duplicate measures.

The TGA is a coagulation assay that measures the amount of thrombin generated in human plasma in real-time by monitoring cleavage of a thrombin-specific fluorogenic substrate (196). Using a TGA allows for several more parameters to be analyzed in addition to the lag time (the time when thrombin begins to appear), including the peak (maximal amount of thrombin formed), the endogenous thrombin potential (ETP, the total amount of thrombin formed), and the rate of thrombin generation (the slope of the line from no thrombin generation to peak thrombin generation). While a standard TGA is initiated using various concentrations of TF to activate the extrinsic pathway, an assay using ellagic acid as an activator was developed with our colleagues at Merck Research

Labs to study effects on thrombin generation during inhibition of the intrinsic pathway.

Similar to its activity in an aPTT, R4cXII-1t dose-dependently increased the lag time, as

well as decreased peak thrombin generation, ETP, and the rate of thrombin generation

in a TGA initiated with ellagic acid (Figure 20). Overall thrombin generation was

decreased to background levels, with a clear saturation point of aptamer inhibition

(3.75μM), while the peak amount of thrombin generated and the rate of thrombin

generation were robustly inhibited. In contrast, a size matched scrambled control RNA

did not inhibit thrombin generation (Figure 20). These data suggest that R4cXII-1 acts as

an anticoagulant by specifically inhibiting FXII function to effectively shut down

intrinsic pathway activation and thus impair thrombin generation triggered by ellagic

acid.
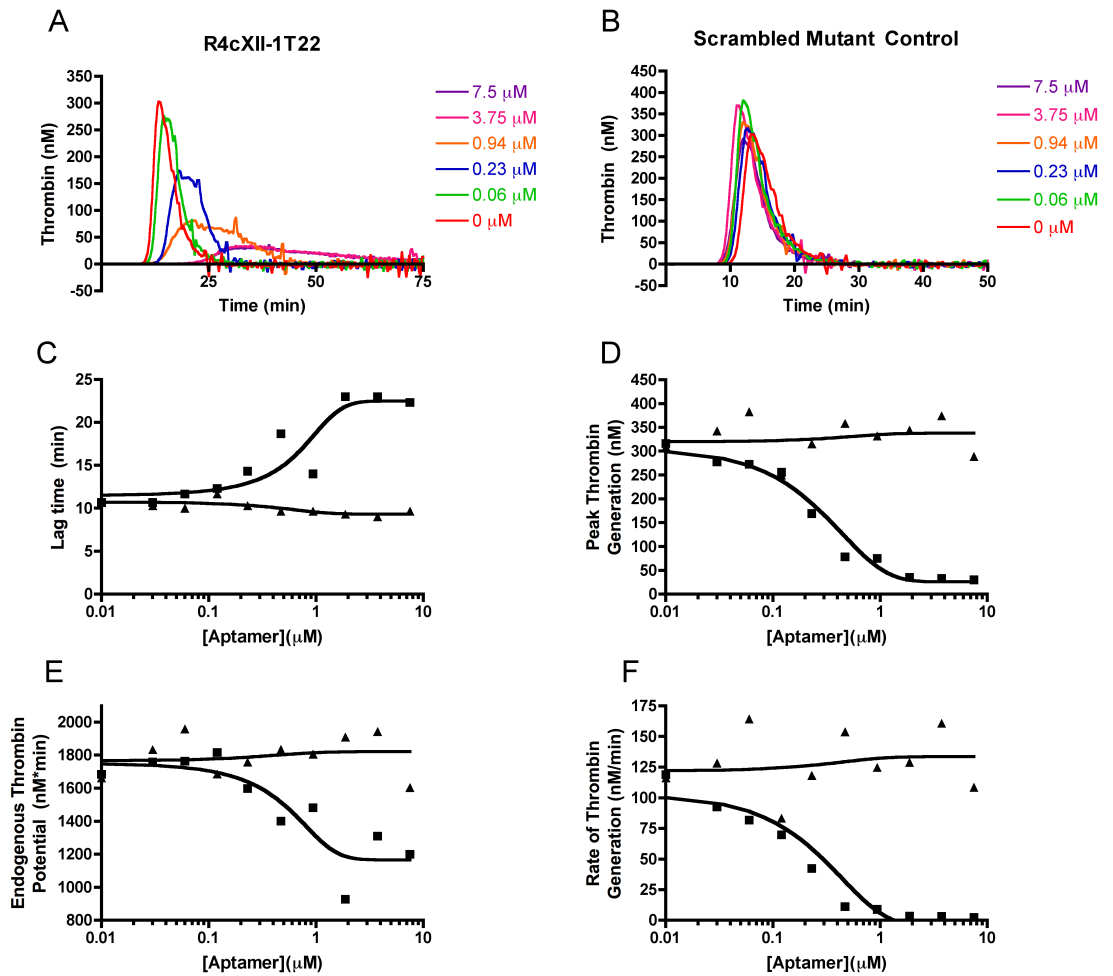
Figure 20. Aptamer R4cXII-1t dose dependently impairs thrombin generation in a TGA assay initiated with ellagic acid

Thrombograms of normal pooled plasma activated with 200x-diluted ellagic acid with various concentrations of A) R4cXII-1t and B) a size matched scrambled RNA. C) Lag time, D) Peak thrombin generation, E) Endogenous thrombin potential, and F) Rate of thrombin generation of R4cXII-1t (■) and a size matched scrambled RNA (▲). Data are representative of three independent experiments.

While the FXII aptamer was isolated solely against the human form of the

coagulation factor, analysis for cross reactivity in animal plasma is a must before

proceeding into *in vivo* models of thrombosis. In general, aptamers have exhibited some

efficacy in a variety of species; with the FIX aptamer cross-reacting in every species tested except for rabbit (data not shown). However, other aptamers exhibit limited cross-reactivity. R4cXII-1 was tested in a number of animal plasmas in an aPTT assay, as well as with different activating reagents. Activation with ellagic acid gave the most reproducible data, with less background control aptamer activity. Aside from human plasma, R4cXII-1t did not exhibit robust anticoagulation above control aptamer in any of the plasma's tested at a fixed concentration (Figure 21). However, there was slight anticoagulant activity over control aptamer in canine, bovine, rat, rabbit, and baboon. Importantly, the plasma samples used here were not treated with CTI, thus contact activation of the plasma sample may already be present.
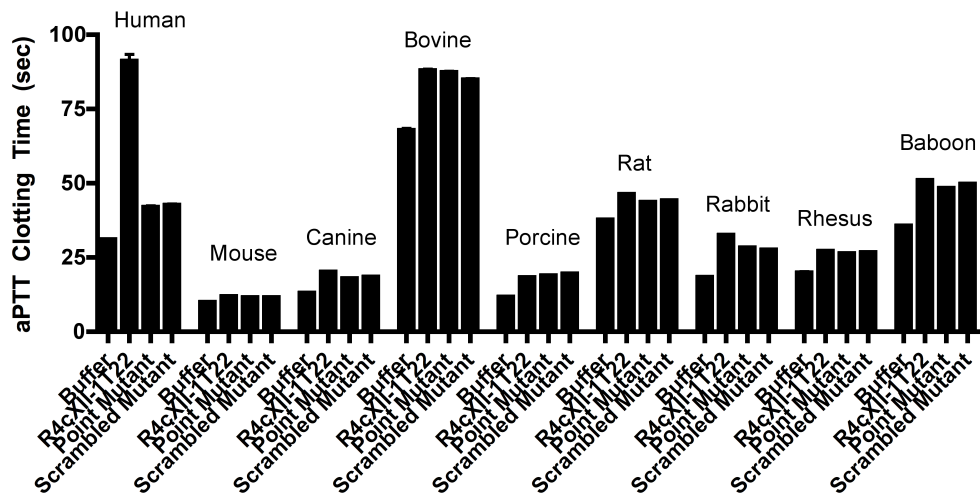
Figure 21. Analysis of R4cXII-1t cross-reactivity utilizing the aPTT across select animal species

Effect of buffer, R4cXII-1t, a point mutant, and a size matched scrambled mutant control in an aPTT clotting assay activated with ellagic acid. None of the plasmas were treated with CTI to inhibit contact activation during the blood drawing process. The data represent the mean ± SEM of duplicate measures.

2.4 Discussion

Herein, we describe the isolation and characterization of a novel FXII/FXIIa inhibitor (Figure 22). A nuclease resistant, 2′F pyrimidine modified RNA aptamer was isolated using *in vitro* iterative selection methods. This aptamer binds with high affinity to both FXII and FXIIa and is a potent anticoagulant in common clinical coagulation assays. Although the aptamer does not inhibit the active site of FXIIa, it functions as an anticoagulant by both inhibiting the autoactivation of FXII and inhibiting FXIIa-mediated activation of FXI. Additionally, the aptamer does not impede the ability of FXIIa to activate PK.

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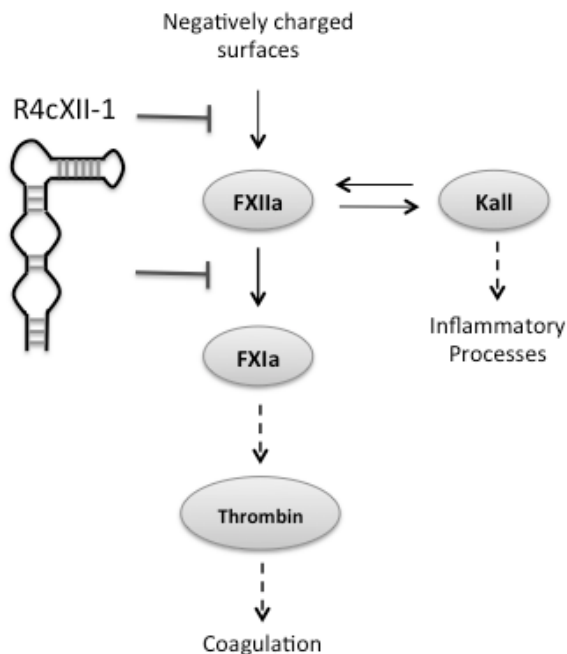
Figure 22. A schematic depicting the role of the FXII aptamer, R4cXII-1, in inhibiting FXII/FXIIa function

The aptamer R4cXII-1 inhibits FXII autoactivation by negatively charged surfaces as well as FXIIa-mediated FXI activation to effectively shut down thrombin generation in a plasma setting. However, the aptamer does not affect the ability of FXIIa to activate the inflammatory mediator prekallikrein. Predicted secondary structure of aptamer adapted from M-fold.

Taken together, the data indicate that the aptamer binds to a region on FXII/FXIIa implicated in anionic and FXI binding, which is spatially separate from where PK binds to FXII. Although a crystal structure of FXII is not available, domain mapping has suggested a closely located binding site for FXI and anionic surfaces in the N-terminus fibronectin type II domain (197-199). Studies on thrombin aptamers have determined that an aptamer binds by presenting an extended surface complementary to the protein

binding site (157) and subsequently sterically interferes with macromolecular

interactions formed between the aptamer binding region and large protein substrates.

While aptamer binding can thus disrupt a specific exosite's function, it can still allow

other distant exosites and active sites to function (181). Interestingly, other putative

binding sites on FXII for anionic molecules exist in separate domains (200). As distinct

anionic compounds bind discretely to FXII and autoactivate FXII at different rates, this

binding to different, and possibly partially overlapping, domains could explain why the

aptamer was not able to inhibit the autoactivation of FXII by various autoactivators to

the same degree. Continued studies into aptamer binding and function using FXII

mutant proteins could help to further delineate the regions involved in macromolecular

substrate interaction (197), especially the differential binding of anionic molecules to

FXII.

The ability to inhibit the intrinsic pathway of coagulation while leaving the

kallikrein-kinin system intact will be beneficial in parsing out the importance of FXIIa

functions *in vivo*. While all members of the contact pathway (FXII, FXI, PK, HK, BK)

have been implicated in thrombosis through studying various knockdown or knockout

animal models, it is not entirely clear which FXIIa-mediated process, inflammation or

coagulation, plays the primary part in contributing to thrombosis (40, 41, 72, 91, 94, 95,

201). Targeted inhibition or animal knockout models have suggested that FXIIa-

mediated FXI activation is responsible for thrombotic protection in injury models due to

the similar thrombotic protection profiles of FXI and FXII deficient animals (40, 78, 201).

Currently, studies using FXIIa inhibitors to assess thrombosis have employed active site

inhibitors, which thus inhibit all amidolytic functions of FXIIa, including PK activation

(41, 202).   Utilizing a specific inhibitor of the intrinsic pathway, while leaving the

kallikrein-kinin system intact, would be valuable in parsing out the various functions of

FXIIa in thrombosis.

The development of a TGA assay triggered by contact activation is instrumental

for studying the functions of contact pathway and intrinsic cascade proteins in plasma

thrombin generation. While autoactivation of FXII serves as the mechanism to initiate

the aPTT, and is oftentimes the diagnostic test used to detect FXII deficiencies in the

clinic during routine preoperative screening, the TGA is commonly triggered by the

extrinsic pathway activator TF, and is thus not sensitive to deficiencies or inhibitors of

FXII (203). Both the aPTT and TGA assays showed a dose-dependent increase in the time

it takes for thrombin or a clot to form with aptamer R4cXII-1 present. However, the

TGA also showed the ability of the aptamer to decrease the total amount of thrombin

formed, the peak amount of thrombin generation, and the overall rate of thrombin

generation. Importantly, the rate of thrombin generation has previously been shown to

be a key contributor in influencing clot stability, with a slower rate of thrombin

generation associated with less stable clots (64, 204). The finding that FXII inhibition

decidedly decreases the rate of thrombin generation in this assay is consistent with

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observations that FXII inhibition or deficiency is antithrombotic, potentially due to formation of unstable clots that can easily break up in thrombosis models under flow(39, 41).

In addition to its role in thrombosis, activation of the contact pathway through FXII occurs upon exposure to foreign substances, such as on the surface of a catheter in invasive surgery or even during the process of drawing blood into a collection tube (205, 206). In these situations, the irreversible FXIIa inhibitor CTI has been effective in inhibiting contact activation (207); however, inhibition of the autoactivation of FXII might result in a better antithrombotic profile. In addition, the ability to fully reverse aptamer activity by addition of a matched oligonucleotide antidote (164) or a universal antidote (165) may be beneficial in these and other settings, especially for allowing further studies into the contact pathway after drawing blood.

Through *in vitro* and animal studies, FXII has been well established as an important mediator of thrombotic effects while not affecting normal hemostatic function. In thrombosis, it is hypothesized that aggregated proteins, activated platelets, and polyphosphate, among other compounds present at a developing thrombus, can contribute to the continued activation of FXII and the intrinsic pathway, leading to excessive thrombin generation to exacerbate the growth of the thrombus (101). Inhibition of the autoactivation of FXII, as well as a significant decrease in the rate of thrombin generated at the site of a growing thrombus through targeted inhibition of the

intrinsic pathway of coagulation, could be a viable antithrombotic strategy in a number

of clinical settings. While clinical studies have not always mirrored the results seen in

FXII deficient animal models, further analysis into the utility of targeted inhibition of the

intrinsic pathway of coagulation is warranted. Currently, an aptamer generated against

FIXa and its matched oligonucleotide antidote is making their way through clinical

development (163, 164). We anxiously await the clinical evaluation of other aptamers

targeting upstream coagulation factors, such as FXII, that may not require antidotes to

be safe and effective antithrombotic agents.

3. Characterization of an anticoagulant aptamer targeting FXI

3.1 Introduction

There is a large unmet need for antithrombotics that are efficacious enough to treat both arterial and venous thrombotic disorders while safe enough to not induce severe bleeding. While currently approved antithrombotics target mainly the common pathway of coagulation, whose action is imperative for hemostasis to occur, human and animal data have suggested that inhibition of the upstream intrinsic pathway proteins, FXI and FXII, have a role in pathological thrombus formation but not hemostasis. This is supported by the fact that humans deficient in FXII and FXI have either no bleeding phenotype or a mild bleeding phenotype, respectively. In addition, genetic mouse knockouts of FXII and FXI do not exhibit prolonged bleeding times, but these mice are protected in several models of thromboembolism (40, 41, 72, 91). Because FXII also has an initiating role not only in coagulation, but also in the inflammatory kallikrein-kinin system, FXI might be a safer target to managing thrombosis without a risk of undesired side effects.

Human FXI is a 160-kDa serine protease glycoprotein that circulates as a disulfide linked dimer of two identical subunits. Each subunit consists of a 35-kDa C-terminal light chain containing the catalytic domain, and an N-terminal 45-kDa heavy chain with four ~90 amino acid tandem repeats termed apple domains (82). The four apple domains confer binding to other proteins: A1 contains binding sites to thrombin

(208), A2 to HK (209), A3 to FIX (51), the platelet receptor GP1bα (52), and heparin (210), and A4 to FXIIa (211). Dimerization occurs at the interface between the two A4 domains (54). The four apple domains form a disk-like structure that supports the catalytic domain (54). In plasma, FXI can be activated by XIIa (49), thrombin and thrombin intermediates (88, 212), as well as autoactivated by FXIa (88). Platelet polyphosphates can potently accelerate the activation of FXI by thrombin, thrombin intermediates, and FXIa, and are thus thought to amplify thrombin generation in a FXI-mediated fashion after extrinsic pathway activation in the hemostatic process (111, 203). In contrast, FXII-mediated FXI activation is not thought to be relevant for hemostasis, but can enhance thrombin generation at the site of a thrombus, thus contributing to pathological thrombosis (96).

All activators of FXI cleave the zymogen at the same activation loop site in the catalytic domain. Activation proceeds through an intermediate structure, 1/2FXIa, with one activated subunit, and is thought to possibly be the primary form of activated FXIa in plasma (56). Dimerization seems to be required for FXI activation, as it is thought that the proteases bind to one subunit while activating the other (60). In addition, dimerization might be important in tethering FXI to the site of injury, as the zymogen subunit can bind to the platelet receptor GP1bα, and leave the active subunit free to bind to and activate FIX. Zymogen FXI binds to platelets better than FXIa, with approximately 1500 binding sites per platelet (61). Whereas optimal binding requires

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HK, zinc ions, residues in the apple 3 domain of FXI, and the N-terminal domain of GP1bα, recent data has suggested that FXI binds to the platelet receptor ApoER2, which colocalizes with GP1bα (55). FXIa also binds to platelets, but with a much lower frequency of 250 binding sites per platelet (62). FXI and FXIa bind to platelets through a separate binding site and mechanism, as FXIa binding to platelets seemingly requires the catalytic domain, but not the apple 3 domain as does FXI (55).

FXI deficiency was first described as hemophilia C in 1953, and is a rare bleeding disorder that affects approximately one in 10,000 people worldwide, but is more common in distinct populations, such as Ashkenazi Jews (65). While hemophilia C does not normally result in spontaneous bleeding, it is a mild to moderate bleeding disorder with increased bleeding tendencies in tissues with high fibrinolytic activity. Historically, it has been difficult to predict bleeding tendency in FXI deficient patients as there has been a poor correlation between bleeding phenotype and plasma FXI levels. For example, some patients with severely low levels of FXI will not bleed after surgery, while patients with normal levels of FXI do bleed, suggesting that bleeding risk may be modified by the levels of other coagulation factors (213). With over 190 reported mutations in the F11 gene (www.factorxi.com), there are two main types of mutation: CRM- (Type I) are mutations causing low FXI plasma levels while CRM+ (Type II) are mostly functional mutations correlating with normal FXI levels but reduced coagulant activity. Treatment is usually not necessary for patients undergoing mild procedures,

but antifibrinolytic agents, as well as fresh frozen plasma or human FXI concentrate is effective in reducing bleeding events.

