Development and Application of Aptamer-Based Therapeutics

by

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University Program in Genetics and Genomics
Duke University

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Jen-Tsan Ashley Chi, M.D., Ph.D.

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 of Duke University

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ABSTRACT

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Abstract

Stroke is the leading cause of morbidity and the third leading cause of death in the United States. Over 80% of strokes are ischemic in nature, produced by a thrombus occluding the cerebral circulation. Currently, there is only one pharmacologic treatment FDA approved for ischemic stroke; recombinant tissue-type plasminogen activator (rtPA). Unfortunately, thrombolysis with rtPA is underutilized, as it must be administered within three hours of symptom onset and it is not uncommon for treatment to result in intracranial hemorrhage. For these reasons, safe and effective treatments of stroke are a medical necessity.

Aptamers are an attractive emerging class of therapeutic agents that offer additional safety because their activity can be reversed with administration of a complimentary oligonucleotide. Accordingly, I hypothesized that aptamers could be used to treat acute ischemic stroke. First, an antithrombotic aptamer previously generated against coagulation factor IXa was used in a murine model of middle cerebral artery occlusion. Upon factor IXa aptamer administration following stroke, neurological function and inflammatory profiles were improved. Moreover, mice previously treated with the aptamer, followed by induction of subarachnoid hemorrhage, had severe mortality levels and hemorrhage grades that were mitigated by administration of the aptamer’s matched antidote.

Second, I generated aptamers against the antifibrinolytic protein plasminogen activator inhibitor-1 (PAI-1), under the hypothesis that aptamer inhibition of PAI-1 would result in a reversible thrombolytic agent. However, after further testing, the aptamers were not found to disrupt the interaction between PAI-1 and its target proteases. Instead, the aptamers were shown to prevent PAI-1 binding to vitronectin,
which translated to restoration of breast cancer cell adhesion in an environment of PAI-1 mediated detachment.

Therefore, aptamer inhibition of factor IXa has demonstrated efficacy in improving outcome following stroke, and should life-threatening hemorrhage arise, an antidote specific to the interventional agent is able to decrease not only hemorrhage grade, but also mortality. This may result in a safer stroke therapy, while a novel aptamer generated against PAI-1 may have application as an antimetastatic agent, which could be used as adjuvant therapy to traditional breast cancer treatment.
This work is dedicated to my parents, Mac and Brenda Walker. Without your love, motivation and support, this would not have been possible.

I love you so very much—thank you.
# Contents

Abstract ................................................................................................................................. iv

Contents .................................................................................................................................. vii

List of Tables ........................................................................................................................... xi

List of Figures .......................................................................................................................... xii

List of Abbreviations ............................................................................................................. xiv

Acknowledgements .................................................................................................................. xx

1. Introduction .......................................................................................................................... 1

  1.1 Thrombosis ....................................................................................................................... 2

    1.1.1 Thrombosis and hemostasis ...................................................................................... 3

    1.1.2 Traditional coagulation cascade .............................................................................. 4

    1.1.3 Cell-based model of coagulation ........................................................................... 6

    1.1.4 Tissue factor-based model of coagulation .............................................................. 8

    1.1.5 Pathological thrombosis ......................................................................................... 9

  1.2 Acute Ischemic Stroke ...................................................................................................... 10

    1.2.1 Pathophysiology ...................................................................................................... 11

    1.2.2 Ischemic stroke vs. hemorrhagic stroke ................................................................. 13

    1.2.3 Current treatments of acute ischemic stroke ......................................................... 15

    1.2.4 Current treatments of intracranial hemorrhage .................................................... 29

  1.3 Aptamers and SELEX ....................................................................................................... 30

    1.3.1 Properties of aptamers as inhibitors ..................................................................... 30

    1.3.2 Aptamers as therapeutic compounds .................................................................. 33

    1.3.3 Antidote control of aptamers .............................................................................. 34

  1.4 PAI-1 ............................................................................................................................... 37
4.3 Results ................................................................................................................................. 100

4.3.1 The aptamer selected against human FIXa anticoagulates mice .............................. 100
4.3.3 FIXa aptamer reduces thrombin activity associated with stroke ......................... 106
4.3.4 Aptamer administration following MCAO reduces inflammatory protein levels.... 108

4.4 Discussion ........................................................................................................................... 110

5. Antidote Reversal of Aptamer Activity Following Subarachnoid Hemorrhage .... 114

5.1 Introduction ......................................................................................................................... 114
5.2 Materials and Methods ....................................................................................................... 117
5.3 Results ................................................................................................................................. 120

5.3.1 Antidote reversal decreases hemorrhage grade ......................................................... 120
5.3.2 Antidote reversal improves survival ........................................................................... 124

5.4 Discussion ........................................................................................................................... 125

6. Conclusions and Perspectives ............................................................................................ 128

6.1 Aptamer Inhibition of PAI-1 ............................................................................................. 128
6.2 Antidote-controlled Aptamer Treatment of Stroke ......................................................... 132
6.3 Summary ............................................................................................................................ 137

Appendix A (GPVI SELEX) ...................................................................................................... 138

Introduction .............................................................................................................................. 138
Materials and Methods ........................................................................................................... 141
Results ....................................................................................................................................... 143

Successive rounds induce shifts in binding to GPVI ............................................................ 143
cR6 reduces collagen-mediated platelet aggregation .............................................................. 145
Discussion & Conclusion ......................................................................................................... 146

Appendix B (Antiplasmin SELEX) .......................................................................................... 150

Introduction .............................................................................................................................. 150
List of Tables

Table 1-1: Clinical trials using streptokinase for treatment of acute ischemic stroke....... 26
Table 2-1: Aptamers selected against PAI-1........................................................................52
Table 5-1: Antidote administration mitigates increase in mortality following aptamer-
exacerbated hemorrhage.............................................................................................. 124
Table A-1: GPVI SELEX Round Parameters.......................................................................144
Table B-1: Antiplasmin SELEX Round Parameters............................................................156
List of Figures