While the function of FXI in hemostasis may depend on the setting, the potential uncoupling of FXI's role in thrombosis and hemostasis is supported by observations from thrombosis models in FXI- deficient mice. Several studies using a ferric chloride model of arterial injury demonstrated that FXI deficiency protected mice from thrombus occlusion at a level comparable to FIX deficiency or high concentrations of unfractionated heparin (40, 72, 73). Attenuation of thrombus development was also observed following arterial injury from mechanical damage to the vessel or ligation (40). This protection was not limited to arterial thrombosis models, as FXI deficiency also conferred a thromboprotective effect in a transient middle cerebral artery stroke model (tMCAO), with a lower volume of infarcted brain tissue and less fibrin deposition in distal microvessels compared to wild type mice without any detectable hemorrhage (41). The potential antithrombotic effect of FXI depletion or inhibition has also been observed in species other than mice. Antibody-mediated FXI inhibition in rabbits increased clot lysis in jugular veins and reduced thrombus propagation on injured neointima of the iliac artery (74, 75), while infusion of FXI antibodies in baboons prevented growth of occlusive thrombi in arterio-venous shunt and vascular graft occlusion models (76, 77). Administration of a FXI specific antisense oligonucleotide in mice reduced levels of FXI while conferring an antithrombotic effect similar to warfarin without abnormal bleeding

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times (78). The same technology has been applied to non-human primates, as antisense

mediated depletion of FXI in cynomolgus moneys results in a prolongation of clotting

times (214). Overall, the *in vivo* evidence strongly suggests that FXI contributes to fibrin

formation and platelet activation, stabilizing occlusive thrombi under high flow

conditions. This differs from its participation in hemostasis, where FXI-driven fibrin

formation is not needed to prevent blood loss following vascular injury in most tissues

(215). The effect appears to be mediated primarily through FXII's activation of FXI and

not via thrombin activation, as FXII deficiency shows similar protective effects in

thrombosis models.

A number of anticoagulant aptamers have been isolated targeting various

proteases of the coagulation cascade. Aptamers are short, single-stranded

oligonucleotides that bind over large surface areas on the target protein and tend to act

as anticoagulant by blocking specific macromolecular interactions. Herein, we describe

the isolation and characterization of aptamers targeting FXI. These aptamers can

partially inhibit FXIa active site cleavage, as well as greatly reduce the ability of FXIa to

activate FIX. In addition, one of these aptamers acts as an anticoagulant to decrease

thrombin generation in a modified thrombin generation assay activated through the

intrinsic pathway.

3.2 Materials and Methods

Materials

The SELEX DNA template sequence was 5'-TCGGGCGAGTCGTCTG-N$_{40}$-CCGCATCGTCCTCCCTA-3' and was purchased from Oligos, etc. (Wilsonville, OR). The 5' and 3' SELEX primer sequence are 5'-GGGGGAATTCTAATACGACTCACTATAGGGAGGACGATGCGG-3' and 5'-TCGGGCGAGTCGTCTG-3', respectively, and were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The 2'Flouro modified cytidine and uridine were purchased from Trilink BioTechnology (San Diego, CA). FXIIa and prekallikrein were purchased from Enzyme Research Laboratories (South Bend, IN). FXI, FXIa, and all other coagulation proteins were purchased from Haematologic Technologies (Essex Junction, VT). Human normal pooled plasma was purchased from George King Biomedical, Inc. (Overland Park, Kansas). AMV-RT enzyme was purchased from Roche Applied Science (Indianapolis, IN). Bovine serum albumin (BSA) was purchased from EMD Chemicals (Gibbstown, NJ), ethylene glycol was purchased from Sigma-Aldrich (St. Louis, MO) and lima bean trypsin inhibitor was purchased from USB Corporation (Cleveland, OH). The Ellagic Acid-based Pacific Hemostasis APTT-XL Reagent was purchased from Thermo Scientific (Waltham, MA), and micronized silica-based TriniCLOT aPTT S was purchased from Trinity BioTech (Bray, Co Wicklow, Ireland). All kinetic assays were performed in 96-well flat bottom microtiter plates (Corning Incorporated, Corning, NY), the absorbance was read at 405nm on a Power Wave XS2 kinetic microplate spectrophotometer (Biotek, Winooski, VT), and the data were

analyzed using the Graphpad Prism Software (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

SELEX

Solution based SELEX was performed as previously described (164). The sequence of the starting RNA library was 5'-GGGAGGACGAUGCGG-N40-CAGACGACUCGCCCGA-3', where N40 is a 40 nucleotide randomized region, and C and U represent 2'F modified ribonucleotides, respectively. A modified RNA library was transcribed *in vitro* using a modified polymerase that can incorporate 2'F cytidine triphosphates and 2'F uridine triphosphates (194). Selection rounds were performed by incubating the modified starting library with FXIa and passing the mixture through a 25-mm 0.45μm nitrocellulose filter membrane (Protran BA 85, Whatman, Inc., Piscataway, NJ) to separate the bound RNA from the unbound RNA. The bound RNA was extracted and RT-PCRed to amplify the bound RNA species. For rounds 1-6, 1 nmol RNA was incubated with FXIa in a low salt Hepes buffer E/F (20mM Hepes, 100mM NaCl, 2mM $CaCl_2$, and 0.01%BSA). For rounds 6-14, 1 nmol RNA was incubated with FXIa in a physiologic salt-Hepes buffer (20mM Hepes, 150mM NaCl, 2mM $CaCl_2$, and 0.01%BSA). Rounds 11, 12 and 14 were cloned and sequenced as previously described to distinguish individual aptamer sequences (152).

RNA Aptamers

For all aptamer sequences, "C" and "U" represent 2'fluorocytosine and

2'fluorouracil. The 70 nucleotide Xla7 (11.7) aptamer sequence is 5' –

GGGAGGACGATGCGGCTCGCTACTATTCCGGATCAGCAGATCACCCCCTCCCCC

CAGACGACTCGCCCGA-3' and the 71 nucleotide sequence for Xla9 (12.7) is 5'-

GGGAGGACGATGCGGTAACGCCACGCTCGACAACGCGTCGAGTGTCCTCCGCC

CCCAGACGACTCGCCCGA-3'. All aptamers were transcribed *in vitro* as previously

described using a modified polymerase that can incorporate 2'F cytidine triphosphates

and 2'F uridine triphosphates (194). Before use, all aptamer preparations were diluted

into a Hepes-based buffer with or without BSA (20mM Hepes, 150mM NaCl, 2mM

$CaCl_2$, and with or without .01%BSA) as indicated. Diluted aptamers were refolded by

heating to 65°C for 5 minutes, and then cooled for 3 minutes at ambient room

temperature.

FXIa small peptide substrate cleavage

FXIa (final concentration 25nM) was incubated with 500nM aptamer, library, or

buffer for 5min at 37°C in a Hepes-based reaction buffer (20mM Hepes pH 7.4, 150mM

NaCl, 2mM $CaCl_2$, .01% BSA). The chromogenic substrate Pefachrome FXIa

(Centerchem, Inc., Norwalk, CT) (final concentration 1mM) was added to a total volume

of 100μL. Substrate cleavage was monitored at an absorbance of 405nm using a kinetic microplate spectrophotometer at 37°C, and rates of substrate cleavage were determined using linear regression analysis.

Restriction Fragment Length Polymorphism

Library and SELEX round DNA (50pmoles) were end-labeled with [γ^{32}P] ATP (Perkin Elmer, Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). [γ^{32}P] DNA was digested with AluI, HhaI, and HaeIII (New England Biolabs, Beverly, MA) for 3 hours at 37°C. Digested radiolabeled DNA was then separated on an acrylamide gel and visualized using a Storm 825 phosphoimager (GE Healthcare, Piscataway, NJ).

Nitrocellulose filter binding assay

A double-filter nitrocellulose binding assay was employed to determine the apparent binding affinity constants (K_d values) of aptamer to protein (152). The 5′ end of the RNA was dephosphorylated using bacterial alkaline phosphatase (Gibco BRL, Gaithersburg, MD), and subsequently end-labeled with [γ^{32}P] ATP (Perkin Elmer, Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Varying concentrations of purified protein were incubated with [γ^{32}P] RNA in Hepes binding buffer with BSA at 37°C for 5 minutes. The RNA-protein complexes were then

separated from the unbound RNA by passing the mixture through a nitrocellulose filter

(Protran BA 85, Whatman Inc., Florham Park, NJ). The levels of bound RNA were

quantified using a Storm 825 phosphoimager (GE Healthcare, Piscataway, NJ). Non-

specific binding of the radiolabeled RNA to the nitrocellulose filter was subtracted, and

the data were fitted using nonlinear regression to determine the apparent binding

affinity for the RNA aptamer-protein interaction (GraphPad Prism, GraphPad Software,

Inc., San Diego, CA).

FXIa-mediated FIX activation

　　　Conversion of zymogen FIX to activated FIXa by FXIa was measured in a two

stage chromogenic assay performed at 37°C in a Hepes-based reaction buffer (20mM

Hepes pH 7.4, 150mM NaCl, 2mM CaCl$_2$, .01% BBF). Aptamer or buffer was incubated

with FXIa (final concentration 5nM) for 5 minutes at 37°C, and reactions were initiated

with the addition of FIX (final concentration 1μM). At timed intervals after the initiation

of the reaction, samples of the reaction mixture were removed and FXIa activity was

quenched by addition of lima bean trypsin inhibitor (final concentration 1mg/mL)

suspended in EDTA (final concentration 2.1mM). FIXa activity was assayed by the

addition of 1mM Pefachrome FIXa 3107 (Centerchem, Inc., Norwalk, CT) suspended in

Hepes-based reaction buffer supplemented with ethylene glycol (20mM Hepes pH 7.4,

150mM NaCl, 2mM $CaCl_2$, .01% BBF, 30% ethylene glycol). The rate of substrate

hydrolysis was recorded at 405nM using a kinetic microplate spectrophotometer at 37°C.

To determine the inhibition constant (K_i), FIX activation reactions were

performed as described above in the presence of varying concentrations of aptamer. The

initial rate of FIX activation by FXIa was determined by linear regression of the rate of

FIXa formed versus time for the first 10 minutes of the reaction. Fractional activity

(V1/V) was calculated by dividing the initial rate of FXI activation in the presence of

aptamer by the initial rate of FXI activation in the absence of aptamer. The K_i was

determined using nonlinear regression analysis.

Cell-based model of thrombin generation

The cell-based model system has been described in detail (64). Briefly, human

monocytes from one individual were isolated and cultured overnight in a 96-well

microtiter plate in 500ng/mL lipopolysaccharide-containing macrophage serum free

media (Gibco, Invitrogen Life Technologies, Grand Island, NY) to induce TF expression.

Platelets were obtained and purified from a normal healthy individual, and diluted to a

final concentration of ~150×10^9 L^{-1}. TF expression on cultured monocytes were checked,

and determined to be on the order of 1pM. Final concentrations of proteins were

approximately at normal plasma levels: antithrombin (3.0μM), TFPI (3nM), FII (1.4μM),

FIX (70nM), FX (135nM), FVIIa (0.2nM), FXI (25nM), FV (20nM), and FVIII (0.3nM).

Aptamers were incubated with FXI for 5 minutes at room temperature. This mixture was added to the culture monocytes, as well as platelets, the purified coagulation proteins, and 3mM calcium chloride. At timepoints, 10μL of the reaction mixture was added to 1mM FIIa Chromozyme (Aniara, West Chester, Ohio) in 1mM EDTA. After 10 minutes, 50% acetic acid was added to stop the reaction, and the rate of substrate hydrolysis was recorded at 405nM using a kinetic microplate spectrophotometer at 37°C.

Clotting Assays

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays were carried out using a model ST4 coagulometer (Diagnostica Stago, Parsippany, NJ). Aptamer diluted in Hepes buffer without BSA (20mM Hepes, 150mM NaCl, 2mM CaCl$_2$) was refolded by heating for 5 minutes at 65°C and then cooled to ambient temperature for 3 minutes. Pooled normal human platelet poor plasma was prepared by drawing blood into sodium citrate tubes. For the aPTT, aptamer (5μL) was then added to 50μL pooled normal human platelet poor plasma and incubated for 5 minutes at 37°C. 50μL APTT-XL Reagent was then added to activate plasma and incubated for 5 minutes at 37°C. Clotting was initiated by adding 50μL of 25mM CaCl$_2$ and the time to clot formation was recorded. Aptamer concentrations represent the final concentration in the entire reaction.

For the PT, refolded aptamer (5μL) was added to 50 μL pooled normal human platelet poor plasma and incubated for 5 minutes at 37°C.   TriniClot PT Excel (Trinity BioTech, Bray, Co Wicklow, Ireland) was added to initiate clotting.

Thrombin Generation Assay

In an Immulon 2HB clear U-bottom 96 well plate (Thermo Labsystems, Franklin, MA), CTI- treated (50μg/mL), pooled normal human platelet poor plasma from healthy, consented volunteers (60μL) was mixed with aptamers (3μL) diluted in Hepes-based buffer without BSA and incubated for 5 min at 37°C. Ellagic acid reagent (final concentration 0.1μM, diluted 1:200 with 4μM phospholipid vesicles (20% phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine) in Hepes-based buffered saline)(15μL) was added to the plasma-aptamer mixture, and incubated for 5 minutes at 37°C. Flu-Ca (Diagnostica Stago)(15μL) was added to start the assay and thrombin generation was measured using a Fluroskan Ascent Reader (Thermo Scientific, Waltham, MA). A thrombin calibrator reagent (Diagnostica Stago, Parsippany, NJ) was used to quantify the amount of thrombin generated in the samples. Data analysis was performed using Thrombinoscope software (Thrombinoscope BV, the Netherlands). Aptamer concentrations represent the final concentration in the entire reaction. The amount of ellagic acid used was standardized to produce a similar curve to a TF-activated TGA. In addition, the amount of CTI present was standardized to inhibit

contact activation of the plasma sample, while still allowing for robust FXII-dependent

activation upon administration of ellagic acid.

Thromboelastogram

Blood was drawn from healthy, consented volunteers under a Duke University

Institutional Review Board Protocol into tubes with 3.2% sodium citrate. Whole blood

(320µL) was mixed with aptamer (10µL) and incubated at room temperature for 5

minutes. Kaolin (10µL)(Haemonetics, Niles, IL) was added, and the entire reaction

(340µL) was added to 20µL CaCl$_2$ in a disposable, plain TEG cup (Haemonetics). Clot

formation was measured at 37°C with a Thromboelastograph Analyzer (Haemonetics)

until a stable clot was formed. Parameters were automatically calculated using the

TEG® Analytical Software version 4.2.3 (Haemonetics).

3.3 Results

3.3.1 SELEX Pool Analysis and Lead Clone Selection

To isolate aptamers targeting FXIa, we utilized a traditional solution based

SELEX against purified FXIa. A library of approximately 10^{14} 2'Fpyrimidine modified

RNA sequences were incubated with FXIa and screened for high affinity binding and

active site inhibition. Because binding of the pool RNA was initially weak, the selection

was begun in a binding buffer with a lower salt concentration (100mM NaCl), and

subsequently increased to physiologic salt concentration (150mM NaCl) once higher

binding affinity was established. After 6 rounds of selection in a lower salt buffer, the

binding affinity of the pool RNA began to increase as high affinity binding sequences

began to dominate the pool. The final rounds were performed at physiological salt

conditions.

Fourteen total rounds of SELEX were performed, with progression monitored in

several ways. First the binding affinity of each round was determined by a

nitrocellulose filter-binding assay; however, this assay was often inconsistent, perhaps

because of the dimer formation of FXIa. Thus, the evolution of the selection was

additionally assessed for functionality and diversity (Figure 23). The functionality of the

pool RNA was monitored by its ability to reduce the active site cleavage rate of a small

substrate. As shown in Figure 23A, the pool began to include a higher number of

sequences that could decrease the rate of active site cleavage of FXIa as early as round 4,

and these sequences were maximally present in rounds 11 and 12. Later rounds began

to slightly loose this functionality, indicating the loss of functional aptamers as

stringency towards tighter binders was increased. Lastly, the diversity of sequences

present in the pool was examined using a restriction fragment length polymorphism

(RFLP) assay (Figure 23B). In this assay, the oligonucleotide pool is digested with a set

of restriction enzymes and separated according to length by gel electrophoresis. The

emergence of separate bands from the starting library RNA was apparent from the

earliest round tested, round 8. Rounds 13 and 14 exhibited an additional band,

suggesting the further evolution of the pool. For comparison of diversity, and to isolate

the more frequent functional sequences present in the earlier pools, rounds 11, 12 and 14

were chosen to clone and sequence.
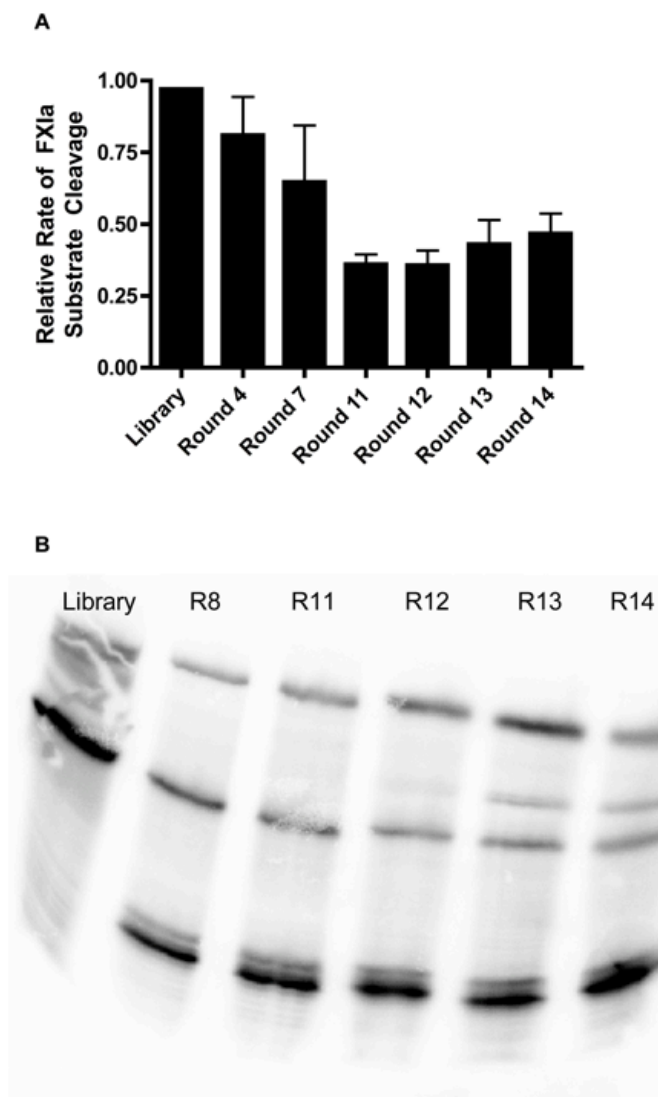
Figure 23. Analysis of FXIa SELEX rounds active site functionality and sequence diversity

A) Chromogenic assay for active site activity. FXIa (25nM) was incubated with SELEX RNA rounds (500nM), and active site activity was measured by a FXIa specific chromogenic substrate. The data were normalized to the rate in the absence of compound and represent the mean ± SEM of duplicate measures. B) RFLP analysis of SELEX rounds. Radiolabeled DNA from each round was digested with the same set of restriction enzymes and separated by gel electrophoresis. The labels above the lanes indicate the round: "R8" means round 8.

The seventy clones selected to sequence from all three rounds were organized into families based upon their primary sequences (Table 7). There was considerable diversity in the pool, with the highest frequency clone present only in 20% of the pooled sequences. While many of the conserved sequences were found in two or all three of the sequenced pools, there were many "orphan" sequences, or sequences that do not fit into a family, but are present at a low frequency in the final pool. The functional data and progression of the SELEX rounds indicate that there is some loss of functional binders, therefore, several of the orphan sequences from rounds 11 and 12 were analyzed, and indicated by their individual clone name.