Figure 1-1: Aptamer inhibition of FIXa and PAI-1 as reversible treatments of stroke ....... 2
Figure 1-2: Traditional waterfall coagulation cascade...................................................... 5
Figure 1-3: Cell-based model of coagulation ..................................................................... 8
Figure 1-4: Fibrinolysis........................................................................................................ 16
Figure 1-5: Schematic of the SELEX process (from Nimjee et al., 2005)......................... 32
Figure 1-6: Antidote control of aptamer activity. From (Nimjee et al., 2005)................... 36
Figure 2-1: Successive rounds against hWT PAI-1 increase in affinity ....................... 53
Figure 2-2: Successive rounds against hSM PAI-1 increase in affinity ....................... 54
Figure 2-3: Individual aptamers bind to WT PAI-1 with high affinity ....................... 55
Figure 2-4: Aptamers do not disrupt the PAI-1/uPA interaction in a direct uPA assay ................................................................. 57
Figure 2-5: Aptamers WT-15 and SM-20 do not disrupt the PAI-1/tPA complex .......... 58
Figure 2-6: Heparin and vitronectin compete with aptamers for binding to PAI-1 in solution .................................................................................................................. 59
Figure 2-7: Aptamers demonstrate reduced binding to mutated PAI-1 that does not bind heparin.................................................................................................................. 60
Figure 3-1: Aptamers prevent heparin-mediated PAI-1 inhibition of thrombin .......... 75
Figure 3-2: Aptamers prevent vitronectin-mediated PAI-1 inhibition of thrombin .......... 76
Figure 3-3: Aptamer interaction with antithrombin III (ATIII) ........................................ 78
Figure 3-4: Aptamer interaction with heparin cofactor-II (HCII) .................................... 79
Figure 3-5: Aptamer interaction with protein C inhibitor (PCI) ...................................... 80
Figure 3-6: Aptamers WT-15 and SM-20 stabilize PAI-1 in an active confirmation without affecting the PAI-1/uPA interaction ............................................................ 82
Figure 3-7: Aptamers SM-20 and WT-15 prevent PAI-1 from binding to vitronectin that is bound in a solid phase ....................................................................................... 84
Figure 3-8: Aptamer SM-20, but not WT-15, restores breast cancer cell adhesion........86

Figure 4-1: Factor IXa aptamer (Ch-9.3t) effectively anticoagulates mice; antidote oligonucleotide (5-2C) use reverses aptamer activity.................................................................101

Figure 4-2: Aptamer administration does not reduce infarct volume..........................103

Figure 4-3: Experimental timeline for neurological evaluation of mice following 60-minute MCAO.................................................................................................................................104

Figure 4-4: Aptamer administration following MCAO reduces neurological deficit.....105

Figure 4-5: Aptamer use mitigates MCAO-associated thrombin generation.................107

Figure 4-6: Use of Ch-9.3t aptamer following ischemia decreases inflammation........109

Figure 5-1: Murine subarachnoid hemorrhage experimental timeline.............................121

Figure 5-2: Antidote administration following aptamer use and subsequent SAH decreases hemorrhage grade....................................................................................................................122

Figure 5-3: Antidote administration mitigates the increase in hematoma size following aptamer use and subsequent SAH........................................................................................................123

Figure A-1: Round binding of GPVI SELEX.................................................................143

Figure A-2: Round cR6 reduces platelet aggregation in the collagen-induced platelet aggregation (CIPA) assay..........................................................145

Figure A-3: Schematic of the GPVI project.................................................................147

Figure B-1: Round binding of antiplasmin SELEX......................................................155
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’</td>
<td>3-prime end</td>
</tr>
<tr>
<td>5’</td>
<td>5-prime end</td>
</tr>
<tr>
<td>5-2C</td>
<td>antidote oligonucleotide 5-2C</td>
</tr>
<tr>
<td>Ch-9.3t</td>
<td>cholesterol-modified aptamer 9.3t</td>
</tr>
<tr>
<td>Ch-9.3tM</td>
<td>mutant cholesterol-modified aptamer 9.3t</td>
</tr>
<tr>
<td>ACA</td>
<td>anterior cerebral artery</td>
</tr>
<tr>
<td>ACT</td>
<td>activated clotting time</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIS</td>
<td>acute ischemic stroke</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP</td>
<td>antiplasmin</td>
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<tr>
<td>aPTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>ATIII</td>
<td>antithrombin-III</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>ºC</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CIPA</td>
<td>collagen induced platelet aggregation</td>
</tr>
<tr>
<td>CRP</td>
<td>collagen-related peptide</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DB</td>
<td>double blind</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DWI</td>
<td>diffusion-weighted imaging</td>
</tr>
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<td>extracellular matrix</td>
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<td>FII</td>
<td>factor II (prothrombin)</td>
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<tr>
<td>FIXai</td>
<td>active-site blocked factor IXa inhibitor</td>
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<tr>
<td>FX</td>
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<tr>
<td>FDA</td>
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</tr>
<tr>
<td>$\gamma^{32}$P</td>
<td>gamma-radiolabeled phosphate</td>
</tr>
<tr>
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<td>Description</td>
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<tr>
<td>GP Ib-IX-V</td>
<td>glycoprotein Ib-IX-V</td>
</tr>
<tr>
<td>GP IIb-IIIa</td>
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<td>GPVI</td>
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<tr>
<td>cGPVI</td>
<td>glycoprotein VI from the University of Cincinnati</td>
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<tr>
<td>hGPVI</td>
<td>histidine-tagged glycoprotein VI</td>
</tr>
<tr>
<td>HCII</td>
<td>heparin-cofactor II</td>
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<tr>
<td>HMWK</td>
<td>high molecular weight kininogen</td>
</tr>
<tr>
<td>IA</td>
<td>intra-arterial</td>
</tr>
<tr>
<td>ICH</td>
<td>intracranial hemorrhage</td>
</tr>
<tr>
<td>sICH</td>
<td>symptomatic intracranial hemorrhage</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>μm</td>
<td>micron</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>μg/mL</td>
<td>micrograms per milliliter</td>
</tr>
<tr>
<td>μg/L</td>
<td>micrograms per liter</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MC</td>
<td>multi-center</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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</table>
mg/kg  milligrams per kilogram
MI     myocardial infarction
min    minutes
mL     milliliter
mM     millimolar
MMP-9  matrix metalloprotease-9
MRA    magnetic resonance angiography
MRI    magnetic resonance imaging
MU     million units
N₂     nitrogen
N₄₀    40-nucleotide random region
NaCl   sodium chloride
ng/mL  nanograms per milliliter
NINDS  National Institute of Neurological Disorders and Stroke
nm     nanometer
nM     nanomolar
O₂     oxygen
OR     odds ratio
PA     plasminogen activator
PAI-1  plasminogen activator inhibitor-1
PAI-1-A active fraction of plasminogen activator inhibitor-1
hSM PAI-1 human stable mutant plasminogen activator inhibitor-1
hWT PAI-1 human wild type plasminogen activator inhibitor-1
PBS    phosphate buffered saline
PC  placebo-controlled
PCI  protein C inhibitor
PCR  polymerase chain reaction
PDI  protein disulfide isomerase
PEG  polyethylene glycol
pg/mL  picograms per milliliter
pH  potential of hydrogen
PK  prekallikrein
PLSD  protected least significant difference
pM  picomolar
PRP  platelet-rich plasma
PWI  perfusion-weighted imaging
RCL  reactive center loop
RNA  ribonucleic acid
rpm  revolutions per minute
RT  room temperature
RT/PCR  reverse transcription/polymerase chain reaction
SAH  subarachnoid hemorrhage
SELEX  systematic evolution of ligands by exponential enrichment
SK  streptokinase
SM  stable mutant
TAT  thrombin-antithrombin
TCCD  transcranial color-coded duplex
TF  tissue factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Description</th>
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<tr>
<td>TF/VIIa</td>
<td>tissue factor/activated factor VII complex</td>
</tr>
<tr>
<td>TNK</td>
<td>tenecteplase</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>rtPA</td>
<td>recombinant tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>VN</td>
<td>vitronectin</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
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First and foremost, I would like to thank God: my heavenly Father, the Lord and Savior Jesus Christ, and the ever-present Holy Spirit residing within. It is only through God’s grace, mercy, direction and comfort that I have been, and will be able to achieve all of the wonderful feats He has for me, while having such a fulfilling, abundant life. This work and my life are testaments that all things are possible with God.

Second only to God is my family. Thank you Mom, Mac, and Ang; one does not choose their parents or siblings, but if I could, I would have chosen you anyway. Mom, thank you for your unwavering love and support, and for the sacrifices you have made over the years that have played such a pivotal role in shaping who I am today. Mac, thank you for being the earthly father I have always needed, sent by God to fill a longing daughter’s heart. Ang, thank you for making me laugh until I cry, playing games, singing songs and quoting movies each time we are together. Thank you for always being true to yourself and others; I have learned more from you than you know. A very large thank you to my extended family, those on both sides of infinity. Your love, support, and prayers have been invaluable; I love you all so very much.

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phases of the Ph.D., but also through life as we go to our Father together for direction. Moreover, you are my companion and confidant, as I find reciprocity in every facet of myself that I share with you. I am looking forward to a lifetime of “iron-sharpening-iron” as we transcend together; ego te amo. Thank you, my affectionately called “Fantastic Four”, Crystal Reynolds, Dr. Charles Withers, Dr. Erikka Dzirasa, and Dr. Kafui Dzirasa. You have helped me navigate through the storms of life, always steering me back to the ultimate compass- God. I love you all so very much; my life has been deeply enriched by your presence.

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To all who have lent a word of encouragement, flashed a smile to lift my spirits, recharged my energy with a hug or sent a word of prayer on my behalf, THANK YOU. Though your name may not have been called, it is forever etched in my heart.
1. Introduction

Stroke is the leading cause of morbidity and the third leading cause of death in the United States (Lloyd-Jones et al., 2008). Over 80% of strokes are caused by a thrombus occluding the cerebral circulation, thus inducing ischemia. Although several experimental treatments are in clinical trials, there is only one pharmacologic treatment FDA approved for ischemic stroke; recombinant tissue-type plasminogen activator (rtPA). Unfortunately, treatment with rtPA is underutilized, as it must be administered within three hours of symptom onset and it is not uncommon for treatment to result in intracranial hemorrhage, which lacks specific treatment. Therefore, safe and effective treatments of stroke are a medical necessity.

Aptamers are an attractive emerging class of therapeutic agents that offer additional safety because their activity can be reversed with administration of a complimentary oligonucleotide. For this reason, I hypothesized that aptamers could be used to treat acute ischemic stroke. First, an antithrombotic aptamer previously generated against coagulation factor IXa was used in a murine model of middle cerebral artery occlusion. When the factor IXa aptamer was administered following stroke, neurological function and inflammatory profiles were improved. Moreover, when the aptamer was administered followed by induction of subarachnoid hemorrhage, thereby recapitulating hemorrhagic transformation following stroke therapy, the hematoma formed was large and resulted in high mortality. However, when the antidote was given, these effects were mitigated, as hematoma size decreased and survival improved.

Second, I generated aptamers against the antifibrinolytic protein plasminogen activator inhibitor-1 (PAI-1), under the hypothesis that aptamer inhibition of PAI-1 would result in a reversible thrombolytic agent (see Fig. 1-1). However, after further
testing, the aptamers were not found to disrupt the interaction between PAI-1 and its target proteases, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Instead, the aptamers were shown to inhibit PAI-1 from binding to heparin and vitronectin. In particular, one aptamer was able to restore breast cancer cell adhesion in an environment of PAI-1 mediated detachment. Therefore, this aptamer may have an application as an antimetastatic agent, which could be used as adjuvant therapy to traditional breast cancer treatment.

Figure 1-1: Aptamer inhibition of FIXa and PAI-1 as reversible treatments of stroke. Aptamer inhibition of factor IXa could prevent microvascular thrombosis, produced by thrombus formation and the influx of inflammatory cells, while aptamer inhibition of PAI-1 could aid in thrombolysis by reducing the inactivation of uPA.
1.1 Thrombosis

1.1.1 Thrombosis and hemostasis

Hemostasis is the physiological process of responding to a vascular insult or injury in proper magnitude; it is the embodiment of “mind like water” within the cardiovascular system. However, thrombosis is the pathological presence of a thrombus within the circulatory system. It is a process arising in response to vessel injury, abnormal blood flow (historically listed as blood stasis), or hypercoagulability; these three provocateurs of thrombosis are collectively known as Virchow’s triad.