Table 7. RNA aptamer sequences from FXIa solution-based SELEX

Clone ID	Variable Region Sequence	Frequency
Xla1	ACCGCATCCGTGAAGATCCCTCTTCTCCCCTCCCCCC	20.0%
Xla8	GCCGTCCTGAGTCGTATGAATTCCGCATGGTCGTCGTGTG	12.9%
Xla2	AATTACCCGCGTCTGTAGTACACATGCTATCCCCTCCCCC	8.6%
Xla7	CAAACTAATTCGGGCCGCGCACTGGGTCCTTCCCCCCCC	8.6%
Xla4	ATCGTGCATTATTTCTGGCTACCAGCCAACGGTCCCCCCC	5.7%
Xla5	ATGTCTGGTAATCACACCACACTGCTCCAGCCTTCCCCCC	5.7%
Xla3	CACTACGAGGCGTCACTTGCCCGAACATCACCTCCCCCCC	2.9%
Xla6	AGCACATAAACGCTGGTGTTTCCACCCACCTCCCCCCCC	2.9%
11.7	CTCGCTACTATTCCGGATCAGCAGATCACCCCCTCCCCC	2.9%
11.23	TATCAGCACGCGTCTGCACTCTAGAGTGTCCCCTCCCCCC	1.4%
12.7	TAACGCCACGCTCGACAACGCGTCGAGTGTCCTCCGCCCC	1.4%

Sequence analysis of the clones indicated a conserved region near the end of the random region, found in most families, consisting of a string of C's. Predicted structural

analysis indicated that this region bound to the 5' constant region, resulting in a similar structure for many of the sequence families (Figure 24). This structure is hallmarked by a central loop with two stem loops emerging from it, as well as a stem and the un-base-paired 3' constant region. Outside of the constant regions and the string of C's, no sequence similarity was found between families.
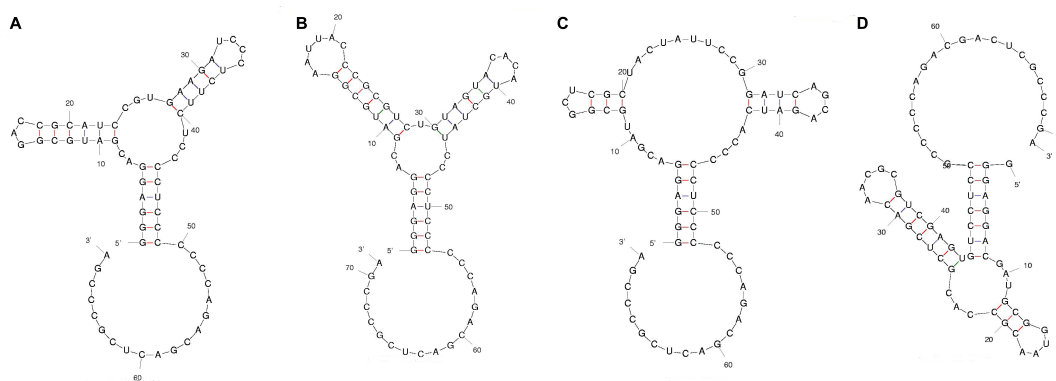
Figure 24. Representative secondary structures of clone families
The M-fold predicted secondary structures of A) Xla1, B) Xla2, C) 11.7 and D) 12.7.

In order to select a lead aptamer for further analysis, a representative clone from each of family was screened in three separate assays: binding affinity utilizing the nitrocellulose filter binding assay, functionality of active site cleavage, and functionality of FXIa-mediated FIXa activation. A summary of these results is found in Table 8. While not found in a high frequency in the selection pool, two of the most potent aptamers, 11.7 and 12.7, were further characterized.

Table 8. FXI aptamer screening results

Aptamers were screened in a binding affinity assay, and two chromogenic assays to determine the rates of active site cleavage of FXIa and FXIa-mediated FIXa generation. For the chromogenic assays, all aptamers were tested at 0.5μM. Data represents one independent experiment.

Aptamer	FXIa Binding affinity K_d approx (nM)	Active Site Cleavage Rate (A405/msec)	FIXa generation rate (A405/mmin)
Xla1	753.9	2.346	0.618
Xla2	394.9	2.021	0.419
Xla3	303.5	4.100	1.314
Xla4	73.1	4.481	1.177
Xla5	65.5	4.436	0.526
Xla6	362.3	2.302	0.823
Xla7	2,182.0	3.974	ND
11.7	27.5	1.603	0.259
11.23	75.6	2.235	1.312
12.7	21.0	1.511	0.178
Library	461.7	4.375	1.81
Buffer	N/A	3.966	1.682

3.3.2 Lead Aptamer Biochemical Characterization

While several of the aptamer clones did not affect active site cleavage, a number of aptamers were able to slow the rate of active site cleavage of FXIa to varying degrees. For all FXI biochemical assays, a non-binding, size-matched control RNA was used to assess the non-specific effects of RNA activity on FXI. Clones 11.7 and 12.7 were maximally able to slow active site cleavage rates by ~65% compared to library control RNA or non-functional aptamers (Figure 25).
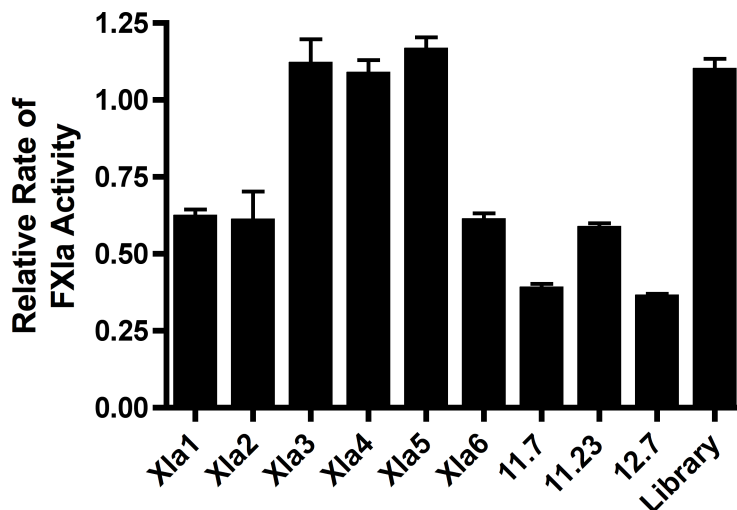
Figure 25. Active site activity assay to determine lead clone candidates
FXIa (25nM) was incubated with FXIa aptamer clones (500nM), and active site activity
was measured by a FXIa specific chromogenic substrate. The data were normalized to
the rate in the absence of compound and represent the mean ± SEM of at least duplicate
measures.

Both of these clones were next analyzed using nitrocellulose filter binding assays

to determine their binding affinity to both zymogen FXI and FXIa (Figure 26). Binding

in general to FXI and FXIa was quite variable and inconsistent, even with control RNA,

compared with binding studies on other proteins, resulting in widely changing

disassociation constants. A compilation of all binding studies suggested that 11.7 binds

to only FXIa, while 12.7 binds to both zymogen FXI and FXIa (Table 9). For both clones,

the B_{max}, or the percentage of RNA present that is folded correctly in order to bind to the

target protein, was also very low, suggesting that only a small fraction of the RNA is

able to bind the protein. Additional nitrocellulose filter binding assays to other proteins

were employed to determine specificity of aptamer binding. Both aptamers did not

exhibit binding toward a panel of coagulation factors, including the structurally related

prekallikrein (data not shown). However, 11.7 did exhibit weak binding to the

enzymatically inactive HK, a protein that circulates in complex with FXI or PK.
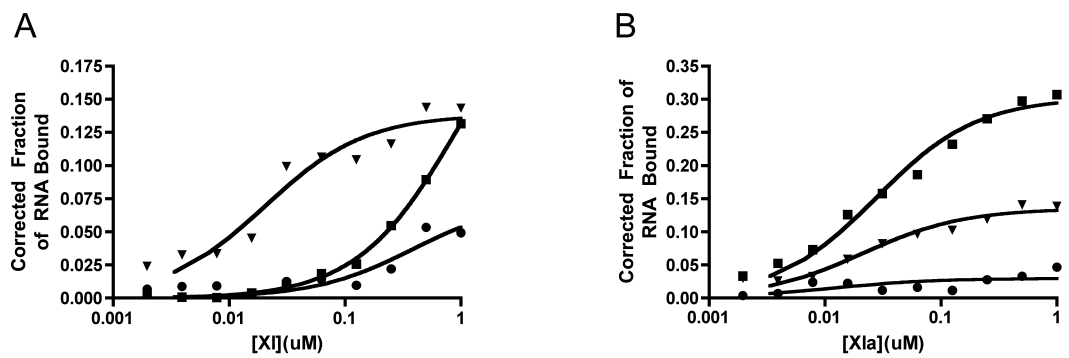
Figure 26. Aptamer 11.7 binds preferentially to FXIa while aptamer 12.7 binds to both zymogen FXI and FXIa
Representative nitrocellulose filter binding assay of 11.7 (■), 12.7 (▼), and library control RNA (●) to A) FXI and B) FXIa.

**Table 9. RNA aptamer binding to soluble FXI and FXIa as determined by a
nitrocellulose filter-binding assay**

Data represent at least duplicate measures.

Aptamer	FXI		FXIa	
	K_d approx (nM)	B_{max} approx (%)	K_d approx (nM)	B_{max} approx (%)
11.7	671.6 ± 265.9	21.3 ± 4.3	52.0 ± 13.5	24.7 ± 2.0
12.7	139.2 ±81.9	10.45 ± 1.7	84.9 ± 32.5	9.1 ± 2.5

In the coagulation cascade, FXI functions to activate FIX to amplify thrombin
generation. Thus, the ability of the lead aptamers to inhibit this reaction was next
determined. This reaction was assessed by monitoring the amount of FIXa formed in a
purified system using FXIa as an activator in the presence or absence of aptamer or
control RNA. The presence of either aptamer in this reaction impaired the initial rate of
FIX activation by greater than ~80%, while the presence of control RNA did not (Figure
27A). To determine the potency of inhibition, the initial rate of FIX activation by FXIa
was measured in the presence of varying concentrations of either 11.7 or 12.7, and was
shown to dose dependently decrease the amount of FIXa formed. An apparent
equilibrium inhibition constant (K_i) of 82.4 ± 12.8nM for 11.7 or 190.1 ± 27.3nM for 12.7
was obtained by plotting the fractional rates of FIX activation versus the concentration of
aptamer used (Figure 27B and C). While 11.7 could maximally inhibit 90% of FIXa
generation, 12.7 had slightly less potency while able to inhibit 75% of FIXa generation.
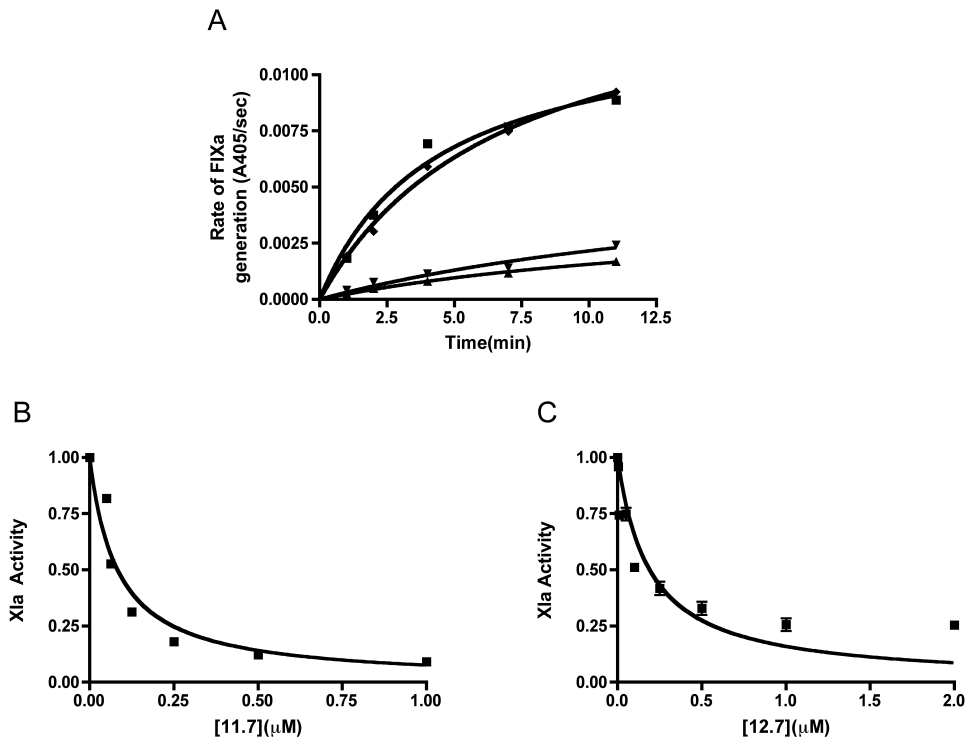
Figure 27. Both lead aptamers reduce FXIa-catalyzed activation of FIX
FIX (1μM) was activated by FXIa (5nM), the reaction was quenched at various
timepoints by the addition of 1mg/mL LBTI, and the amount of FIXa was quantified
using a chromogenic substrate. A) The rate of FIXa generation at various timepoints was
determined in the presence of 500nM 11.7 (▼), 12.7 (▲), control library RNA (◆), or
buffer (■). Lines represent nonlinear regression fits of the data. The data are
representative of two independent measures. Fractional FXIa activity of B) 11.7 and C)
12.7. Fractional activity was calculated by dividing the initial rate of FXI activation in the
presence of R4cXII-1 by the initial rate of FXI activation in the absence of aptamer. The K_i
was calculated by nonlinear regression analysis. The data represent the mean ± SEM of
at least duplicate measures

Since both aptamers can reduce FXIa active site cleavage and FIXa generation,

they were next tested to see if they could reduce thrombin generation in a reconstituted

cell-based model of coagulation. This assay was developed to assess thrombin

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generation using cultured monocytes as a source of tissue factor, and purified platelets

as a surface for physiologic amounts of procoagulant proteins to generate thrombin (64,

216). Preliminary results suggest that both aptamers are potent inhibitors of thrombin

generation in this assay (Figure 28). This assay does not include FXII, thus any FXI

activity is thought to occur from the thrombin feedback loop.
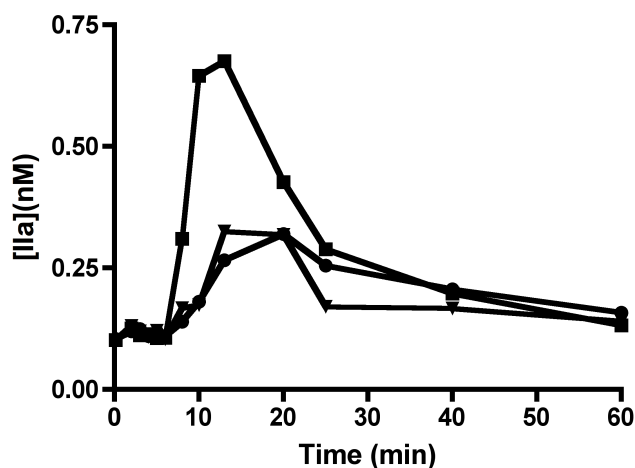
Figure 28. Both aptamers inhibit thrombin generation in a reconstituted cell-based model
Purified platelets and physiologic amounts of procoagulant factors are added to human monocytes induced to express tissue factor and thrombin generation is determined by a chromogenic substrate. The amount of thrombin formed with pre-incubation of FXI with 11.7(●), and 12.7 (▼) with control library RNA (■) was determined. The data are representative of two independent experiments.

3.2.3 Lead Aptamer Functional Analysis

While both aptamers exhibit biochemical inhibition of FXIa function and

downstream thrombin generation, we next tested each aptamer for anticoagulant

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activity in a plasma-based common clinical coagulation test, the activated partial

thromboplastin time (aPTT). This assay measures the time it takes for a fibrin clot to

form in plasma, and is sensitive to deficiencies or inhibition of the intrinsic and common

pathways of coagulation. This assay uses a negatively charged substance, such as kaolin

or ellagic acid, to activate FXII and prime the cascade, and, upon addition of Ca^{2+} to

initiate clotting, records the time for a fibrin clot to form. Aptamer 12.7 shows a slight

increase of clotting time over control RNA in an aPTT activated with ellagic acid (Figure

29A), although this increase plateaus once aptamer saturation of target protein is

reached. In addition, 11.7 does not show activity in this assay. As a control, both

aptamers do not inhibit clotting time in a PT, which measured extrinsic pathway

inhibition (Figure 29B). Use of a different activator (micronized silica), abolished the

ability of 12.7 to prolong clotting time in an aPTT (data not shown).
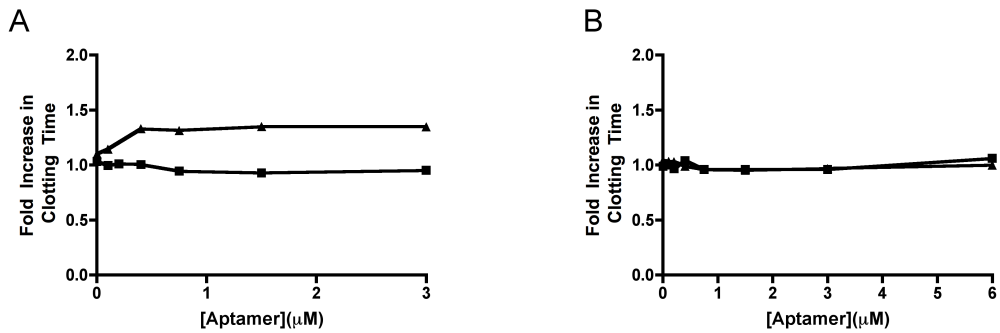
Figure 29. Lead aptamer screening in functional clotting assays
Dose titration of 11.7 (■) and 12.7 (▲) in A) an aPTT clotting assay activated with ellagic acid or B) a PT clotting assay activated with TF. The data were normalized to the clot time of a control library RNA.

Because of the minimal prolongation of clot time seen in an aPTT, 12.7 was

examined in the more sensitive thrombin generation assay (TGA) to determine

functionality in plasma. This is a coagulation assay similar to the cell-based model

system that measures thrombin generation in human plasma in real-time by monitoring

cleavage of a thrombin-specific chromogenic substrate. While the cell-based model

system used cultured macrophages to initiate thrombin generation through the extrinsic

pathway, a TGA was developed with our colleagues at Merck Research Labs using

ellagic acid as an activator to study thrombin generation during inhibition of the

intrinsic pathway. Using a TGA allows for several more parameters to be analyzed in

addition to the lag time (the time when thrombin begins to appear), including the peak

(maximal amount of thrombin formed), the endogenous thrombin potential (ETP, the

total amount of thrombin formed), and the rate of thrombin generation (the slope of the

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line from the lag time to peak thrombin generation). Contrary to the aPTT data, 12.7 exhibited potent inhibition of thrombin generation in this assay, including prolongation of the lag time by several minutes (Figure 30). Importantly, in contrast to the aPTT, which uses sodium citrate pooled human plasma, this assay uses human plasma that has been treated with corn trypsin inhibitor (CTI), a FXIIa inhibitor that is added during the blood drawing process to inhibit any contact activation that may occur. While 12.7 dose dependently inhibited thrombin generation and all analyzed parameters, including the ETP, the peak amount of thrombin formed, and the rate of thrombin generation, size matched library control RNA exhibited agonist activity and dose dependently increased the rate of thrombin generation and the peak amount of thrombin formed. While the mechanism of this is undetermined, other control RNA with no inhibitory characteristics also exhibited this trend (data not shown). However, control RNA had no effect on the ETP of the sample, indicating that the same amount of thrombin was generated across doses. In contrast, 12.7 completely abolished ETP, with no thrombin formed at the two highest concentrations tested (Figure 30D).
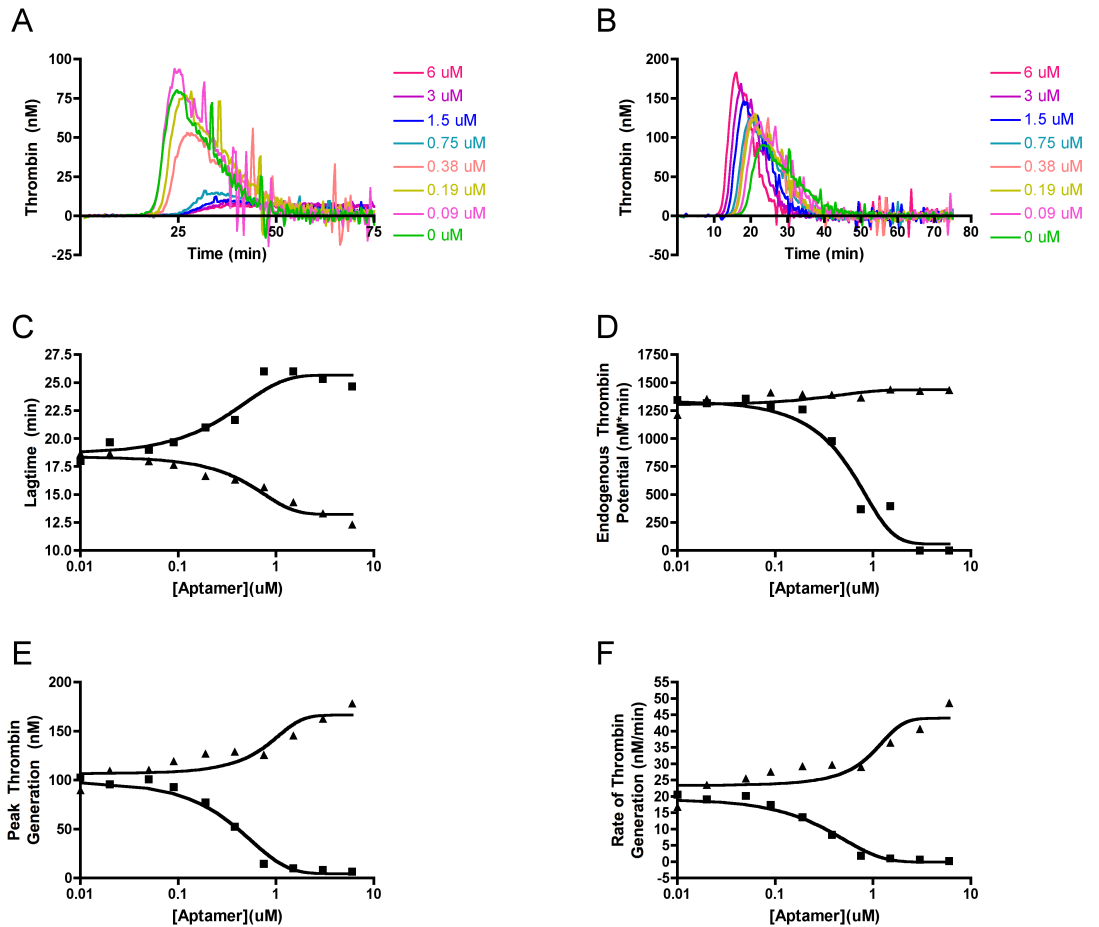
Figure 30. Aptamer 12.7 dose dependently impairs thrombin generation in a TGA assay initiated with ellagic acid

Thrombograms of normal pooled plasma activated with ellagic acid with various concentrations of A) 12.7 and B) size-matched library control RNA. C) Lag time, D) Endogenous thrombin potential E) Peak thrombin generation, and F) rate of thrombin generation of 12.7 (■) and library control RNA (▲). Data are representative of one independent experiment.