Vascular injury can arise as a result of many factors such as smoking, hypertension, hypercholesterolemia, and hyperlipidemia. Platelets circulate throughout the vascular system, searching for these areas of endothelial injury. Once recognized, platelets become activated and the coagulation cascade begins on their surface (Hoffman et al., 2001). This leads to the formation of a fibrin meshwork that captures blood cells, forming a thrombus.

Both turbulence and stasis are abnormal forms of blood flow. Turbulence can arise as a result of normal vessel bifurcation or pathological ulcerated atherosclerotic plaques. In addition, turbulence can result from blood hyperviscosity, for example, due to sickle cell anemia. When blood flow moves from laminar flow to turbulent flow, countercurrents and local pockets of stasis can occur. Stasis is a major factor in the development of thrombi and can arise from several sources. For example, irregular contraction due to atrial fibrillation or a faulty valve can reduce the cardiac ejection fraction, causing the blood that is not ejected to pool, creating a thrombogenic environment. In addition, a non-contractile myocardium following infarction can lead to formation of a mural thrombus. Blood can also become trapped in an aneurysm or
remain stagnant downstream of an occluded vessel. When blood is no longer in circulation and becomes static, thrombi are prone to form.

The last portion of Virchow’s triad of thrombosis, hypercoagulability, is either primary in nature, due to genetics, or secondary, which is acquired. Primary causes include Factor V Leiden, which is due to a single amino acid change that renders factor Va resistant to inactivation by activated protein C; a prothrombin gene mutation in the 3’ untranslated region that increases prothrombin levels; or elevated homocysteine levels due to a mutation in the methy1tetrahydrofolate reductase gene. Also, although rare, genetic deficiencies of the anticoagulants antithrombin III, protein C, and protein S will also lend towards a perpetual hypercoagulable state. Acquired causes of hypercoagulability include high estrogen levels, which occur during pregnancy or use of oral contraceptives/hormone replacement therapy; procoagulant tumors; acute inflammation and infection; sepsis; endotoxemia; heparin-induced thrombocytopenia, and antiphospholipid antibody syndrome.

1.1.2 Traditional coagulation cascade

In 1964, two groups published nearly identical models of coagulation, both based on a ‘cascade’ or ‘waterfall’ effect of one protein activating a zymogen, which once activated, leads to the activation of an additional ‘downstream’ protein (Davie et al., 1964; Macfarlane, 1964). In this model, the intrinsic pathway (or the contact activation pathway) and the extrinsic pathway (or the tissue-factor pathway) converged to a common pathway, which lead to the activation of thrombin and thus formation of fibrin (see figure 1-2).
The intrinsic pathway, which models the activated partial thromboplastin time (aPTT) coagulation assay, begins with the exposure of collagen due to vascular injury. According to this cascade, a complex of high molecular weight kininogen (HMWK), prekallikrien (PK) and factor XII (FXII; also known as Hageman factor) is formed; FXII and prekallikrien become activated to FXIIa and kallikrien, respectively. This complex then activates factor XI (FXI), which then activates factor IX (FIX). Activated factor IX (FIXa) then joins with factor VIII (FVIII) that has been activated by the extrinsic pathway to form the tenase complex that activates factor X (FX).
The extrinsic pathway, which models the activated clotting time assay (ACT), begins with the exposure of tissue factor, again, from vascular injury. This tissue factor (TF) forms a complex with, and activates, Factor VII (FVII). The TF-FVIIa complex then activates FX and FVIII. According to this model, Factor X is activated by two different mechanisms; at this junction, the two pathways converge into the common pathway where FXa joins with FVa to form the prothrombinase complex, which cleaves prothrombin into thrombin. Though a major advance in the field of coagulation, this cascade is primarily the result of in vitro testing, and as such is unable to fully explain hemostasis in vivo. For example, as written, the two portions of the cascade should be equal in importance. However, deficiencies in high molecular weight kininogen, prekallikrien, and factor XII do not cause bleeding disorders, demonstrating that they are not essential to hemostasis. In addition, activation of factor X by TF-FVIIa of the extrinsic pathway cannot compensate for deficiencies in FVIII and FIX in patients with hemophilia (Hoffman et al., 2001). For these reasons, Hoffman and Douglas developed a model of coagulation that better paired in vivo observations with previously available data.

1.1.3 Cell-based model of coagulation

The cell-based model of coagulation is based upon the hypothesis that coagulation is regulated more so by the cellular surfaces upon which activation of the coagulation proteins occurs, rather than the kinetics and levels of the factors themselves. In this model, coagulation occurs in three semi-concurrent phases: initiation, amplification, and propagation (see figure 1-3).

The initiation phase begins on the surface of a tissue-factor bearing cell, for example, a fibroblast or monocyte, that is exposed due to vascular injury or disruption of the fibrous cap of an atheroma. The now exposed tissue factor activates FVII by
mechanisms not fully elucidated. TF-FVIIa then activates factor IX and factor X; FXa activates and joins with factor V to form the prothrombinase complex, which forms a small amount of thrombin.

That amount of thrombin is enough to catalyze several additional reactions, forming the amplification stage of coagulation. Here, platelets that were recruited to the site of vascular injury through platelet receptors glycoprotein VI (GPVI) and glycoprotein Ib-V-IX (GP Ib-V-IX) binding to the exposed extracellular matrix proteins collagen and von Willebrand factor (VWF; bound to collagen), respectively, serve as the next cellular surface upon which coagulation proceeds. Once activated, platelets release alpha granules that contain factor V. The small amount of thrombin generated during the initiation phase further activates these platelets through cleavage of Par1, and catalyzes the activation of factors XI, V, and VIII. FVIII in complex with VWF binds to platelets; during thrombin activation of FVIII, the bond between FVIII and VWF is broken, but FVIIIa remains on the platelet surface, along with the previously mentioned, now activated FV (FVa).