While the aPTT and TGA indirectly measure thrombin generation in a plasma-based setting, an assay that uses whole blood, and thus also platelets, was utilized to measure clot strength kinetics. This assay, the thromboelastogram, uses kaolin to

activate citrated blood, and measures the time it takes for a clot to form (lagtime), the

rate of clot formation (αangle) and the strength of the clot (maximum amplitude). Both

lead aptamers were tested in this assay, and both 11.7 and 12.7 could prolong the time it

takes for a clot to form over size-matched control RNA (Figure 31). Aptamer 12.7 also

significantly decreased the angle, or the rate of clot formation.
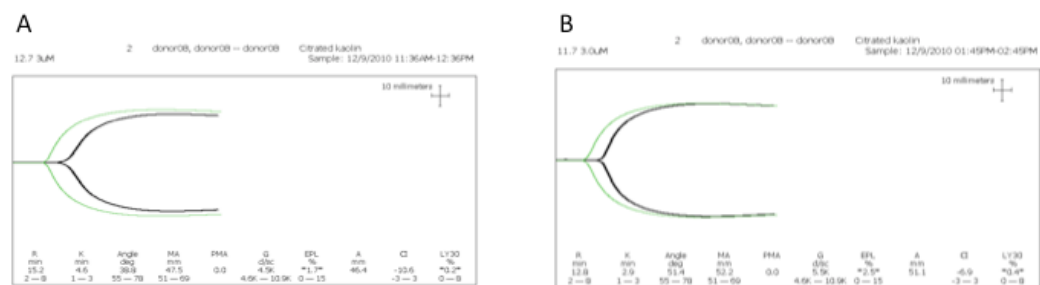
**Figure 31. FXI aptamers can impair clot strength parameters in a
thromboelastogram activated with kaolin**
Thromboelastogram tracings for A) 12.7 (green) and size-matched control scrambled
RNA (black) and B) 11.7 (green) and size-matched control scrambled RNA (black). The
data are representative of two independent observations.

While the FXI aptamer was isolated solely against the human form of the

coagulation factor, analysis for cross reactivity in animal plasma is a must before

proceeding into *in vivo* models of thrombosis. In general, aptamers have exhibited some

efficacy in a variety of species; with the FIX aptamer retaining binding in every species

tested except for rabbit (data not shown). However, other aptamers exhibit limited

cross-reactivity. Both lead FXI aptamers were tested in a number of animal plasmas in

an aPTT assay, as well as with different activating reagents. Unfortunately, no

anticoagulant effect was seen in any of the plasmas tested (data not shown).

Importantly, the animal plasma samples were not treated with CTI, thus contact

activation of the plasma sample may already be present, and subsequent aptamer

inhibition may not be detected. This experiment will be repeated once we obtain CTI-

treated, non-contact activated plasma from each animal species.

3.4 Discussion

We utilized a solution based SELEX to isolate aptamers that bound to FXIa and

inhibited coagulant function. A number of aptamers were characterized that could

reduce the rate of FXIa active site cleavage of a small chromogenic substrate, as well as

inhibit FXIa-mediated FIX activation (Figure 32). All of these functional aptamers

evolved a similar predicted secondary structure, however, binding affinities varied

widely, and were generally low compared to other aptamer selections. Two lead

aptamers exhibited a robust decrease in thrombin generation in a reconstituted cell

based model system. Plasma or blood based assays utilizing the FXIa aptamers did not

initially have a robust affect on thrombin generation; however, contact activation during

a blood draw could decrease the function of these aptamers in plasma or blood that was

not treated with a contact pathway inhibitor. Addition of a FXIa aptamer in a plasma

assay using contact-inhibited plasma during the blood draw showed potent inhibition of

thrombin generation.
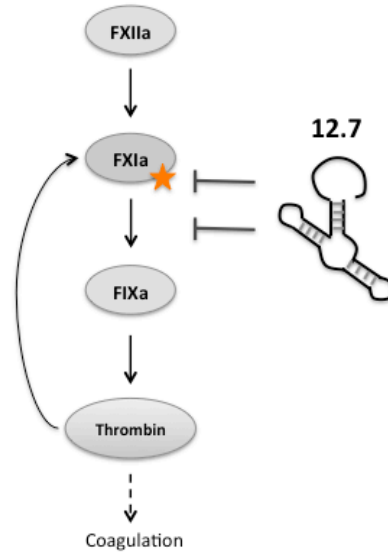
Figure 32. A schematic depicting the role of the FXI aptamer, 12.7, in inhibiting FXI/FXIa function

The aptamer 12.7 inhibits FXIa active site cleavage as well as FXIa-mediated FIX activation to effectively shut down thrombin generation in a plasma setting. Predicted secondary structure of aptamer adapted from M-fold.

Initial binding of the starting RNA library to FXIa was low at physiologic salt concentrations; therefore, SELEX was initially started in a lower salt containing buffer in which the library RNA exhibited binding (data not shown). After six rounds, the pool had evolved with a higher affinity binding than the starting library in both lower salt and physiologic salt buffers; thus, the selection was then continued at physiologic salt concentrations. Throughout the selection, background binding varied widely in separate experiments and in separate lots of FXIa. While each lot of FXIa was believed

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to be >95% pure by the company it was purchased from, during the preparation process, the zymogen protein is activated by FXIIa, a protein that can bind to library RNA fairly easily. While there are not any of the original lots of protein available to subsequently test for purity, contamination of a high nucleic acid binding protein could have been responsible for the varied background binding. Subsequent lots showed much reduced and more consistent background bindings, suggesting that purification methods have been optimized.

Because of the inconsistencies with the nitrocellulose filter-binding assay, the SELEX rounds were also monitored functionally in a simple chromogenic assay determining the rate of FXIa active site cleavage. While aptamers do not normally bind within and inhibit the active site, some aptamers have been found to reduce this activity (184). It is thought that the aptamer either binds in such a way to partially occlude or allosterically affect the active site, thus reducing rates of substrate cleavage. Beginning with Round 4, the selection pool contained sequences that could slightly reduce FXIa active site cleavage. Further evolution of the pool resulted in further reduction of active site cleavage. However, as stringency in binding was increased in later rounds, the pool began to loose the frequency of functional binders. Analysis of individual clones identified the most functional aptamers from the two most potent rounds in this assay.

Cloning and sequencing three rounds from this selection led to identification of numerous aptamer families and orphan sequences. While some selections are driven

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nearly to completion, with the identification of one or two closely related sequences that tend to dominate the pool (152, 191), this selection had many aptamer families present to screen for functionality. Testing these aptamers in a panel of biochemical assays identified several of the most potent aptamers, and several aptamers that had no functionality. Interestingly, all of the functional aptamers contained the same predicted structure, while the non-functional aptamer structures varied. In analyzing the sequences of the aptamer families, most sequences evolved a string of C's near the end of the variable region that bound to the 5' constant region. The library used for this selection was shorter than previous libraries (71 nucleotides versus 80 nucleotides). While both libraries contained 40 nucleotide random regions, the shorter library contained a truncated 3' constant region. In another selection using this library, the same string of C's near the end of the variable region was also evolved. This suggests that the constant regions in this shorter library may not exhibit stable binding, and thus the selection had to evolve stable aptamer structures first before then selecting for high affinity binders. Since the binding affinity was so low in this selection, and this was the first selection using this library to isolate aptamers against coagulation factors, the initial library may not have been able to exhibit high structural diversity due to low stability binding of the constant regions, especially at physiologic salt concentrations.

Similar to the selection rounds, binding analysis of the two most potent functional clones exhibited lower binding affinities (high nanomolar range) and B_{max}

percentages (10-20%) than normally seen with other aptamers to coagulation factors

(152, 162). As most coagulation proteins are not dimers, the nitrocellulose filter-binding

assay may not be a suitable assay to determine binding with a dimerized protein. It is

possible that the protein could fall apart, or bound RNAs could be eluted off the protein

due to a structural change upon binding to the nitrocellulose filter. Separate binding

assays to compare aptamer binding to FXIa are currently planned, as well as binding

assays to a non-dimeric form of the protein, as well as the isolated catalytic domain. In

addition, the predicted secondary structure of the RNA's present a large un-basepaired

3' region. While no other structures are predicted, it is our experience that un-

basepaired regions are not very stable. A native gel binding assay with 11.7 to FXIa

showed three aptamer states, where only one of those states were able to bind to FXIa,

corresponding with the determined B_{max} where only 30% of the aptamer is folded

correctly to bind to FXIa.   Truncation of the aptamers, especially eliminating the 3' un-

basepaired region, could lead to further stabilization of the aptamer structure capable of

binding to FXIa, thus increasing the B_{max} of the aptamer.

The crystal structure of FXIa has been solved, along with the locations of the

binding site of the proteins that FXIa interacts with. These binding sites are located in

four apple domains, on top of which the catalytic site sits. The decision to perform

SELEX on FXIa rather than FXI preceded from the observation that zymogen FXI does

not bind FIX. Cleavage of the activation loop by FXIIa or thrombin in the catalytic

domain results in significant conformational changes. Specifically, an arginine residue

which is normally buried in the A3 domain near the FIX binding site is subsequently

exposed upon FXI activation, acting as a switch to shift the position of the apple domain

disk relative to the catalytic domain, thus exposing the FIX binding site (54). As both

lead aptamers reduce active site cleavage and block FIX activation, they most likely bind

near or in this region, as aptamers tend to home in on sites that mediate protein-protein

interactions (157). Interestingly, 11.7 binds only to FXIa and is a more potent inhibitor of

FIX activation in a purified protein setting than 12.7, which can bind to both FXI and

FXIa. This suggests that 11.7 binds more directly than 12.7 in the FIX binding site that is

occluded in zymogen form. Other proteins bind the regions surrounding the FIX

binding site, such as GP1bα, which binds in the same apple domain, and thrombin and

HK, which bind in separate apple domains. As aptamers tend to bury large surface

areas, biochemical analysis to determine if these aptamers block the function of these

other proteins would help to map the aptamer binding site, as well as further elucidate

the mechanism of aptamer inhibition.

Functionally, there was initially a discrepancy between the lack of potency in

plasma-based assays versus the potent inhibition of FXI function in biochemical assays

or the reconstituted cell-based model of thrombin generation. This could be due to prior

contact pathway activation in the plasma samples. The process of drawing blood can

activate the contact pathway through the exposure and subsequent activation of FXII to

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foreign surfaces. Therefore, FXIa can already be present and bound to FIX in a plasma

preparation from a blood draw without an inhibitor of FXIIa present, and subsequent

inhibition of FXIa in these samples might not be viable or as potent as inhibition in CTI-

treated samples, where presumably no FXI has been activated. CTI is an irreversible

FXIIa inhibitor, and inclusion has been shown to inhibit contact pathway activation

during the blood drawing process. This discrepancy can be seen when comparing the

results from the aPTT and TGA with 12.7. While 12.7 has minimal activity in the aPTT,

which uses plasma without CTI, there is robust inhibition of thrombin generation in the

TGA, including the lag time, using plasma prepared with CTI. Further analysis to

determine if CTI-treated plasma would have a great aptamer effect in the aPTT is

warranted. In addition, 11.7 has not been evaluated in a CTI-based plasma assay; thus,

its functionality in plasma is unknown, although it was able to decrease thrombin

generation in the cell-based model. However, 11.7 could also be less potent in plasma

than 12.7 because it only binds to activated FXI while 12.7 binds to both FXI and FXIa.

Therefore, 12.7 would already be bound to FXI in plasma and able to immediately block

FIX activation. In addition, several aptamers that bind to the zymogen as well as the

activated protein have been shown to not only inhibit downstream function of their

target protein, but also to inhibit their protein's activation (181, 184). While it has not

been directly studied if these aptamers can block FXIIa-mediated FXI activation, the

aptamer would presumably be more potent if it could block these two subsequent

stages. In addition, the aPTT and TGA used here only measures FXI as a function of the

contact activation pathway, and does not incorporate feedback activation by thrombin

or interactions with platelets. The cell based model system, however, does not include

FXII, thus the inhibition of thrombin generation seen in this system indicates that the

aptamer does affect either or both of these functions. Further biochemical assays, as well

as a TGA assay run with platelet-rich plasma, can determine the extent of inhibition of

either of these functions.

Overall, we have isolated an aptamer inhibitor of FXIa that is a potent inhibitor

of thrombin generation. Additional biochemical assays would assist in mapping the

binding site of the aptamer, as well as give additional information into the mechanism of

thrombin inhibition. As the conditions for plasma preparation are continually being

determined for each species, further assessment of aptamer activity in plasma and blood

based assays, as well as assessment of cross-reactivity of the aptamer in animal plasmas

will be necessary in order to determine if this aptamer is potent enough to move

forward into clinical trials. As FXI may have a slightly more prominent role in

coagulation than FXII, as evident by FXI deficiency being accompanied with a mild

bleeding disorder, the development of a specific FXIa inhibitor is promising step in

finding a therapeutic to inhibit thrombosis. An aptamer inhibitor of FXI is attractive, as

any potential bleeding effects due to therapy could be quickly reversed with an aptamer

antidote, thus increasing the safety and providing an effective therapeutic to treat

thromboembolism.

4. Comparing the sensitivity of coagulation assays for target validation

The results in this chapter were performed as part of a collaboration with the scientists at Merck Research Labs in Rahway, NJ.

4.1 Introduction

Clinically, a number of hemostatic tests are available that serve as a simple indicator of the clotting function of blood in a human being by determining the time that it takes for a plasma sample to clot. These tests are used to identify or assess bleeding risk in patients with genetic clotting factor defects or those under anticoagulant therapy. In reality, these clotting times, while commonly used, do not always correlate with bleeding phenotypes and cannot detect the use of all anticoagulants (196, 217). Thrombin is the pivotal step in the coagulation cascade; thus, any alteration in any of the components responsible for making thrombin can greatly affect bleeding rates. Aside from the clotting time, the rate of thrombin generation or the peak amount of thrombin generated can have a profound affect on the stability of a clot (64). Thus, a more comprehensive view of thrombin dynamics could more predictably predict bleeding rates in patients with genetic defects or under anticoagulant therapy.

The thrombin generation assay (TGA) offers this more comprehensive and global assessment of thrombin formation, as it uses a small chromogenic substrate to detect thrombin generation in a plasma sample. While the analysis of thrombin generation has been around for decades, the development of an automated system has been hampered

by the difficulties of measuring optical density (thrombin formation) during a change in turbidity of the plasma sample due to clot formation (196). The introduction of a slow-reacting thrombin-specific fluorogenic substrate circumvented this problem, and the fluorescence signal is converted to the amount of thrombin generated as a function of time by comparing thrombin generation in a calibrated parallel sample (196). In this way, several thrombograms, the graph that measures thrombin generation overtime, can be measured simply and reliably in many samples at the same time with minimal manpower, thus making it an excellent candidate for use in the clinic.

In general, a thrombogram in normal plasma follows the same trend: after activation of the plasma sample, there is a lagtime during which the coagulation cascade is generating undetectable amounts of thrombin and priming the cascade for a large burst of thrombin. Once detectable amounts of thrombin have formed and the burst of thrombin generation has begun, the lagtime has ended, and is generally correlated with the formation of a fibrin clot or the clotting times that are measured in the mechanical aPTT and PT assays. During the burst of thrombin generation, thrombin is formed very quickly, with a steep rate of reaction, culminating in a peak amount of thrombin being formed. After the peak is reached, thrombin generation slowly tails off, as coagulation factors in the sample have been used or are inactivated. These measurable parameters (lagtime, peak thrombin generated, total amount of thrombin formed, etc.) are

dependent upon the type of activator used; commonly, low amounts of TF (1pmol) are

used and are generally thought to be the most physiological setting for activation (196).

While the TGA has been slowly making its way into the clinic, it has found a

home in research settings, as it can easily be used to study the contribution of clotting

factors to thrombin generation. In addition, this assay is well suited for *in vitro* drug

development to identify drugs that affect thrombin dynamics for antithrombotic

therapy. Analysis of current anticoagulant drugs have determined that inhibition of the

ETP to approximately 20-40% of normal results in stable anticoagulation (218), while

inhibition of the ETP to less than 20% of normal is correlated with bleeding phenotypes

(217). Therefore, this assay has been used to determine a target therapeutic window of

20-40% ETP for efficacious and safe antithrombotic administration.

While the above studies have primarily been carried out with deficiencies or

drugs that target the common or extrinsic pathway of coagulation, recent insight into the

actions of the intrinsic pathway, primarily FXIIa-mediated FXI activation, have led to the

hypothesis that these factors may be involved in pathological thrombus formation, but

not hemostasis (40). While the common TGA is activated through the extrinsic pathway

without the contribution of FXII, a standardized TGA activated through the intrinsic

pathway would be beneficial to determine the effects on thrombin generation during

inhibition of these factors, as well as identify potential therapeutics.

Here, we developed a standardized modified TGA (mTGA) assay that is activated by ellagic acid. We use a panel of anticoagulant RNA aptamers that specifically target five clotting factors (FII, FX, FIX, FXI, and FXII) to assess inhibition of the intrinsic and common pathways of coagulation in this assay, as well as compare thrombin inhibition in the mTGA to the standard, TF-activated TGA. Aptamers are single stranded, highly structured oligonucleotides that act as protein antagonists by binding to large surface areas on their target protein to directly inhibit protein-protein interactions (157). The aptamer platform is a promising tool for anticoagulant therapy, as aptamer administration can be rapidly and effectively reversed by a sequence specific or universal aptamer antidote (160, 165). Currently, an aptamer generated against FIXa and its matched oligonucleotide antidote are making their way through clinical development (163, 164). Using these aptamers as specific anticoagulant tools, it was found that this assay can detect inhibitors of the intrinsic and common pathway, and will be useful in target validation for analysis of an anticoagulant in human as well as animal plasmas.