Thus far, FVa and FVIIIa are present on the surface of the activated platelet. Factor IXa that was previously activated on the tissue-factor bearing cell diffuses to the activated platelets, joins with FVIIIa on the platelet surface to form the tenase complex, and activates FX. The newly formed FXa (as opposed to the FXa formed on the tissue-factor bearing cell, which was unable to diffuse to the platelet surface due to rapid inhibition by tissue factor pathway inhibitor) combines with the platelet-bound FVa to form the prothrombinase complex, which is generates the “thrombin burst” necessary for fibrin formation.
1.1.4 Tissue factor-based model of coagulation

Expanding upon the cell-based model of hemostasis, Furie et al. developed a coagulation model that primarily highlights the role of tissue factor; here, they hypothesize that activation of encrypted tissue factor by protein disulfide isomerase (PDI) initiates coagulation, as opposed to solely relying on tissue factor exposed by vascular injury (Furie et al., 2008). According to this model, tissue factor is present in circulating blood bound to microparticles—vesicles less than 1000 nm in diameter that display proteins of the blood cells from which they were derived. However, this tissue factor exists in an encrypted form that renders them unable to initiate coagulation. Platelets exposed to areas of vascular injury become activated by collagen and express P-selectin; the circulating microparticles then bind to the P-selectin on platelets via
microparticle P-selectin glycoprotein ligand-1. Now in proximity to each other, the encrypted tissue factor on the microparticle is activated by PDI that was secreted by activated platelets, and is sequestered to the area of injury, ready to promote coagulation (Furie et al., 2008).

Also, in direct contrast to the cell-based model, Furie et al. propose that the platelet surface is not required for coagulation. For support, they cite a Par4-null mouse that displayed normal fibrin generation (Vandendries et al., 2007) and concluded that because those murine platelets could not become activated by thrombin, additional membrane surfaces must be required for coagulation (Furie et al., 2008).

1.1.5 Pathological thrombosis

Although platelet activation and coagulation are necessary for maintenance of hemostasis, these processes may be initiated in a pathological manner, leading to thrombosis. The most common root of pathologic thrombus formation is atherosclerosis, the progressive hardening and narrowing of the blood vessels. According to the response to injury model proposed by Ross et al., atherosclerosis begins as a response to endothelial injury by monocytes, platelets, smooth muscle cells, and lipoproteins (Ross, 1993). Circulating monocytes bind to the injured vascular endothelium and enter into the subendothelial intima through tight junctions, transforming the monocytes into phagocytic macrophages. These macrophages then ingest lipids, primarily in an oxidized form, becoming foam cells. When combined with the proliferation of smooth muscle cells, fatty streaks characteristic of early atherosclerosis are formed.

Free radicals released from macrophages within the atheroma lead to further endothelial injury, propagating a positive feedback loop for foam cell formation. As this process continues, larger atheromas and less stable plaques are formed. Depending on its location, the plaque may be more prone to fissuring and rupture; for example, those
near bifurcations are increasingly vulnerable. When this disturbance occurs, blood becomes exposed to collagen in the extracellular membrane, as well as tissue factor sequestered by foam cells and present in the atheroma. These lead to the activation of platelets and coagulation, forming a thrombus.

Another category of agents hypothesized to incite thrombosis is what Furie et al. call “pathologic microparticles”. These are similar to the microparticles previously mentioned, with the exception that tissue factor is present in an active conformation. These pathologic microparticles may be abundant in the circulation and could serve as a biomarker for increased thrombotic risk. Additionally, the microparticles derived from tumor cells or inflammatory cells may account for increased thrombosis during cancer/paroxysmal nocturnal hemoglobinuria (Furie et al., 2008).

1.2 Acute Ischemic Stroke

When pathological thrombi are present in the peripheral circulation, deep venous thrombosis or arterial claudication occurs. In a like manner, if thrombi occlude the coronary circulation, a myocardial infarction (MI) develops, and if in the pulmonary circulation, pulmonary embolisms arise. Of particular interest are thrombi present in the cerebral circulation, leading to acute ischemic stroke (AIS).

According to the 2009 report of heart disease and stroke statistics, approximately 795,000 strokes occur each year in US; this averages to someone suffering from a stroke every 40 seconds (Lloyd-Jones et al., 2008). Moreover, this stroke claims a life every 3-4 minutes, rendering stroke the third leading cause of mortality in US (behind heart disease and cancer) (Lloyd-Jones et al., 2008).
1.2.1 Pathophysiology

Initial injury/cytotoxic edema

In most cases, neuronal activity is due to a neurotransmitter binding to its receptor; this binding changes the permeability of ion channels, allowing for the influx of positive ions (depolarization). Maintenance of the ion gradient necessary for these voltage changes requires energy in the form of ATP, which is generated from oxygen and glucose. Neuronal injury therefore depends on the duration and magnitude of ischemia, as cells deprived of blood are deprived of oxygen and glucose. Without proper energy, neuronal activity decreases and cellular processes begin to malfunction. For example, without oxygen, the cell enters anaerobic metabolism, the by-products of which, lactic acid and hydrogen ions, lead to free radical formation. These free radicals then damage cellular membranes, genetic materials and proteins, leading to irreversible neuronal injury and ultimately cell death. When lacking proper amounts of ATP, ion gradients are unable to be maintained. In response to this, the cell enters into anionic depolarization, equilibrating the intracellular environment with the extracellular environment. This process allows for the influx of sodium, chloride, and calcium; and the efflux of potassium. This disturbance then leads to the release of glutamate and aspartate, excitatory neurotransmitters that instigate additional free radical formation as well as further sodium and calcium influx. Increased intracellular calcium causes the release of arachadonic acid and activation of phospholipases, both of which increase free radical formation. In an attempt to bring the cell into osmotic equilibrium, water enters into the cell, leading to acute cytotoxic edema; this develops over minutes to hours, and may be reversible in nature.
**Vasogenic edema**

In the presence of massive neuronal injury, tight junctions in the damaged endothelium become increasingly permeable, allowing for the extravasation of plasma-protein (e.g., albumin) containing fluid into the extracellular space. This process, called vasogenic edema, typically occurs hours to days after the initial ischemic insult and is considered irreversible. Due to the increased presence of volume within the rigid container of the cranium, intracranial pressure increases. This may induce brain shifting and possibly herniation into the foramen magnum, causing permanent damage to brain cells and possibly death.

**Reperfusion injury**

To avoid further neuronal injury, glucose and oxygen levels must be restored through reperfusion of the ischemic area. Paradoxically, restoration of blood flow to these reversibly damaged areas is also associated with tissue damage, termed reperfusion injury. During reperfusion, red blood cells carry oxygen that damages cell membranes and proteins, which leads to the release of free radicals. In addition, white blood cells entering areas of vascular injury sustained during ischemia release additional free radicals and proinflammatory cytokines that activate leukocytes and upregulate adhesion molecules. This leads to the accumulation of leukocytes, which in small capillaries can cause additional ischemia, known as the “no-reflow” phenomenon. This phrase was originally coined by Ames et al. in 1968 to describe the incomplete restoration of normal blood flow following a period of ischemia (Ames et al., 1968).

An ideal approach, then, is to combine a reperfusion agent with a treatment that reduces reperfusion injury. In response, neuroprotective agents that serve as free radical scavengers and leukocyte adhesion inhibitors have been developed and have had
positive results in preclinical studies. However, in clinical trials, many of these approaches were not successful as patient outcome was rarely improved (Krams et al., 2003; Wang et al., 2007).

1.2.2 Ischemic stroke vs. hemorrhagic stroke

Of all strokes, 87% are ischemic in nature. The remaining 13% are due to hemorrhage, 10% of which arise from extravasation of blood into the cerebral tissue (intracranial hemorrhage; ICH), and 3% from blood that has entered into the subarachnoid space (subarachnoid hemorrhage; SAH) (Lloyd-Jones et al., 2008).

Ischemic stroke etiologies

There are three origins of ischemic stroke: primary large vessel occlusive disease, embolism, and primary small vessel occlusive disease. The most common cause of large vessel disease is atherothrombosis, which is also the most common overall cause of stroke. Approximately 32% of strokes are caused by an embolus of some sort, and the overwhelming majority are cardiogenic in nature. Another common source of emboli is arterial atherothrombosis generated from within the cerebral circulation. Small vessel or “lacunar strokes” typically arise from microatheromas or lipohyalinosis. Additional etiologies of primary small vessel occlusions include eclampsia and arteritis.

Regions of ischemic tissue

Juxtaposed to the occluded vessel is the core of the infarct; this region typically receives blood flow that is less than 10-25% its normal value. Here, neurons and glial cells rapidly deplete their energy stores and become necrotic. Though neurons are typically less forgiving of severe ischemia, glial cells may be spared if reperfusion is restored.
The core of the infarct is surrounded by a penumbral region that is injured, but supplied by enough collateral vessels that the damage is deemed reversible. This area typically experiences mild to moderate ischemia and as a result, can remain viable for as long as a few hours following the initial insult. However, because the anastamosing collateral supply is not sufficient to indefinitely meet energy demands, penumbral cells may also enter necrosis if not reperfused in a timely manner. Because this tissue can be “rescued”, restoring blood flow to the penumbra is the primary target of reperfusion therapy.

**Hemorrhagic stroke (ICH/SAH)**

Intracranial hemorrhage (ICH) has a 30-day mortality of 35-52%, with half of those deaths occurring within the first two days (Adams et al., 2007). Furthermore, greater than 80% of patients with ICH remain disabled six-months after the initial insult, highlighting the severe morbidity and mortality that arises from this disease (Adams et al., 2007). Similarly, subarachnoid hemorrhage (SAH) presents with a global mortality of 32-67%, with half of these patients dying within two weeks and less than one third of survivors able to resume their normal daily activities (Ferro et al., 2008).

Upon casual inspection, patients with ICH/SAH present very similarly to those with acute ischemic stroke (AIS) in that both present with a sudden, focal neurological deficit. However, a patient with ICH is more likely than a patient with AIS to have a smooth progression to increasingly severe symptoms that develop over minutes to hours, as well as vomiting and headache. Additionally, SAH typically presents with headache, neck stiffness, and other signs of meningeal irritation. However, the definitive diagnosis of both ICH and SAH is made through imaging; as such, computed tomography (CT) or magnetic resonance imaging (MRI) should be performed as soon as possible (Broderick et al., 2007; Ferro et al., 2008). While ICH presents as intracerebral
hematomas, SAH is diagnosed by the presence of subarachnoid hematic densities on CT and is confirmed through lumbar puncture. Once confirmed, the patient undergoes intra-arterial angiography, primarily to investigate evidence of a ruptured aneurysm, which causes ~75% of all subarachnoid hemorrhages.

1.2.3 Current treatments of acute ischemic stroke

FDA approved treatments

_Intravenous pharmacologic thrombolysis: tPA_

Thrombolytic therapy was first explored in the 19th century after the liquefaction of clotted blood and spontaneous dissolution of fibrin thrombi was observed (Lyden, 2005). However, it was not until the 1940’s when interest in clot lysis during streptococcal infection allowed for improved understanding of the mechanism of fibrinolysis. This led to a 1947 report that animal tissues also contained an agent (then termed fibrokinase) that could activate plasminogen (Collen et al., 2004). Although many plasminogen activators were reported (including urokinase plasminogen activator, tissue plasminogen activator, vascular plasminogen activator, and blood plasminogen activator), it was determined in 1980 that the latter three were one in the same (Rijken et al., 1980). Therefore, there are only two endogenous plasminogen activators; urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Collen et al., 2004).

Tissue-type plasminogen activator (tPA) is a serine protease that functions to increase the circulating level of plasmin. Plasmin is formed by the proteolytic cleavage of plasminogen, its inactive precursor. This is the process by which tPA serves to “activate” the plasminogen. Once formed, plasmin is able to break up the fibrin meshwork that forms the thrombus structure; this process is called fibrinolysis (see Fig.
1-4). When this occurs, the thrombus disintegrates and blood once again flows freely within the blood vessel.

![Diagram of Fibrinolysis]

**Figure 1-4: Fibrinolysis**

Alteplase (recombinant tPA) underwent extensive preclinical testing before it was considered for treatment of AIS. The thrombolytic effects of tPA were first demonstrated using a rabbit model of pulmonary artery thrombosis in 1980 (Matsuo et al., 1981); this spawned the testing of tPA in animal models of acute myocardial infarction (AMI). Once determined that tPA was able to induce recanalization of thrombosed coronary arteries in closed- and open-chest canine models (Bergmann et al., 1983; Gold et al., 1984), tPA was tested in human pilot studies for treatment of AMI in 1983, which eventually lead to larger clinical trials and its FDA approval. In 1985, Zivin et al. published the groundbreaking manuscript that illustrated tPA’s efficacy in not only acute myocardial infarction, but also acute ischemic stroke. This paper demonstrated that tPA could improve neurological function after thrombolysis of autologous blood clots injected into the carotid circulation of rabbits (Zivin et al., 1985). Following this result, further studies were conducted in various preclinical models that trended towards demonstrating that tPA was effective, but also carried a risk of ICH. Tissue-type plasminogen activator was then tested in many human preliminary trials
beginning in 1990, which ultimately led the National Institute of Neurological Disorders and Stroke (NINDS) to design a pilot study in 1992 that consisted of two parts: part I investigating the effects of tPA administered within 90 minutes from stroke onset and part II, when given within 91-180 minutes (Brott et al., 1992; Haley et al., 1992). These dose escalation studies formed the foundation that the early major clinical trials (ECASS, NINDS, etc.) were laid upon, and led to the 1994 FDA approval of tPA for use in acute ischemic stroke.