4.2 Materials and Methods

Materials

The 2′F modified cytidine and uridine were purchased from Trilink BioTechnology (San Diego, CA). CTI collection tubes were purchased from

Haematologic Technologies (Essex Junction, VT). Human normal pooled plasma was purchased from George King Biomedical, Inc. (Overland Park, Kansas). The ellagic Acid-based Pacific Hemostasis APTT-XL Reagent was purchased from Thermo Scientific (Waltham, MA).

RNA Aptamers

For all aptamer sequences, "C" and "U" represent 2'fluorocytosine and 2'fluorouracil. The FII aptamer sequence (58nt) is 5'-GGCGGUCGAUCACACAGUUCAAACGUAAUAAGCCAAUGUACGAGGCAGACGACUCGCC-3' (181), the FX aptamer sequence (80nt) is 5'-GGGAGGACGATGCGGACATCTCCGCACTCAGTTTGAGCACCAAACCAACAGCTCCCAGACGACTCGCTGAGGATCCGAGA-3', the FIX aptamer sequence (80nt) is 5'-GGGAGGACGATGCGGACTTGTCGGATAGGCCCTGATCCTAGTACACAAGACCAAGCAGACGACTCGCTGAGGATCCGAGA-3', the FXI aptamer sequence (71nt) is 5'-GGGAGGACGATGCGGTAACGCCACGCTCGACAACGCGTCGAGTGTCCTCCGCCCCCAGACGACTCGCCCGA-3' and the FXII aptamer sequence (51nt) is 5'-GGCTCGGCTGCCAGCAGGTCACGAGTCGCAGCGACTCGCTGAGGATCCGAG-3'. The FII and FXII control aptamer sequence (51nt) is 5'-GGGGCAGCCGTGGACCGACTGCCGCATGCCATTGACAGTCCGATGCCAGGC-3', the FIX and FX control aptamer sequence (80nt) is 5'- GGGAGGACGAUGCGG-N$_{40}$-

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CAGACGACUCGCUGAGGAUCCGAGA-3' and the FXI control aptamer sequence (71nt) is 5'-

GGGAGGACGATGCGG-N$_{40}$-CAGACGACTCGCCCGA-3'.

All aptamers were transcribed *in vitro* as previously described using a modified

polymerase that can incorporate 2'F cytidine triphosphates and 2'F uridine

triphosphates (194). Before use, all aptamer preparations were diluted into a Hepes-

based buffer (20mM Hepes, 150mM NaCl, 2mM CaCl$_2$). Diluted aptamers were refolded

by heating to 65°C for 5 minutes, and then cooled for 3 minutes at ambient room

temperature.

Clotting Assays

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays

were carried out using a model ST4 coagulometer (Diagnostica Stago, Parsippany, NJ).

Pooled normal human platelet poor plasma was prepared by drawing blood into

sodium citrate tubes. For the aPTT, aptamer (5μL) was then added to 50μL pooled

normal human platelet poor plasma and incubated for 5 minutes at 37°C. 50μL APTT-

XL Reagent was then added to activate plasma and incubated for 5 minutes at 37°C.

Clotting was initiated by adding 50μL of 25mM CaCl$_2$ and the time to clot formation was

recorded. Aptamer concentrations represent the final concentration in the entire

reaction.

For the PT, refolded aptamer (5μL) was added to 50 μL pooled normal human

platelet poor plasma and incubated for 5 minutes at 37°C.   TriniClot PT Excel (Trinity

BioTech, Bray, Co Wicklow, Ireland) was added to initiate clotting.

Thrombin Generation Assay

In an Immulon 2HB clear U-bottom 96 well plate (Thermo Labsystems, Franklin,

MA), CTI- treated (50μg/mL), pooled normal human platelet poor plasma from healthy,

consented volunteers (60μL) was mixed with aptamers (3μL) diluted in Hepes-based

buffer without BSA and incubated for 5 min at 37°C. Ellagic acid reagent (final

concentration 0.1μM, diluted 1:200 with 4μM phospholipid vesicles (20%

phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine) in

Hepes-based buffered saline)(15μL) was added to the plasma-aptamer mixture, and

incubated for 5 minutes at 37°C. Flu-Ca (Diagnostica Stago)(15μL) was added to start the

assay and thrombin generation was measured using a Fluroskan Ascent Reader

(Thermo Scientific, Waltham, MA). A thrombin calibrator reagent (Diagnostica Stago,

Parsippany, NJ) was used to quantify the amount of thrombin generated in the samples.

Data analysis was performed using Thrombinoscope software (Thrombinoscope BV, the

Netherlands). Aptamer concentrations represent the final concentration in the entire

reaction. The amount of ellagic acid used was standardized to produce a similar curve to

a TF-activated TGA. In addition, the amount of CTI present was standardized to inhibit

contact activation of the plasma sample, while still allowing for robust FXII-dependent

activation upon administration of ellagic acid.

Thromboelastogram

Blood was drawn from healthy volunteers into standard sodium citrate tubes.

Whole blood (320μL) was mixed with aptamer (10μL) and incubated at room

temperature for 5 minutes. Kaolin (10μL)(Haemonetics, Niles, IL) was added, and the

entire reaction (340μL) was added to 20μL CaCl$_2$ in a disposable, plain TEG cup

(Haemonetics). Clot formation was measured at 37°C with a Thromboelastograph

Analyzer (Haemonetics) until a stable clot was formed. Parameters were automatically

calculated using the TEG® Analytical Software version 4.2.3 (Haemonetics).

4.3 Results

4.3.1 Modified TGA assay validation

In order to evaluate inhibition of the intrinsic pathway of coagulation, as well as

the common pathway in the TGA, the standard TF-activated TGA was modified so that

plasma samples were activated with diluted ellagic acid aPTT reagent, thus

autoactivating FXII to stimulate thrombin generation through the intrinsic pathway.

During the development of this assay, it was recognized that activation of the plasma

sample occurs relatively easily during the blood drawing or plasma preparation process,

as samples with only added phospholipids and calcium were able to generate thrombin

without an activator present (data not shown). Activation of the plasma sample is

thought to occur through the contact pathway, as FXII can be readily autoactivated upon

exposure to foreign surfaces, resulting in activated FXII and FXI in the plasma sample.

While this does not pose a problem in the standard TF-activated assay, as calcium is

needed for extrinsic pathway reactions, as well as the activation of FIX by FXI, prior

activation of the intrinsic pathway in the plasma sample will confound results in an

intrinsic pathway based assay. Therefore, a standardized amount of corn trypsin

inhibitor (CTI), an irreversible FXIIa inhibitor, was used to inhibit any FXIIa that may

become activated during the blood draw or plasma preparation process. Importantly,

only enough CTI was used to inhibit activation of FXII in this process, and not further

inhibit FXII during activation in the assay.

To assess inhibition in the mTGA assay, a panel of RNA aptamers was used to

determine thrombin generation during inhibition of each coagulation factor in the

intrinsic and common pathways. Each of these aptamers have slightly different binding

properties to their respective target (Table 10); however, each is specific for binding to

only their specific protease. Although the exact mechanisms of all of these aptamers

have not been evaluated, aptamers tend to bind to exosites on the surface of their

protein, and thus inhibit protein-protein interactions that are essential for their

coagulant function (157, 181).

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Table 10. Summary of aptamer properties

Aptamer Target	Length (nt)	K_d Zymogen (nM)	K_d Protease (nM)
FII	58	10.0	1.0
FX	80	136.0	39.0
FIX	80	0.7	0.4
FXI	71	139.2	84.9
FXII	51	12.9	0.4

Inhibition of each coagulation factor in the common (FII and FX) and intrinsic (FIX, FXI, FXII) pathways were detected in the modified TGA (mTGA) upon administration of aptamers. Dose titrations of each aptamer exhibited a distinct inhibition profile: full inhibition of FII, FX, and FIX completely abolished thrombin generation while inhibition of FXI and FXII reduced, but did not completely inhibit thrombin generation (Figure 33). Further analysis and comparison of individual TGA parameters during aptamer inhibition is discussed below.
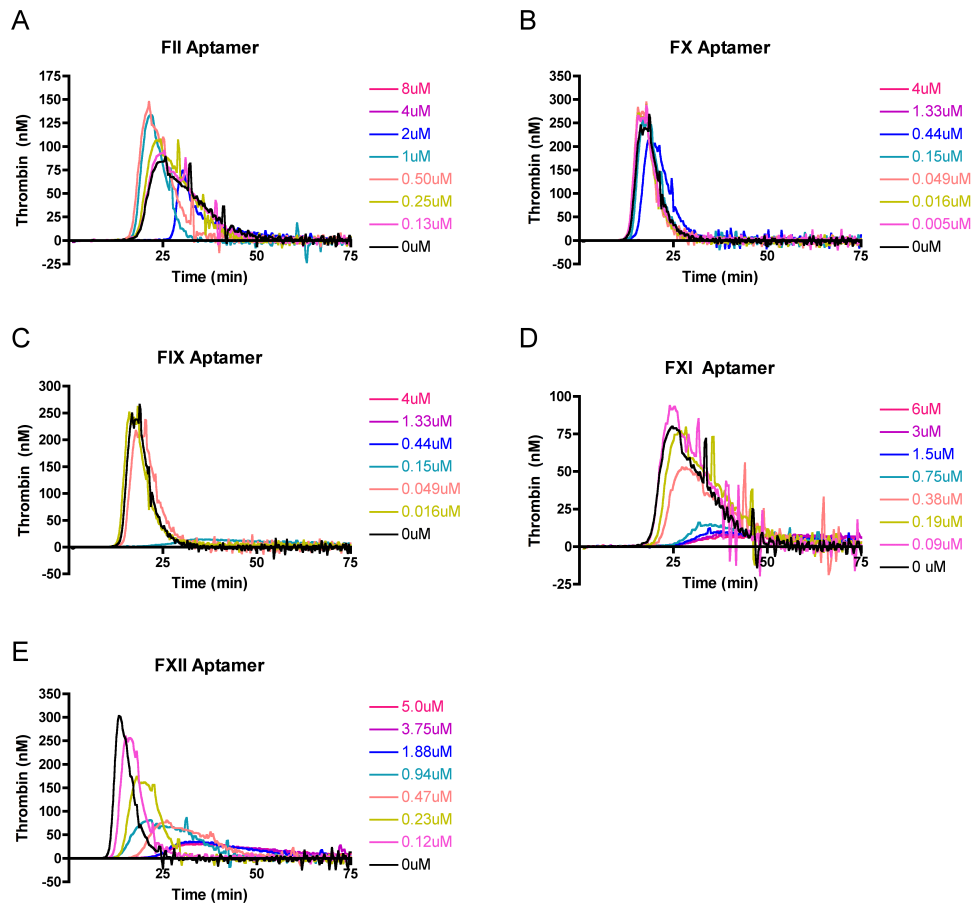
Figure 33. Dose titrations of the panel of aptamers in the mTGA
Thrombograms of normal pooled plasma activated with 200x-diluted ellagic acid with indicated concentrations of A) FII Aptamer, B) FX Aptamer, C) FIX aptamer, D) FXI Aptamer, and E) FXII Aptamer. Preliminary data represent one assay.

Comparison of the mTGA versus the standard TF-activated TGA was analyzed

by comparing parameters of both assays during inhibition of either FX (common

pathway) or FIX (intrinsic pathway). As a common pathway coagulation factor,

inhibition of FX in both assays gave comparable results, with the FX aptamer in TF-

activated (Figures 34A,C,E) and ellagic acid activated (Figure 34B,D,F) exhibiting similar

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potency in thrombograms (Figure 34A,B), ETP (Figure 34C,D) and the peak amount of

thrombin generated (Figure 34E,F). Both assays were able to detect a complete

inhibition of thrombin generation at saturating concentrations of the FX aptamer, with a

slightly steeper dose response curve in the modified TGA versus the low TF activated

TGA.
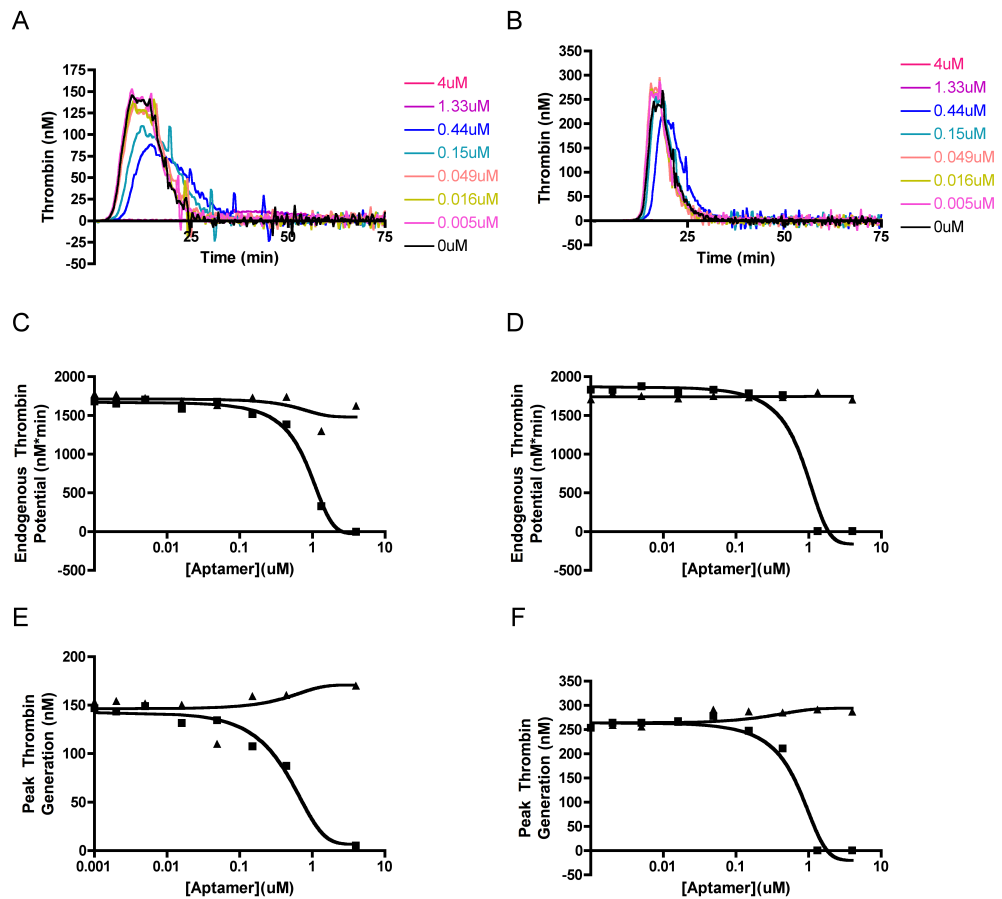
Figure 34. Comparison of the inhibition of the common pathway with the FX aptamer during the standard TF-activated TGA versus the modified ellagic acid-activated TGA

Normal pooled plasma was mixed with the FX aptamer and activated with either low (1pM) TF (A,C,E) or 200x-diluted ellagic acid (B, D, F). A) and B) represent thrombograms of the FX aptamer at indicated concentrations. C) and D) are the ETP and E) and F) are the peak thrombin generated of the FX aptamer (■) and a library size-matched control RNA (▲). Preliminary data represent one assay.

Comparison of the standard and mTGA assays during inhibition of the lower

intrinsic pathway of coagulation was assessed using an aptamer targeting FIX. FIX can

be activated through FXI (intrinsic pathway) or at a smaller extent through the TF/FVIIa

complex (extrinsic pathway). Activating the TGA with ellagic acid forces thrombin

generation to proceed mainly through the intrinsic pathway, thus analyzing FXI

activation of FIX, while activating the TGA with TF allows for the TF/FVIIa to activate

FIX as well as activate the common pathway directly through FX. The mTGA was more

sensitive to FIX inhibition, as saturating concentrations of the aptamer were able to

completely inhibit thrombin generation (Figure 35B,D,F). In contrast, high amounts of

aptamer were not able to fully inhibit thrombin generation in the TF-activated assay

(Figure 35A, C, E).
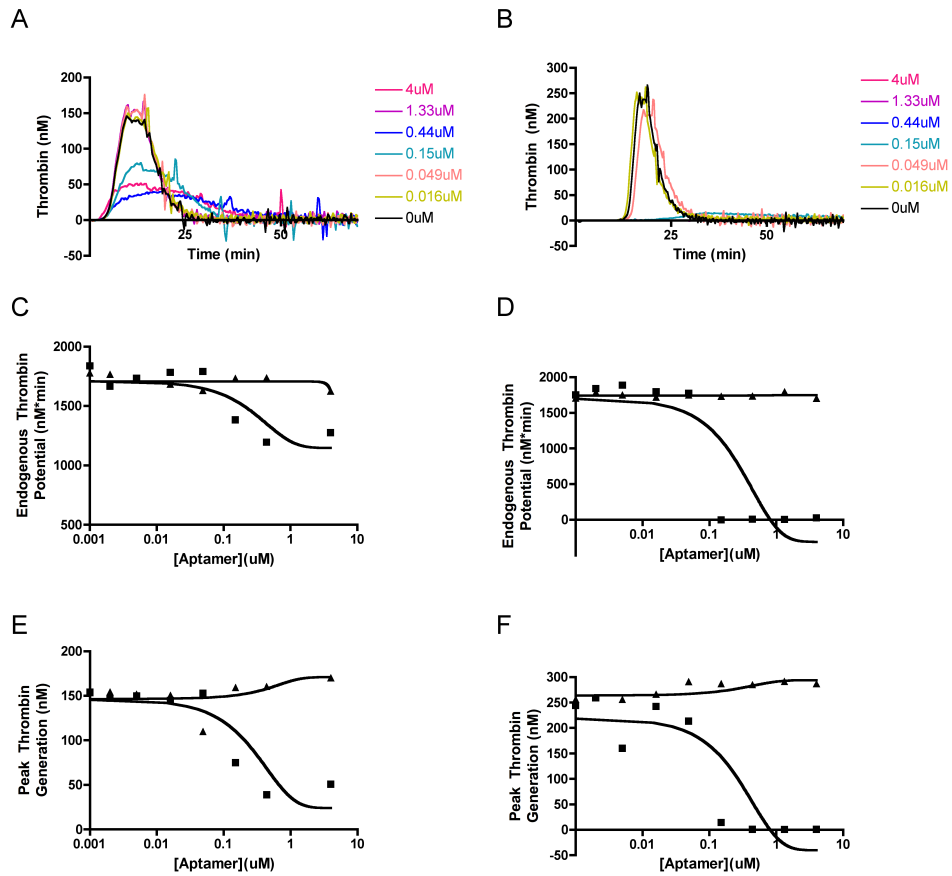
Figure 35. Comparison of the inhibition of the intrinsic pathway during the standard TF-activated TGA versus the modified ellagic acid-activated TGA
Normal pooled plasma was mixed with the FIX aptamer and activated with either low (1pM) TF (A,C,E) or 200x-diluted ellagic acid (B, D, F). A) and B) represent thrombograms of the FX aptamer at indicated concentrations. C) and D) are the ETP and E) and F) are the peak thrombin generated of the FIX aptamer (■) and a library size-matched control RNA (▲). Data represent one assay.

4.3.2 Modified TGA parameter analysis upon aptamer inhibition

Analysis of thrombogram parameters can further validate the modified TGA

assay, as well as give insight into the mechanisms of inhibition of each individual

coagulation factor when thrombin generation is initiated by the intrinsic pathway. First,

the ETP for each aptamer was analyzed (Figure 36). The ETP represents the total

amount of thrombin generated over time, and is determined by calculating the area

under the thrombin generation curve. Full inhibition of the common pathway, as well

as FIX and FXI, completely inhibited thrombin generation in this assay, as the ETP of

each sample was zero or a negligible amount. Inhibition of FXII with the RNA aptamer,

however, only decreased the ETP, but did not completely shut down thrombin

generation.