Endovascular mechanical thrombolysis: Merci retriever

The Merci retriever is a corkscrew shaped thrombus retrieval device intended to be used in pts who are ineligible for intravenous (IV) tPA or those who fail IV tPA. It was FDA approved in August 2005 for recanalization of intracranial arteries, though its clinical utility for larger arteries has not yet been fully established. When comparing the Merci device to intra-arterial thrombolysis with uPA (results of the PROACT II trial), mortality and recanalization rates were similar (Josephson et al., 2008).

Mechanical thrombolysis has many advantages over intravenous thrombolysis with tPA. Most notable is extension of the three-hour time window for efficacy without compromising safety. Both major clinical trials with the Merci retriever (MERCI and MultiMERCI) enrolled patients up to eight hours from symptom onset (Smith et al., 2005). In addition, the Merci device can be used following thrombolysis failure, as 73% achieved reperfusion (with a rate of symptomatic ICH of 6.7%) following IV tPA with subsequent embolectomy, according to the MultiMERCI trial (Smith, 2006). A pooled analysis of MERCI and MultiMERCI demonstrated that the Merci retrieval system increases recanalization and improves outcome and survival without increasing symptomatic intracranial hemorrhage (sICH) when used for internal carotid artery embolectomy (Flint et al., 2007), and it can be safely used in patients with abnormal
hemostasis (as defined by a platelet count less than 100,000 and elevated coagulation times) (Nogueira et al., 2008). Currently, novel mechanical thrombolytic devices such as photoacoustic agents, lasers and aspiration tools are undergoing evaluation in clinical trials.

**Experimental treatments**

*Antithrombotic agents for treatment of stroke*

Currently, anticoagulation for treatment of acute ischemic stroke has not demonstrated any benefit, even when administered within three hours (Khaja et al., 2007). In fact, heparin use was associated with a negative risk profile, as anticoagulation following stroke increased the risk of symptomatic ICH; as such, its use is not recommended for treatment of AIS, and its administration is contraindicated within the first 24 hours following intravenous tPA (Adams et al., 2007).

Several clinical trials have been conducted evaluating antithrombotics, primarily antiplatelet agents, for treatment of AIS. For example, the much anticipated GP IIb-IIIa inhibitor abciximab was recently assessed for acute treatment of stroke. Following initially promising Phase II results (AbESTT) demonstrating a non-significant shift in improving 3-month modified Rankin scores (P>0.05 after adjusting for severity of stroke and age); the Phase III trial, AbESTT-II, began. However, this trial was halted prematurely due to an increased rate of bleeding (Adams et al., 2008).

Although use of aspirin in conjunction with tPA is not recommended (though it may replace tPA if the patient is not eligible for thrombolytic therapy), daily use of aspirin, if commenced within 48 hours following stroke onset, has been shown to reduce the risk of recurrent stroke within 14 days according to the International Stroke Trial (IST) and Chinese Acute Stroke Trial (CAST) (Sandercock et al., 2008).
Thus far, no antithrombotic agent has been recommended for use as an acute treatment of stroke. In considering its use, the benefit of reperfusion must be evaluated in light of the increased risk of hemorrhage. An ideal agent would therefore inhibit thrombosis without affecting hemostasis. If this were not possible, antidote reversal of the drug’s activity would confer additional safety. As such, further evidence is needed to determine the safety and efficacy of antithrombotic agents as solitary treatments of AIS or as adjunct therapy to tPA.

Intra-arterial pharmacologic thrombolysis

Delivery of a thrombolytic agent at the site of arterial occlusion using a microcatheter (intra-arterial [IA] thrombolysis), in general has higher recanalization rates than IV tPA, but concerns exist regarding the time delay from symptom onset to treatment, as angiography is required (which demands interventionalists and specialized equipment that may not be readily available). According to the 2007 AHA/ASA recommendations for treatment of stroke, IA thrombolysis is recommended for patients with middle cerebral artery (MCA) occlusion that are not expected to do well with IV tPA (Adams et al., 2007). For example, patients that present 3-6 hours after symptom onset, have large vessel occlusions that are more resistant to thrombolysis, and suffer from greater neurological deficits. These recommendations are based on the Phase III PROACT II trial when IA prourokinase was administered within six hours of symptom onset (Furlan et al., 1999). However, because prourokinase is not available, these results were extrapolated for use of rtPA and urokinase in the US. Currently, SYNTHESIS EXP is an ongoing Phase III clinical trial that was expanded from the SYNTHESIS trial, which compares IV tPA to IA tPA (and/or mechanical embolectomy).

Combination therapy: Antithrombotics + IV tPA

As previously stated, antithrombotic therapy may serve as a beneficial adjunct to
IV tPA for treatment of ischemic stroke, as platelet aggregation can increase resistance to thrombolysis, and inhibition of microthrombi may lead to improved reperfusion. Although abciximab alone for treatment of AIS is not recommended, combined use with a thrombolytic agent could decrease the necessary dose of both and may favorably affect outcome, as it is used with tPA for treatment of myocardial infarction (Ohman et al., 1997). The current clinical trial (ROSIE: ReoPro Retevase Reperfusion of Stroke Safety Study—Imaging Evaluation) is evaluating a combination of abciximab and reteplase, using MRI (or CT if patient is ineligible for MRI) to determine evidence of reperfusion.