Figure 36. Comparison of the endogenous thrombin potential (ETP) during aptamer inhibition in the modified ellagic acid-activated TGA

Normal pooled plasma was mixed with either the indicated aptamer (■) or a size-matched control RNA (▲) and activated with 200x-diluted ellagic acid. The ETP was calculated using the Thrombinoscope software. A) FII Aptamer, B) FX Aptamer, C) FIX Aptamer, D) FIX Aptamer, E) FXII Aptamer. The preliminary data represent one or more assays.

Next, the peak amount of thrombin formed was analyzed during aptamer administration. The peak is measured by calculating the maximum amount of thrombin formed, which is the peak of the thrombin generation curve. Analysis of this parameter in the mTGA behaves similarly to the ETP with aptamer inhibition of FIX, FX, and FII

exhibiting a steep dose response curve (Figure 37A,B,C). Inhibition of FXI and FXII

more slowly reduced the peak, with maximal inhibition able to severely diminish the

peak without completely abolishing thrombin generation (Figure 37D,E). This is in

contrast to the ETP, as the FXII aptamer was able to greatly reduce the peak amount of

thrombin formed without having such affect on the ETP (Figure 37E).

Figure 37. Comparison of the peak amount of thrombin formed during aptamer inhibition in the modified ellagic acid-activated TGA

Normal pooled plasma was mixed with either the indicated aptamer (■) or a size-matched control RNA (▲) and activated with 200x-diluted ellagic acid. The peak amount of thrombin generated was calculated using the Thrombinoscope software. A) FII Aptamer, B) FX Aptamer, C) FIX Aptamer, D) FIX Aptamer, E) FXII Aptamer. The preliminary data represent one or more assays.

Next, the rate of thrombin generation upon aptamer administration was

analyzed. This parameter measures the slope of the amount of thrombin formed from

the first amount of thrombin generated (lagtime) to the maximum amount of thrombin

generated (peak). Similarly to the ETP and peak amount of thrombin generated,

inhibition of the common pathway (FII and FX) exhibited a steep dose-response

inhibition, as smaller aptamer doses had no effect on the rate of thrombin generation,

but the rate of thrombin generation was suddenly abolished at higher doses (Figure 38).

Inhibition of the intrinsic pathway (FIX, FXI, FXII) exhibited a much slower rate of

reduction of thrombin generation. For every aptamer, administration of the control

aptamer was able to increase the rate of thrombin generation dose-dependently, while

having differing affects on the other parameters.
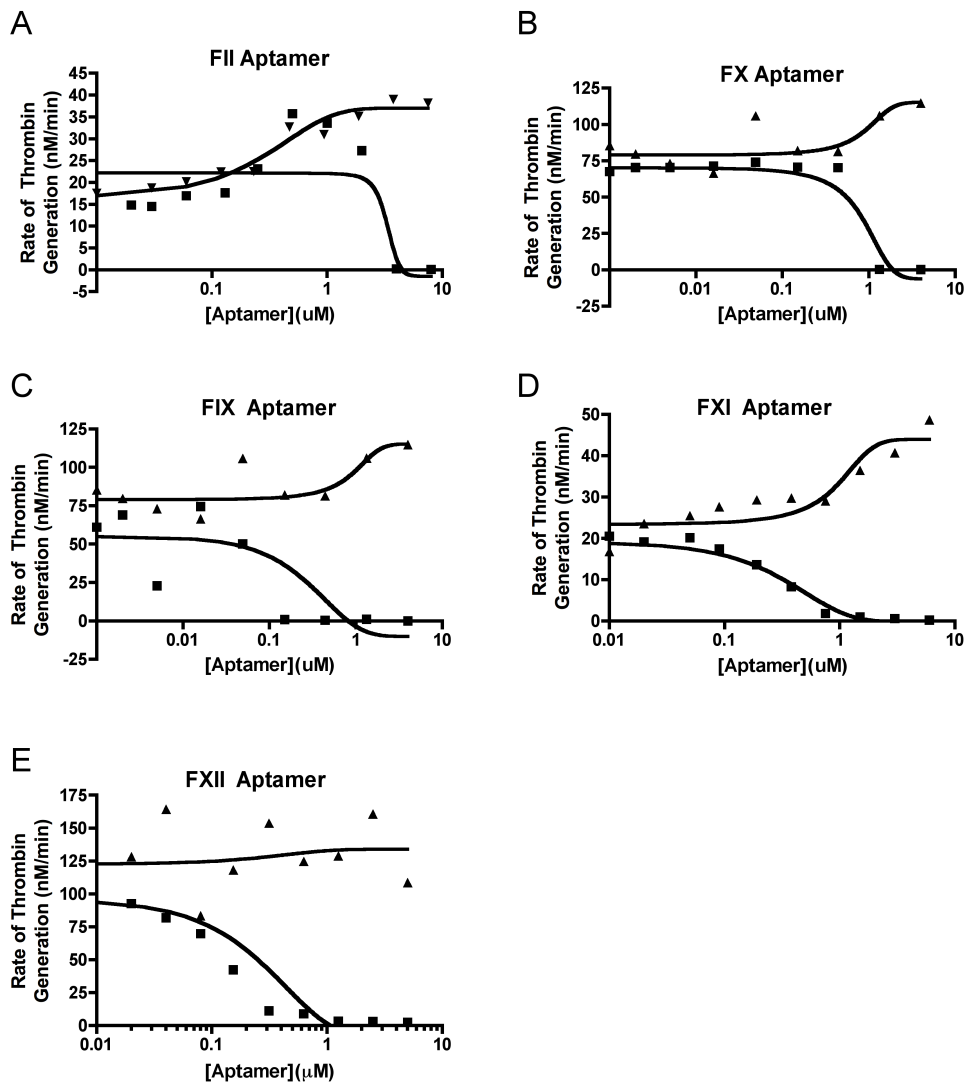
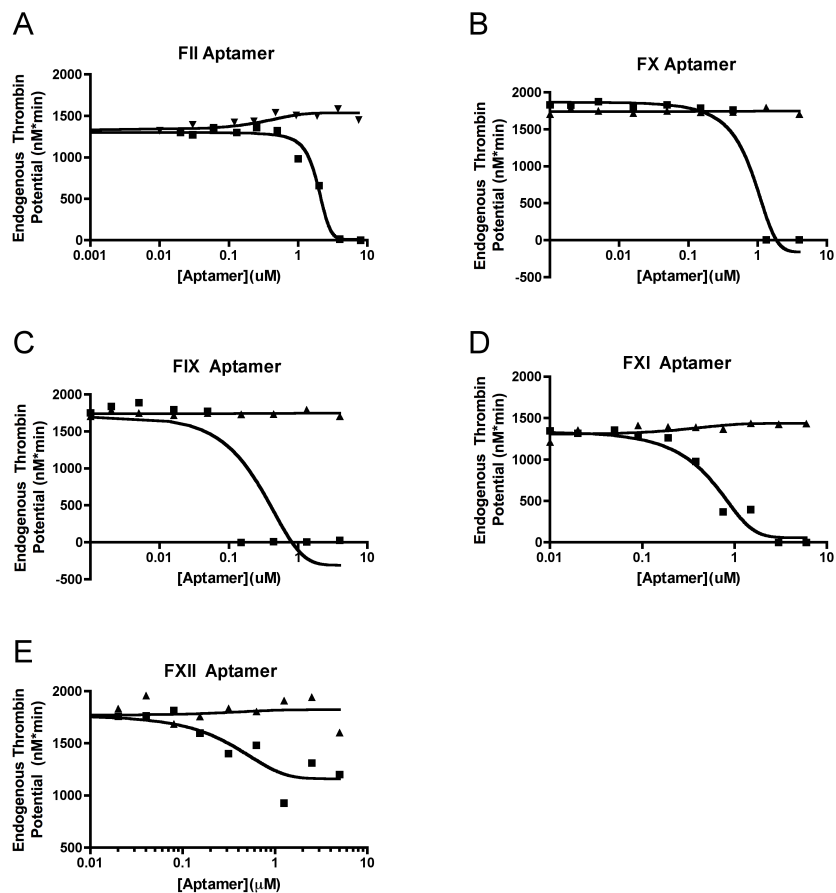**Figure 38. Comparison of the rate of thrombin generation during aptamer inhibition in the modified ellagic acid-activated TGA**

Normal pooled plasma was mixed with either the indicated aptamer (■) or a size-matched control RNA (▲) and activated with 200x-diluted ellagic acid. The rate of thrombin generation was calculated using the Thrombinoscope software. A) FII Aptamer, B) FX Aptamer, C) FIX Aptamer, D) FIX Aptamer, E) FXII Aptamer. The preliminary data represent one or more assays.

The lagtime measures the time it takes for the first amount of thrombin to be formed, thus it is considered to be the time at which clotting first occurs. Full inhibition of the common pathway does not form thrombin, thus, the lagtime cannot be computed at the higher aptamer concentrations and these data points are missing (Figure 39A and B). Inhibition of FIX greatly increases the lagtime, while inhibition of FXI and FXII does not prolong the lagtime as severely compared to the other aptamers (Figure 39).
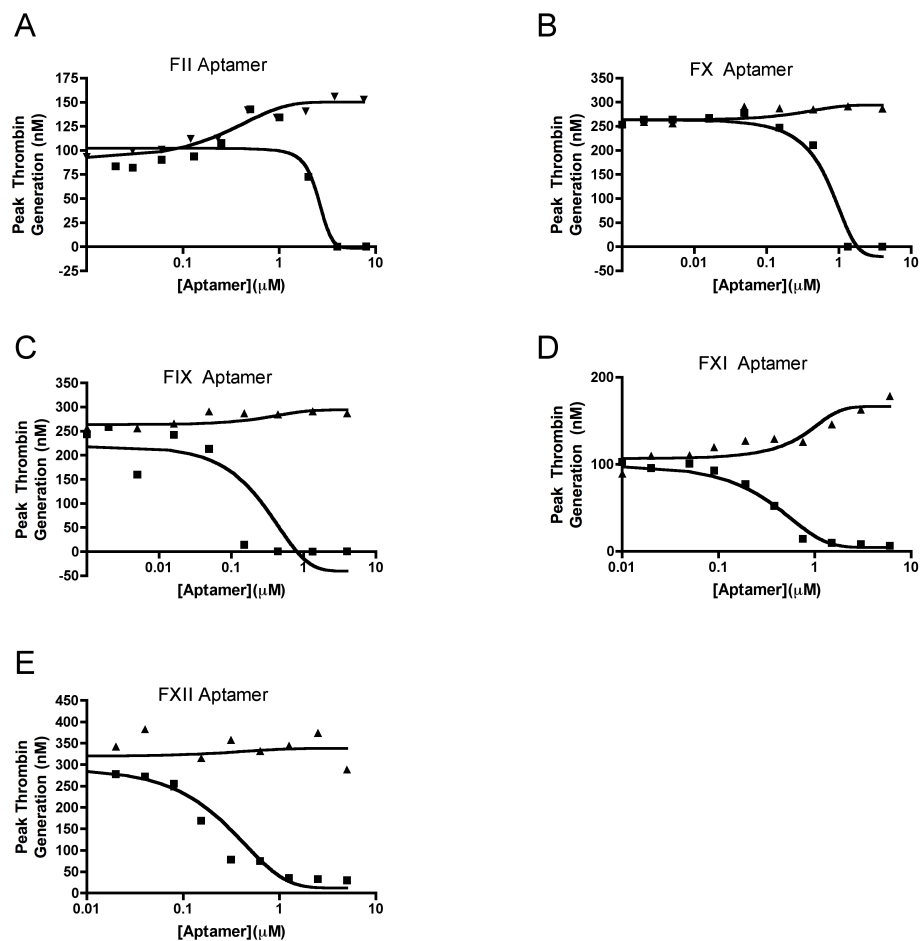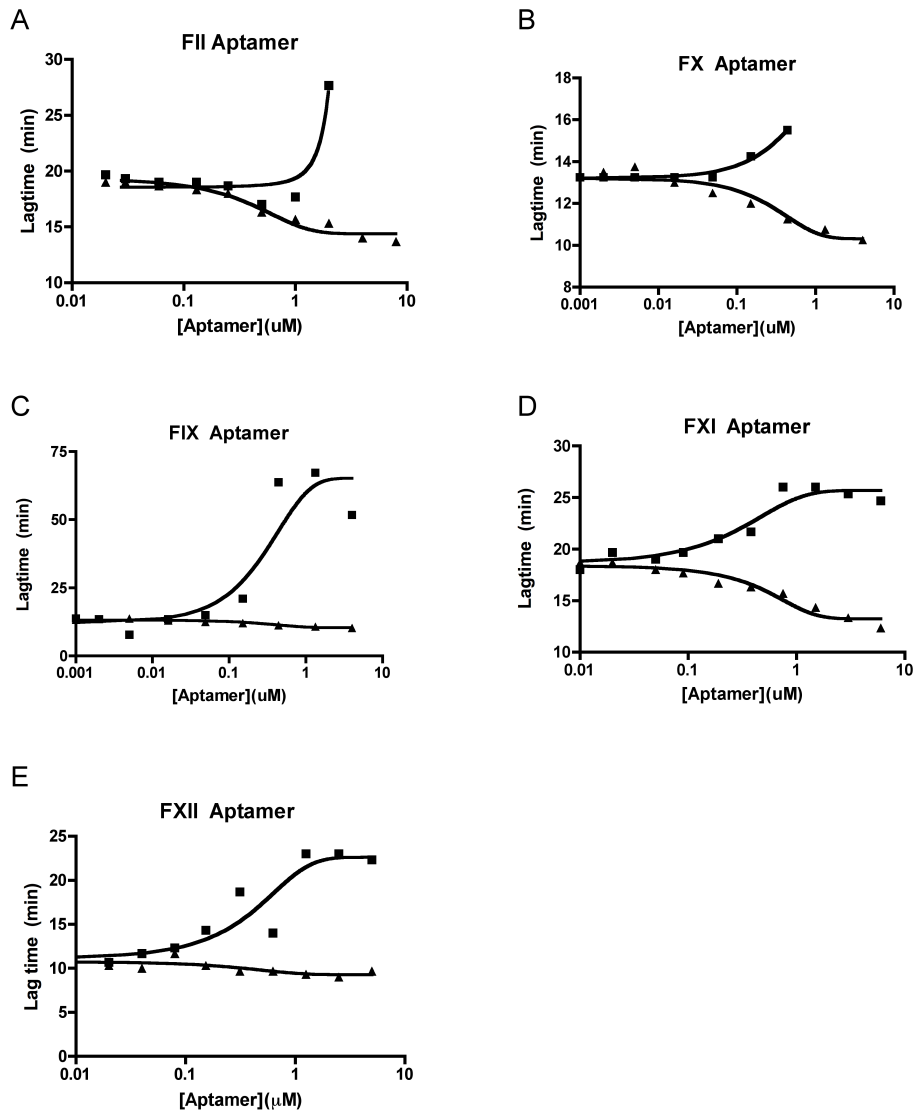
Figure 39. Comparison of the lagtime during aptamer inhibition in the modified ellagic acid-activated TGA

Normal pooled plasma was mixed with either the indicated aptamer (■) or a size-matched control RNA (▲) and activated with 200x-diluted ellagic acid. The lagtime was calculated using the Thrombinoscope software. A) FII Aptamer, B) FX Aptamer, C) FIX Aptamer, D) FIX Aptamer, E) FXII Aptamer. The preliminary data represent one or more assays.

4.3.3 Comparison of aptamer inhibition and mTGA validation in human plasma and blood-based coagulation assays

Two common coagulation tests, the activated partial thromboplastin time (aPTT) and the thrombin generation assay (TGA), also measure the lagtime of clot formation in plasma. The aPTT assay uses the same activator, ellagic acid, to activate FXII and thus initiate clotting through the intrinsic pathway. The PT uses TF to activate the extrinsic pathway, and thus measures the function of the extrinsic pathway of coagulation. All aptamers prolonged the clotting time in the aPTT, with inhibition of the common pathway having a maximum effect (Figure 40A). Similar to the mTGA, inhibition of the intrinsic pathway could prolong clot time maximally with FIX, intermediately with FXII, and only slightly with FXI (Figure 40A). In the PT, inhibition of the intrinsic pathway did not have an effect, while inhibition of the common pathway produced a robust effect in clot time, with the FII aptamer inhibiting clot formation past the assay maximum (Figure 40B).
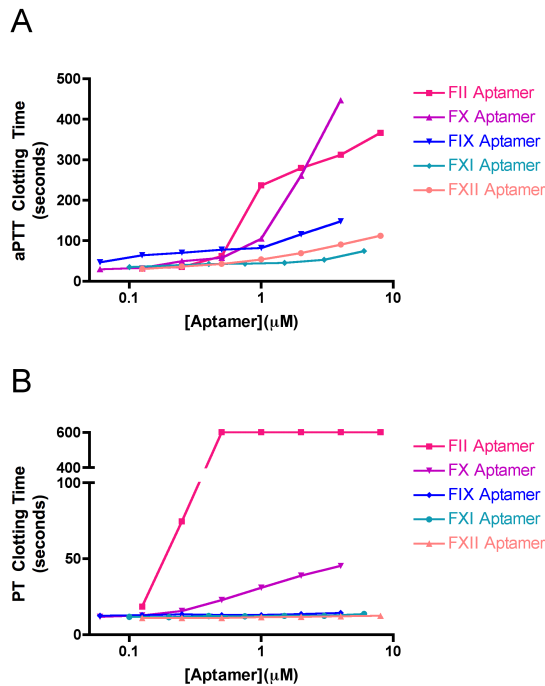
Figure 40. Comparison of clotting times during aptamer inhibition in an aPTT and PT with human plasma

Normal pooled plasma was mixed with the indicated aptamer and activated with either A) aPTT reagent or B) PT reagent. The data represent one or more assays.

In addition to the plasma based assays, an experiment utilizing whole blood and the presence of platelets would also give detailed information about the dynamics of inhibition in a complete setting. Thromboelastography uses whole blood to study the kinetics of clot formation by using mechanical motion to analyze clot strength. The two parameters studied included the lagtime (the time until clot formation) and the maximum amplitude (the strength of the clot formed). Table 11 summarizes the data from aptamer dose-titrations performed with kaolin as the intrinsic pathway activator.

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Whereas all of the aptamers except the FXII aptamer could prolong the lagtime, none of

the aptamers had a large effect on the maximum amplitude of the system.

Table 11. Summary of preliminary TEG parameters with the panel of aptamers in human whole blood

Aptamer Target	FII	FX	FIX	FXI	FXII
Lagtime (Doubling Time)	4μM	2.6μM	0.17μM	5.1μM	No effect
Maximum Amplitude	No effect	Slightly reduced	No effect	Slightly reduced	No effect

In order to determine the most sensitive coagulation assay for target and

inhibitor validation, the panel of aptamers were compared in the TGA, the aPTT/PT, and

the TEG for either doubling time (aPTT/PT and TEG) or IC50 (calculated from the peak

amount of thrombin generated in the modified TGA) (Table 12). Importantly, the

modified TGA was the only assay in which CTI was added to the collection tube during

the blood draw so as to inhibit contact activation of the sample, resulting in more

sensitivity to aptamer inhibition with all but the FII aptamer.

Table 12. Summary of aptamer effects in clinical coagulation assays on human plasma or blood

Aptamer Target	FII	FX	FIX	FXI	FXII
aPTT (Doubling Time)	0.7μM	0.4μM	0.1μM	5μM	1μM
PT (Doubling Time)	0.15μM	0.5μM	No effect	No effect	No effect
TEG (Doubling Time)	4μM	2.6μM	0.17μM	5.1μM	No effect
mTGA (IC50)	3.0μM	0.3μM	<0.1μM	<1.0μM	0.2μM

4.3.4 Comparison of aptamer inhibition and mTGA validation in animal plasma and blood-based coagulation assays

Before a drug can proceed into human clinical trials, it must first be extensively studied in animals for safety and efficacy. Because most coagulation assays are blood or plasma based, they are easily adapted for assessing drug inhibition with animal plasmas. All of the aptamers used here are isolated solely against the human form of the coagulation factor; thus, analysis for cross reactivity in animal plasma is a must before proceeding into pre-clinical trials. In general, aptamers have exhibited some efficacy in a variety of species; however, the cross-reactive species varies from aptamer to aptamer. The panel of aptamers was tested in rabbit plasma or blood in the aPTT/PT, TEG, and mTGA assays for cross-reactivity (Table 13). Only the FII and FX aptamers were able to exhibit cross reactivity in rabbit plasma as determined by the aPTT and the mTGA; however, these aptamers did not exhibit high efficacy in either the PT or the TEG.

Aptamers targeting the intrinsic pathway (FIX, FXI and FXII) did not show reproducible

efficacy in rabbit plasma.

Table 13. Summary of aptamer effects in clinical coagulation assays on rabbit plasma or blood

*Doubling time was not reached at the highest concentration tested.

Aptamer Target	FII	FX	FIX	FXI	FXII
aPTT (Doubling Time)	1.5µM	1.5µM	No effect	No effect	8µM
PT (Doubling Time)	>8µM*	>4µM*	No effect	No effect	No effect
TEG (Doubling Time)	2.7µM (IC50)	No effect	No effect	No effect	No effect
mTGA (IC50)	5µM	1.2µM	No effect	No effect	No effect

4.4 Discussion

The standard TF-activated TGA offers a comprehensive view of thrombin

dynamics, generating more information on how deficiencies or drug-inhibition of

coagulation factors affect thrombin generation than any other common clinical

coagulation test. However, the standard TGA proceeds without contribution of the

intrinsic pathway of coagulation (FXII). While physiologic hemostasis is thought to

occur mainly through the extrinsic pathway, thus mirroring the standard TGA, intrinsic

pathway activation is thought to occur through continued thrombin generation at the

site of the growing thrombus (39). To this end, a modified TGA assay that stimulates

thrombin generation through the intrinsic pathway was developed in order to study

thrombin generation during intrinsic pathway inhibition as well as identify potent

inhibitors of this pathway.

Aptamers targeting both the common pathway (FII and FX) and the intrinsic

pathway (FIX, FXI, and FXII) were used to validate the modified TGA, compare the

inhibition of thrombin generation in both assays, as well as compare thrombin

generation during inhibition of each pathway and individual clotting factor. First, the

strength of activator used to initiate the intrinsic pathway was calibrated to exhibit a

similar pattern of thrombin generation seen in the standard TF-activated TGA. The

mTGA was able to detect significant decreases in thrombin generation upon inhibition

of all targets tested, in both the intrinsic and common pathways. Inhibition of FII and

FX of the common pathway completely abolished thrombin generation as expected since

these factors are essential for thrombin and fibrin clot formation (1). Thrombin

generation during inhibition of the intrinsic pathway varied depending upon the target.

While inhibition of FIX and FXI severely reduced thrombin generation to negligible

amounts, inhibition of FXII could only lower the amount of thrombin formed. Like FXII,

FXI has been shown to be autoactivated upon contacting certain negative surfaces, such

as dextran sulfate. While it was not tested whether ellagic acid could autoactivate FXI

itself, some FXI activation would bypass the need for FXII to generate thrombin. In

addition, some contact activation of the plasma sample, as occurs during the blood

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drawing process, could also contribute to downstream activation of clotting factors that would affect thrombin generation upon inhibition of FXII.

The standard TF-activated TGA was compared to the ellagic acid-activated mTGA during inhibition of the common pathway (FX) and the intrinsic pathway (FIX). Comparison of the ETP and peak amount of thrombin formed during inhibition of FX was similar between the two assays. As the intrinsic and extrinsic pathway converge upon activation of FX, this result suggests that both assays proceed similarly once the common pathway is activated. On the other hand, there were differences in thrombin generation upon inhibition of FIX. FIX is thought to be primarily activated by FXI, however, the TF/FVIIa complex can activate small amounts of FIX in the initiation phase of thrombin generation as well as directly activating FX. This was mirrored during comparison of the two assays, as the TF-activated TGA only lowered thrombin generation, with a substantial amount of thrombin still able to be formed. In the mTGA, which is activated through the intrinsic pathway, thrombin generation was essentially abolished, as only negligible amounts of thrombin were able to be detected upon full FIX inhibition. This result highlights the differences in the two assays, and validates the mTGA in proceeding primarily through the intrinsic pathway.

Further analysis into the individual parameters of the mTGA upon inhibition with each aptamer exhibited certain trends consistent with the roles of each factor in thrombin generation. Inhibition of FII and FX of the common pathway was able to

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completely abolish thrombin generation to the point where no parameters could be computed. Both of these aptamers exhibited a steep dose titration curve, as thrombin generation seemingly was either all-on or all-off. This result was in part mirrored in an assay utilizing a cell-based model of thrombin generation, an *in vitro* system using cultured macrophages as a source of tissue factor, physiologic amounts of purified proteins, and platelets that serve as the surface for thrombin generation to occur. In this assay, only small levels of FX were needed to normalize parameters, similar to the steep dose titration seen here as well as previous data using the extrinsic activated TGA (64) (217).

As FIX has roles in both the intrinsic and extrinsic pathway, its thrombin generation profile was comparatively "in the middle" between common pathway inhibitors and other intrinsic pathway inhibitors. Inhibition of FIX led to a marked increased in the lagtime, and high concentrations of the aptamer were able to completely inhibit thrombin generation. However, this aptamer had a slightly slower onset of action compared to the FII and FX aptamers. In contrast, inhibition of FXI and FXII could not completely inhibit thrombin generation, although both aptamers severely impaired the peak and rate of thrombin generation, consistent with the finding that inhibition of factors contributing to tenase complex formation primarily affects these parameters (64). While FXI could also greatly inhibit the ETP, FXII had a much smaller effect on the ETP, perhaps reflecting that FXI could be activated in the plasma sample by

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several ways. In contrast to the other aptamers, inhibition of FXI and FXII only slightly

increased the lagtime of the sample, the parameter that is solely measured in the aPTT.

Thus, it seems that inhibition of FXI and FXII is better analyzed in the mTGA than

traditional clotting time assays, as both of these aptamers could maximally affect other

thrombin generation parameters with minimal disturbance of the lagtime.

Because of the sensitivity of this assay to contact activation, a standardized

amount of CTI was included in the collection tube during the blood draw in order to

inhibit contact activation during the blood drawing and plasma preparation process.

While human blood is easily activated during the blood drawing process, rodent blood

is highly attune to contact activation as well. In our experience, each animal species

requires a certain amount of CTI present during the blood draw to inhibit contact

pathway activation. Rabbit was chosen as the species of choice due to quantity of blood

drawn and regularity in the blood drawing process, so as to obtain a decent number of

plasma samples that have not been contact activated. In comparison, it is difficult to

obtain the volume of plasma needed that is not contact activated in rats, as more than

half of the samples drawn are contact activated (verbal communication).

In comparing aptamer efficacy across the panel of coagulation assays performed

with rabbit blood or plasma, only the common pathway inhibitors (FII or FX) were able

to exhibit cross-reactivity. Intriguingly, both aptamers were only able to inhibit

thrombin generation effectively in the aPTT or mTGA in rabbit plasma - both assays that

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are activated by ellagic acid. Inhibition of FII in the kaolin-activated TEG was able to prolong the time for a clot to occur, but did not reach doubling time at the concentrations tested. The FX aptamer, however, did not show any effect in the TEG in rabbit plasma. In addition, both aptamers did not have an effect in the TF-activated PT assay. This suggests that rabbit plasma may act differently than humans, with the extrinsic system perhaps not being as important in initiating coagulation. In addition, the fact that none of the three intrinsic pathway aptamers were able to cross react in this species suggests that these proteins may be quite different from the human proteins, and perhaps act in a different manner. Studies of cross-reactivity with the entire panel of aptamers in rat and non-human primates are currently underway.

Overall, the modified TGA is the most sensitive and informative assay used to study both intrinsic and common pathway inhibition. Inhibition of any of these coagulation factors can affect a number of parameters that are easily analyzed using the mTGA, while each of the other assays tested here primarily measure the lagtime. While this study focused on the use of RNA aptamers targeting each specific coagulation factor, data with small molecule and antibody inhibitors also exhibited similar trends, albeit with different mechanisms of inhibition (data not shown). Further analysis and comparison between thrombin inhibition patterns and mechanism of small molecules versus aptamer could give insight into coagulation target validation for the type of therapy being developed. In addition, further validation and analysis of coagulation

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factor inhibitors in animal models versus mTGA assessment will also aide in the ability

of this assay to identify potent antithrombotics.

5. Conclusions and Perspectives

5.1 The rationale behind developing aptamer inhibitors of the intrinsic pathway of coagulation

Traditionally, the hemostatic system of proteins and zymogens involved in maintaining the integrity of the vasculature has been organized into a cascade model hallmarked by two converging pathways initiated by either the extrinsic or the intrinsic pathway and resulting in thrombin and fibrin clot formation. While the extrinsic pathway is triggered by the binding and subsequent activation of circulating FVII to exposed TF on a damaged vessel wall and thought to be the dominant cause of coagulation, the intrinsic, or 'contact' pathway, is activated when FXII is exposed to any number of artificial or physiologic negatively charged surfaces circulating in the blood and subsequently activates FXI to initiate the cascade.

Although the intrinsic pathway can activate clotting efficiently *in vitro*, such as in clotting assays that use glass to activate FXII and initiate clotting, these tests do not necessarily correlate with *in vivo* observations from hemophilic or factor deficient patients. While patients with hemophilia A or B (FVIII or IX deficiency) or common factor deficiencies (X, V, or II) have severe bleeding phenotypes or are incompatible with life (77), patients with deficiencies of FXII and FXI either have no bleeding tendencies or an injury-related variable bleeding tendency, respectively (219), suggesting that FXII and FXI are not required in hemostasis. Mice lacking FXI or FXII exhibit no bleeding phenotype, and, interestingly, both are protected against thrombus formation in several

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models of arterial thrombotic injury. These studies suggest that, while thrombus

initiation is not interrupted on the vessel wall after injury in both FXI and FXII deficient

mice, thrombi that are formed are unstable and break up before occlusion of a blood

vessel can occur (40, 72, 73, 91). Protection from thrombosis is not limited to arterial

injury, as FXII or FXI deficiency also confer a thromboprotective effect in a transient

middle cerebral artery stroke model (MCAO) (41, 92). The antithrombotic effect of FXI

deficiency is also apparent across species, as inhibition of FXI with antibodies in rabbits

and baboons resulted in reduced thrombus propagation and prevented growth of

occlusive thrombi in either injured arteries or on arterio-venous shunts, respectively

(74-77, 93). Taken together, this work increasingly suggests that inhibition of the

upstream, intrinsic coagulation factors XI and XII can hinder thrombosis without

affecting normal hemostatic events (220). It then follows that the pharmacological

inhibition of FXI and FXII in humans could produce a safe and effective anticoagulant to

treat and prevent thromboembolic disorders (Figure 41).
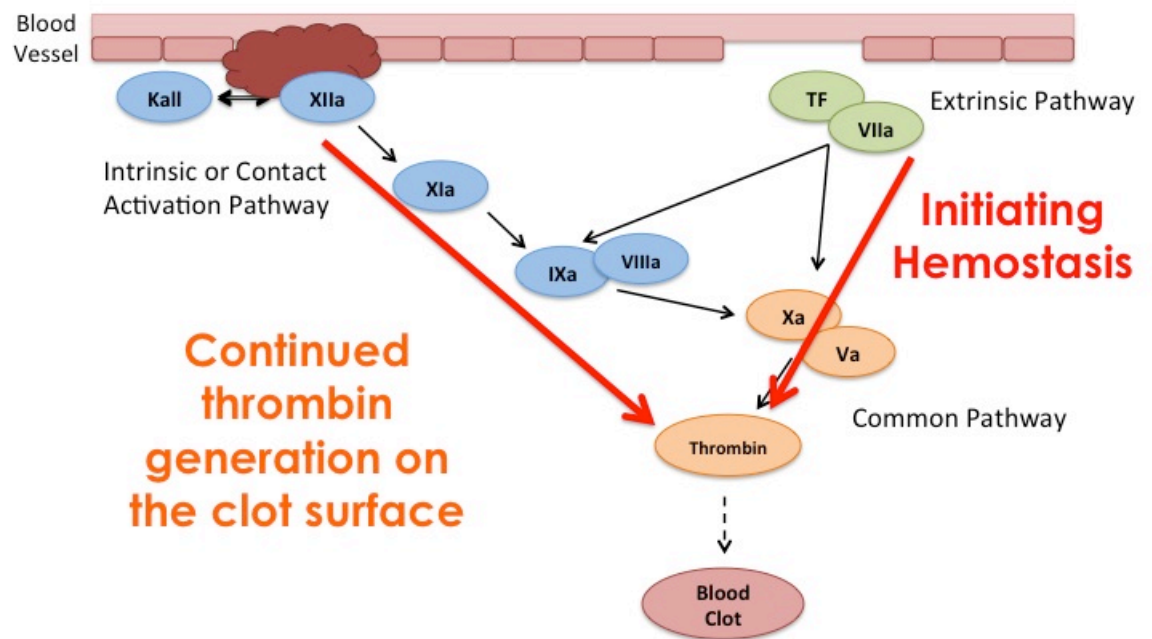
Figure 41. Decoupling hemostasis and thrombosis
While the extrinsic pathway is essential for initiating hemostasis at the site of blood vessel wall injury, continued thrombin generation on the clot surface due to the intrinsic pathway contributes to the stability of the blood clot. Pharmacologic inhibition of FXI and FXII of the intrinsic pathway could lead to a safe and effective antithrombotic that interrupts thrombosis without interrupting hemostasis.

The overall goal of this project was to develop potent and specific inhibitors of

the intrinsic pathway utilizing the aptamer platform of therapeutics and evaluate this

inhibition as a potential anticoagulant strategy. Currently approved anticoagulants

inhibit either downstream or multiple members of the coagulation pathway. While

these drugs are effective anticoagulants, they are not always safe, and drug

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administration without a direct antidote can require constant monitoring and confer a higher risk of bleeding complications. Striking the correct balance between inhibiting thrombus formation (efficacy) and reducing the risk of severe bleeding (safety) is crucial in developing a novel anticoagulant. This ratio of efficacy to safety can be achieved by two ways: targeting factors that cause bleeding, but having the ability to reverse the drug's action, or targeting factors involved in pathological thrombus formation that do not play a major part in hemostasis. While aptamer-antidote pairs targeting downstream members of the coagulation pathway (including FII, FX, and FIX) have previously been described to be effective anticoagulants yet will require antidotes to be safely administered, this project was successful in isolating effective aptamer inhibitors of two upstream members of the intrinsic pathway, FXI and FXII, which may not require antidotes to be safe and effective anticoagulants.

5.2 Discovery and pre-clinical development of intrinsic pathway aptamer inhibitors

The discovery and initial development phase of aptamer therapeutics includes performing a directed evolution selection to isolate a specific, high affinity aptamer, and subsequently testing this aptamer in a number of biochemical assays to identify its mode of action, as well as plasma or blood based assay to asses its anticoagulant potential. Both aptamers described in this process were able to be initially biochemically characterized, as well as demonstrated anticoagulant ability and reduction of thrombin generation in a plasma-based assay (Figure 42).

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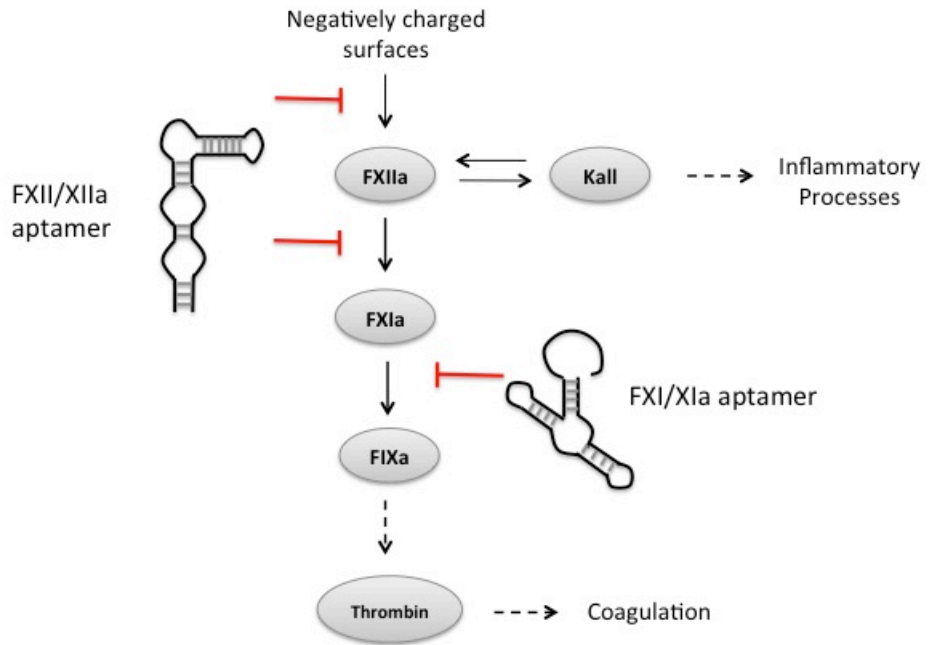
Figure 42. Inhibiting the intrinsic pathway of coagulation with RNA aptamers.
Both FXII and FXIa aptamers specifically inhibit their respective targets to decrease thrombin generation and fibrin clot formation.

While this strategy has been used successfully to identify aptamer anticoagulants

specifically targeting a number of other coagulation factors, the plasma-based

characterization of intrinsic pathway inhibitors was hindered by several circumstances.

The first issue has been discussed at length in the above chapters: the ability of the

contact pathway to be readily activated during the blood drawing process. This

property hindered the use of *in vitro*, plasma-based assays to be utilized to screen a large

number of intrinsic pathway inhibitory aptamers for anticoagulant activity. Instead, more time intensive biochemical assays were employed to screen these aptamers for functionality before moving into more complex plasma-based assays. It was not until the introduction of the use of CTI, which inhibits contact activation in the sample during the blood drawing process, that we were able to see potent inhibitory effects upon aptamer administration in a plasma-based setting. It was hypothesized that previous contact activation of the plasma sample led to downstream activation of coagulation factors, perhaps culminating with FXIa binding to FIX. It has been determined that only a small amount of thrombin formed can lead to the fibrin clot formation measured in clotting time assays. Upon re-calcification, any residual FXIa bound to FIX can now activate FIX; however, inhibition of FXII/FXIIa or FXI/FXIa would not have a large effect on clotting times in a plasma sample that can already produce a small amount of thrombin due to basal activation. Further studies into the difference of using contact pathway inhibited plasma versus untreated plasma will continue to clarify these differences and allow us to better screen and characterize our aptamer libraries against FXI, FXII and kallikrein.

Aside from hindrances during the blood drawing process, *in vitro* plasma or blood-based assays that closely mimic physiologic responses are lacking in the laboratory and the field. While it is widely accepted that the extrinsic pathway is the main contributor to coagulation *in vivo*, the aPTT and PT use extreme amounts of either intrinsic or extrinsic pathway activators in order to reach a standardized clotting time

utilized in the clinic for reproducibility. These activators are mostly non-physiologic substances or are more concentrated than in any physiologic setting; thus, they fully and quickly activate the clotting cascade. As these assays were primarily based off the initial cascade model of coagulation, they do not take into account the importance of surfaces for clotting to occur, as platelets are replaced by phospholipids and endothelial cells are presumably not present. It is our observation that inhibition of the common pathway proteases (FII and FX) can completely disrupt clotting in these assays, while intrinsic pathway inhibitors have a lesser effect on clotting times, with clots eventually being able to form. Therefore, while this assay can easily identify common pathway inhibitors, the use of intrinsic pathway inhibitors may not cause a large effect on clotting times, and thus it might be harder to identify inhibitors of these targets.

In addition, the traditional clotting assays and other plasma or blood based *in vitro* assays used in this study do not take into account the influence of blood flow on the system. Blood flow continuously supplies the area of clotting with new material, including zymogens, platelets, and inhibitors, as well as carrying away activated proteases, platelets, and inhibitors. Blood flow is a currently underexplored but important mediated of fibrin deposition and can greatly influence the stability of the clot (5). As it is hypothesized that FXI and FXII might play a larger role in continued thrombin generation than initial clot formation, inhibitors of these two proteases in a flow-based system might play a larger role than indicated using non-flow assays. As

more is known about the difference between thrombotic environments versus

hemostatic environments, *in vitro* assays need to be developed to mimic these settings.

In addition to choosing the most physiologic *in vitro* assays to more effectively

examine thrombosis versus hemostasis, the choice of animal species and animal model

during pre-clinical drug development can greatly influence the result. Because aptamers

bind tightly over a large surface area on the protein's surface, they do not always exhibit

a high rate of cross-reactivity to other species, where even one amino acid change over

the binding region can disrupt aptamer-protein binding. Anecdotally, it is thought that

if the aptamer inhibits an established coagulation reaction found across species, such as

the binding of FV to FX to form the prothrombinase complex, then this reaction, and

thus the specific exosites on each protein, should be highly conserved. Therefore, an

aptamer that binds to that exosite and inhibits that reaction theoretically has a higher

chance of cross-reacting with different species. In general, aptamers tend to target

exosites on the surfaces of proteins. We found (summarized in Chapter 4, as well as

unpublished data) that aptamers targeting the downstream coagulation factors (FII, FX

and FIX), all which block exosites on their target protein, have a higher rate of cross-

reactivity over the animal species tested than the intrinsic pathway-targeting aptamers.

This mirrors what is thought to have occurred evolutionarily – with the emergence of

the common pathway proteases first, followed by the more recent emergence of the

intrinsic pathway. Thus, our data indirectly hints that the protein exosites on proteins

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higher up the cascade may not be as conserved across species. Further biochemical studies, including crystal structures of aptamers bound to proteins, would be needed to examine this theory.

Because the proteins for FXI and FXII may not be as conserved across species, established animal models of thrombosis or hemostasis used to evaluate inhibition of FXI and FXII are brought into question. If the function of FXI and/or FXII are slightly different in an animal than a human, then determination of the therapeutic potential of an inhibitor may not correlate. Animals may not be "small humans" where the pathways are identical between the two species. In this instance, it has been observed that different species have different clotting times, with rabbits and rats clotting very quickly compared with humans or non-human primates. This indicates that the coagulation system may have some very real differences, if not just the concentration of protein present in the blood. Both of these species need to have higher amounts of CTI present in order to prevent the contact pathway from becoming activated, indicating that this pathway might play a greater role in coagulation than in humans. Therefore, the use of these animal models to characterize inhibitors, as well as the murine data using FXI or FXII deficient mice, may not replicate what is or will be seen in humans. The selection of the right animal model is *key* in developing these aptamers further as therapeutics. Currently, the FXI and FXII aptamers do not potently cross react in any animal species besides human. Additional experiments using correct blood drawing

processes are underway to test these aptamers in more closely related species where the cost to do animal model experiments have been decreasing, including non-human primates.

Aside from testing the aptamers against different animal serums, strategies to re-design the aptamer to be cross-reactive can be undertaken. The human binding aptamer can be partially randomized and re-selected against the animal protein in a "doped-pool" SELEX. The goal of this SELEX would be that slight changes in the final aptamer sequence will bind to the same exosite on the human protein and with a higher affinity to the animal protein. This selection assumes that high-quality, purified animal proteins are available. Unfortunately, these are not currently available commercially for FXI or FXII.

In subsequent selections, the consideration of cross-reactivity can be included in the selection protocol, therefore considering pre-clinical studies during the drug discovery process. The first instance of this was demonstrated using a toggle selection approach, whereby a thrombin aptamer was isolated that could bind to both human and pig thrombin by alternating selection rounds with each purified protein (151). Again, while this strategy is effective, it is limited with the ability to obtain highly purified animal protein.

Aside from toggle SELEX, there are other strategies that can be employed to increase cross-reactivity. If no purified protein is available, a complex convergent toggle

selection may be employed. The complex SELEX approach has previously been utilized to select for a library of aptamers that bind to a mixture of proteins including the plasma proteome (152). Theoretically, a complex toggle approach would alternate selection rounds between human and animal plasma proteomes, with a resulting pool of aptamer sequences that can bind to both proteomes. This strategy can be employed in several different ways to increase the probability of isolating an aptamer that can bind to both species – the plasma proteomes can be combined to form a mixture of human and animal proteins, or a complex selection can be performed on the animal proteome followed by a convergent selection with the purified human target protein. Any number of these selections could 1) increase the chance of obtaining a species cross-reactive aptamer and 2) not require the use of high quantities of purified animal proteins.

5.3 Clinical development of aptamer therapeutics

Before aptamers begin preclinical and clinical trials, several optimization steps must occur in order to make the compound cost-effective and able to be stably administered. First, in order to decrease manufacturing and material costs, as well as potentially decrease any non-specific, RNA length dependent issues, the aptamer is truncated to the shortest length possible that retains binding affinity and anticoagulant function. The FXII and FXI aptamers were isolated using an 80 and 71 nucleotide modified RNA library, respectively. While these libraries are able to isolate functional aptamers, the functional portion of the aptamer may not include all nucleotides.

Truncation is aided by comparing each family of aptamer sequences within a selection. While these families differ considerably in base composition, there may be conserved strings of bases found throughout several different families. Using this strategy, the FXII aptamer was truncated from 80 nucleotide to 52 nucleotide while keeping intact a conserved string of bases. Previously, 40 nucleotides was the maximum length for a cost-effective aptamer, that length has gone up as manufacturing costs have been reduced, and a 50-mer is possible for cost-effect, large scale production. The FXI aptamer has not yet been truncated.

In addition to truncation, the aptamer is further optimized by replacing as many 2'F pyrimidine modifications and 2'hydroxyl nucleotides to a 2'O-methyl modification in order to further increase stability as well as reduce the likelihood of activating innate immune responses. This reduces production costs while still protecting the aptamer from endonuclease cleavage. In addition, stabilization of the aptamer by optimizing the stems present in the RNA structure, such as switching a U-A base pair to the more stable G-C base pair, can also be performed if needed. Oftentimes, this can aid in truncation, as two consecutive U-A base pairs making up a stem can sometimes be altered to one G-C base pair without disrupting the stability of the aptamer.

In preparation for animal or human administration, a high molecular weight moiety, such as PEG, will be appended to the end of the aptamer in order to increase the size of the aptamer and thus reduce clearance time facilitated by the kidneys. While this

modification does not usually alter the structure of the aptamer, there have been

instances where addition of a PEG can partially disrupt aptamer binding. While this is

rare, the PEG can be appended to the other end of the aptamer, or a longer linker can be

added to retain proper aptamer folding and function. In this way, the half-life of the

aptamer can be increased from minutes to days. Overall, this strategy has been

successful in optimizing aptamers for animal studies, and an anticoagulant aptamer

targeting FIX modified with this scheme is currently in Phase 2 clinical trials.

5.4 Applications and the therapeutic potential of an intrinsic pathway inhibitor

Overall, inhibition of the intrinsic pathway could be the "holy grail" of

anticoagulant therapy, as inhibition of either FXI or FXII seems promising in inhibiting

thrombosis without affecting hemostasis. There is some debate that FXI may be the

preferred target, as it is thought to be primarily a coagulation protein with no known

large roles in other physiologic processes. FXII, on the other hand, has emerging roles in

inflammation as well as potential basal growth factor activity stemming from low levels

of zymogen activity. In this setting, an aptamer therapeutic might be beneficial, as

aptamers can block distinct functions based on the binding site location on the target

protein. To this end, while the FXII aptamer described here inhibits FXI activation, it

does not effect prekallikrein activation, and thus potentially might affect coagulation but

not inflammatory processes. As other roles of FXII are elucidated, aptamer effects can be

studied, including FXII's emerging role as a growth factor. Because of the low risk of

bleeding and central role as a contact activator, inhibition of FXI or FXII can be utilized a drug therapy in many ways, including long term therapy to prevent thrombosis in at-risk patient populations or in combination therapy to inhibit blood contact activation when using artificial surfaces, such as catheters or a heart-lung machine.

The development of an intrinsic pathway aptamer as a long-term anticoagulant has several benefits and drawbacks. Although inhibition of FXI or FXII is not anticipated to be correlated with major bleeding events, some bleeding events associated with surgical procedures in specific tissues have been reported in FXI-deficient patients. Aptamers inherently have the ability to be reversed, and this technology has been tested in humans to be fast-acting and effective (163). While minimal events are expected with intrinsic pathway inhibition, a readily available antidote can further increase the safety of these drugs by rendering the target protein completely functional within minutes of antidote administration.

While long-term anticoagulation has traditionally been effectively administered orally, aptamers are currently only parentally available, which limits their use to injections or intravenous use, especially during surgical procedures. Preliminary data has suggested that aptamers can also be administered subcutaneously with a longer onset of action, which is an acceptable mode of administration for a long-term anticoagulant (189). However; while aptamer effects can be seen for weeks after subcutaneous administration, a rebound anticoagulant effect is exhibited after antidote

use. However, this should not be a hindrance to an intrinsic pathway inhibitor where

antidote use is expected to be minimal or for acute settings where re-dosing of antidote

is possible.

Aptamers inhibiters of other major coagulation proteases have previously been

described (164, 181, 182). While all are functional inhibitors of their protease target and

can independently anticoagulate blood or plasma, their concurrent administration

synergizes with greatly increased anticoagulant effects (data not published). Current

studies are ongoing to determine an effective anticoagulant strategy for use in cardio-

pulmonary bypass, with preliminary data suggesting that inhibiting two sequential

steps in the coagulation cascade is the most potent anticoagulation combination. The

addition of an intrinsic pathway aptamer inhibitor could also be beneficial in this highly

thrombogenic, artificial setting, where blood flows through tubing and over an

oxygenator. Use of the FXII aptamer to inhibit contact activation by these materials

could lead to reduced activation of coagulation factors, and potentially lower dosing

requirements for other aptamers. Currently, the use of two highly potent, non-aptamer

anticoagulants is limited, as synergy can oftentimes result in uncontrollable hemorrhage.

Importantly, concomitant use of several aptamers is ultimately safer, as you can quickly

reverse all aptamers using an universal antidote, or reverse the action of just one

aptamer by using a sequence specific antidote. In addition, full or partial reversal of

aptamer function can be reproducibly performed. This level of control with an

anticoagulant therapeutic is unparalleled, and can lead to multiple applications in the surgical field.

In the laboratory, aptamers are useful tools to examine diverging functions of a protease, as they can inhibit specific functions while leaving others intact. The FXII aptamer described in this project inhibits coagulation (FXI activation) but not inflammation (prekallikrein activation). While *in vivo* the aptamer may effect both functions of FXII because it inhibits the first step of FXII autoactivation, this aptamer can be used to examine the difference between FXIIa-mediated reactions in the laboratory. As FXII and FXIIa has increasingly been shown to be involved in a number of different processes, this aptamer could be valuable in discerning the influence of its separate functions. Similarly, FXI has binding sites for multiple coagulation proteins, including the platelet receptor GP1bα, and can be activated by other proteases other than FXII. While aptamer effects on these interaction has not yet been assessed, further biochemical characterization will allow for detailed mechanisms in aptamer inhibition, and the potential to aid in further discerning the biologic function of FXI.

5.5 Summary

Overall, the novel aptamer inhibitors of FXII and FXI described here are promising as potential therapeutics. As both of these aptamers can decrease thrombin generation in plasma-based assays, further *in vivo* analysis will determine if these aptamers can affect the FXII-mediated FXI activation that contributes to attenuated

thrombin generation in a thrombotic environment. As a class of drugs, aptamers lend

themselves well to anticoagulation, as they can inhibit specific protein-mediated

reactions, thus eliminating off target effects for a protein, especially FXII, whose role in

several pathways is not yet elucidated. In addition, aptamers have the ability to be

quickly reversed while leaving their target protein intact, thus increasing their safety

during potential bleeding events. In the setting of FXII and FXI, this is attractive not

only for FXI aptamer therapy, where mild bleeding events may occur, but also for

potential combination therapy with a more potent antiplatelet agent or antithrombotic

agent, which, while effective, has been hindered by a lack of safety due to increased

bleeding events. Lastly, this project highlights the need for better physiologic laboratory

assays in which to study potential drug therapeutics. We were successful in developing

a standardized assay that can detect changes in thrombin generation during inhibition of

the common or intrinsic pathway of coagulation. As many common coagulation tests

were based off of outdated coagulation models, the introduction of new or updated tests

should be welcomed to the laboratory.

Appendix A

Throughout this project, it was noticed that the aptamer controls could exhibit nonspecific effects in plasma-based assays, notably the aPTT and TGA. These effects, however, were completely opposite in each assay, as control RNA acted as an anticoagulant in an aPTT and an agonist in a TGA. The data and some speculation as to why this occurs are found here.

In an aPTT, 2'F pyrimidine modified, nonspecific RNA can dose-dependently increase clotting times in an aPTT activated by either micronized silica or ellagic acid (Figure 43). This activation is also length dependent, as shorter RNA's exhibit less of an effect on clotting times, with a steep drop off in nonspecific prolongation for RNAs less than 70 nucleotides. Therefore, our aptamers were truncated whenever possible to less than 70 nucleotides to decrease nonspecific effects.
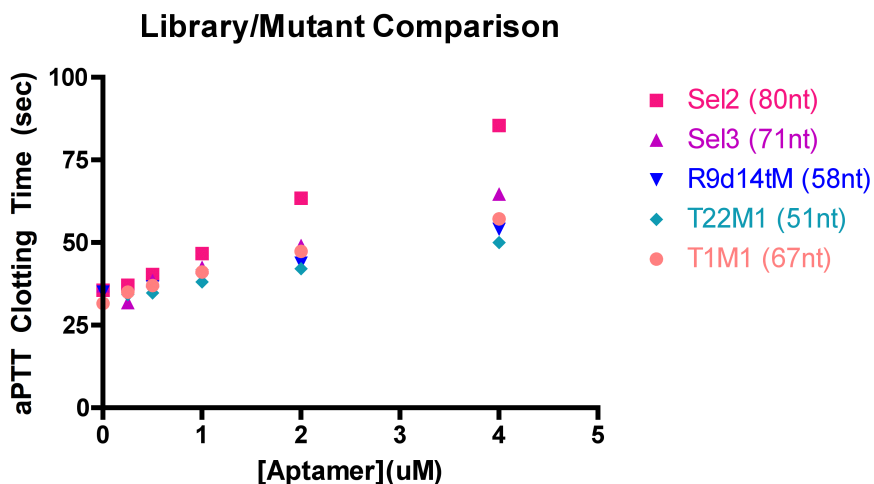
Figure 43. Control RNA comparison in an aPTT assay

Normal plasma was incubated with indicated control aptamers, and activated with micronized silica to obtain clotting times. The data represent the mean ± SEM of duplicate measures.

It was quickly noticed that the same RNA constructs used in the aPTT acted as agonists in the modified TGA, as they also could dose-dependently increase the rate and peak amount of thrombin generated (Figure 44). This agonist effect, however, did not alter the ETP of the sample. Anecdotally, the control RNA had a larger effect when the baseline peak thrombin generation of the sample was low (around 100nM), and it also seems that size has an inverse effect, with the longer RNA's exhibiting less of an effect than the shorter RNA's. This data, however, needs to be repeated.
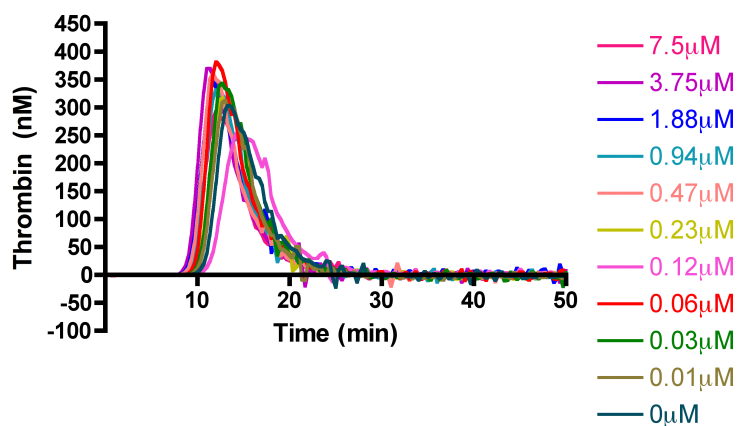
Figure 44. An RNA control exhibits antagonistic effects in a modified TGA assay

A 50 nucleotide scrambled control RNA was incubated with plasma at the indicated concentrations before addition of diluted ellagic acid activator to begin thrombin generation. The data are representative of three independent measures.

It was determined that these opposite, nonspecific effects depends on the potency of the activator (data not shown). The TGA uses 200x diluted ellagic acid to initiate thrombin generation with the aPTT uses "full strength" ellagic acid. As tested by an aPTT analyzing lag times, as the concentration of ellagic acid increases, less agonistic effects of control RNA were seen, with a switch to a prolongation of lag time as the reagent neared full strength (personal communication with Merck). This hints at possibly two different mechanisms of nonspecific interactions.

Control aPTT experiments looking at FXIIa and FXIa activated clotting times suggested that the agonist effect may be mediated by FXIa. It is known that polyanions, such as polyphosphates, can act as a cofactor to enhance FXI activation by thrombin (111). Similarly, incubation of either purified protein or plasma with control aptamer or polyphosphate accelerated clotting times in a FXIa-dependent manner, while no difference in lagtime was seen in FXIIa-initiated clotting times (Figure 45). As a control, the FXII aptamer inhibited clotting times in FXIIa activated plasma, but acted similarly to nonspecific RNA in FXIa activated plasma.

A

B
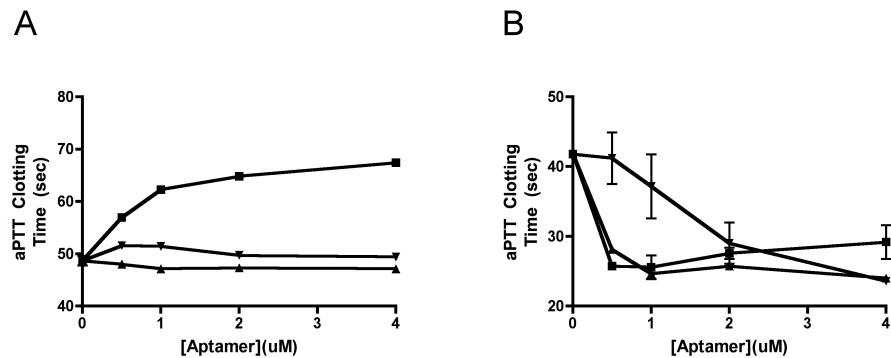
Figure 45. Analysis of polyanions on FXIIa or FXIa clotting times
The effect of R4cXII-1(■), scrambled control RNA (▲) or polyphosphates (▼) in a
clotting assay activated with A) 250nM FXIIa or B) 40nM FXIa. The data represent the
mean ± SEM of duplicate measures.

While the general mechanism on why these nonspecific effects occur is not

known, the data hints at two different mechanisms that depend on the concentration of

activation reagent used. At high concentrations of activator where activation is forced to

proceed through the intrinsic pathway, nonspecific RNA could interfere with binding

properties of the activator to FXII (it is well established that nonspecific RNA binds to

FXII), and thus slightly inhibit clotting. When the activator is diluted, perhaps allowing

for other feedback mechanisms to contribute to thrombin generation, nonspecific RNA

could be acting as an agonist in a FXI-dependent manner. Further analysis will help to

determine the specifics of these nonspecific, yet assay-specific, RNA effects.

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Biography

Rebecca Leigh Smock Woodruff was born on July 9, 1985 in Richmond, Virginia. She attended the University of North Carolina at Chapel Hill from 2003 through 2007 and obtained a Master's of Science in Biology and a minor in Chemistry. During her time there, she was a varsity swimmer for all four years, and team captain during the 2006 and the ACC-Champion 2007 seasons. In addition, Rebecca began her research career at UNC in Ralph Baric's lab studying the SARS coronavirus. Rebecca initiated her graduate studies at Duke University in the fall of 2007 under the Cell and Molecular Biology Department. After choosing to conduct her studies under the mentorship of Bruce Sullenger, she affiliated with the University Program in Genetics and Genomics, and designed her thesis work on developing the aptamer technology as an anticoagulant therapy.

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