Argatroban is a direct thrombin inhibitor with a short half-life that is used as treatment for heparin-induced thrombocytopenia and during percutaneous interventions in the US. After determining that administration up to 12 hours following stroke was safe (although ineffective) in the 2004 ARGIS-1 trial (LaMonte et al., 2004), argatroban was combined with tPA in patients with AIS due to MCA occlusion within three hours of symptom onset. Though neurologic outcomes were not significantly improved and sICH was increased (Sugg et al., 2006), as only 15 patients were enrolled, a larger patient population was deemed necessary to uncover the true effects; that Phase II clinical trial (ARTIS) is currently ongoing and recruiting patients.

**Combination therapy: IV tPA + IA tPA**

Initially, the safety of using IV tPA with IA tPA was assessed in the Emergency Management of Stroke Bridging (EMS) trial which demonstrated higher rates of recanalization, albeit without improving outcome (Lewandowski et al., 1999). To improve neurological function, the trial design moved away from concomitant IV tPA and IA tPA, and began an approach utilizing the treatments in series in the Interventional Management of Stroke (IMS) trial. First, the standard dose of IV tPA was administered, followed by angiography. If evidence of a thrombus remained, the patient
would undergo IA tPA within five hours. This trial (and the subsequent IMS-II) not only established the safety of this procedure, but demonstrated lower mortality rates in experimental arm, with similar rates of sICH (2004; 2007). IMS-III, the Phase III extension, is currently ongoing.

**Sonolysis and thrombolysis**

Ultrasound improves thrombolysis by inducing cavitations within the fibrin meshwork such that plasma is able to flow through the thrombus; this allows tPA to penetrate deeper into the clot, leading to more complete and faster dissolution (Francis et al., 1995). One of the first clinical trials to utilize ultrasound in combination with thrombolysis was the CLOTBUST trial; here, two hours of transcranial Doppler ultrasound was administered to patients with MCA occlusions receiving IV tPA. A statistically significant increase in recanalization and a nonsignificant trend towards clinical improvement after three months was noted with ultrasound, versus patients receiving IV tPA only (Alexandrov et al., 2004). TRUST is an ongoing clinical trial similar to CLOTBUST that is evaluating a shorter length of isonation (1 hour versus the 2 hours in CLOTBUST).

Expanding on these results, microbubbles, which are air- or gas-filled microspheres with specific acoustic properties that enhance the contrast between fluid and tissue, are also in evaluation in conjunction with ultrasound and thrombolysis for treatment of AIS. Microbubbles are attractive because they are able to decrease the amount of energy needed to cause cavitation; also, the bubbles absorb energy until they burst, which may also help accelerate clot lysis. In a 2006 study, Molina et al. demonstrated the safety and feasibility of this approach, as a significantly greater number of patients achieved complete recanalization when they received IV tPA,
ultrasound, and microbubbles (Molina et al., 2006). NANOART is a similar ongoing trial that utilizes gas-filled nanobubbles as opposed to air-filled microbubbles.

Sekoranja et al. assessed a combination of IV tPA, IA tPA and ultrasound with microbubbles in a pilot study; here, patients with middle cerebral artery occlusions received IV tPA, followed by transcranial color-coded duplex (TCCD) sonography 30-minutes after the start of the tPA infusion to assess for reperfusion. If recanalization occurred, the patient was continued on IV tPA; if the MCA remained occluded, IV tPA was stopped and the remaining tPA was given intra-arterially. Results of the trial concluded that combined IV/IA thrombolysis, guided by TCCD, is safe and efficacious, as low rates of sICH occurred in each group (6% in both IV tPA alone and IV/IA tPA groups), and reperfusion occurred in 51% of IV tPA vs. 88% in the IV tPA followed by IA tPA group (Sekoranja et al., 2006).

Extension of the three-hour time window

In addition to safety, a major deterrent from tPA use is the narrow three-hour time window for administration. ECASS I and II, as well as ATLANTIS A and B, were early clinical trials used to establish the safety and efficacy of tPA; unlike NINDS however, these trials allowed for a six-hour time window for tPA administration. A meta-analysis of ECASS I and II, ATLANTIS A and B, and NINDS demonstrated a definite benefit of tPA when administered up to 4.5 hours after symptom onset; however, this additional 90-minutes may come with an increased risk of sICH (Hacke et al., 2004). Similarly, the third international stroke trial (IST-3) is a Phase III, ongoing clinical trial that plans to recruit 6000 patients. This trial is specifically assessing patients receiving 0.9 mg/kg rtPA between three and six hours after symptom onset. In a like manner, the ECASS III trial is currently investigating use of 0.9 mg/kg rtPA within 3-4.5 hours.
As extension of the time window for thrombolysis may come with increased risk of hemorrhage, investigators have used information on the penumbra from imaging to guide the decision to treat with thrombolytic agents in the 3-6 hour time window. For example, Ribo et al. used radiological evidence of a mismatch in diffusion-weighted imaging (DWI) and perfusion-weighted imaging (PWI) as inclusion criteria for ultrasound assisted-thrombolytic therapy of patients 3-6 hours after symptom onset. In this trial, recanalization rates were similar (though outcome was better) in the group chosen to undergo thrombolysis within 3-6 hours based on the presence of a diffusion/perfusion mismatch versus patients receiving traditional IV tPA within three hours of symptom onset (Ribo et al., 2005). The Diffusion and perfusion imaging Evaluation For Understanding Stroke Evolution (DEFUSE) trial performed MRI before tPA was administered; they noted retrospectively that patients with a diffusion/perfusion mismatch did best following early reperfusion with tPA, while patients with fatal ICH fit a “malignant” MRI profile (large DWI and PWI lesions) (Albers et al., 2006). Similarly, the recent results of the Echoplanar Imaging Thrombolytic Evaluation Trial (EPITHET) trial demonstrated that patients with a radiologic DWI/PWI mismatch have favorable results (i.e., increased reperfusion and improved neurologic outcome) following tPA when administered 3-6 hours after symptom onset (Davis et al., 2008).

Another measure of the mismatch between perfusion and diffusion can be assessed using magnetic resonance angiography (MRA) parameters with DWI. A post-hoc analysis of patients with MRA-DWI mismatch (defined as either a DWI lesion volume less than 25 mL in patients with proximal vessel occlusion; or a DWI lesion volume less than 15 mL in patients with proximal vessel stenosis or an abnormal finding of a distal vessel) demonstrated a strong correlation between the presence of a
MRA/DWI mismatch and improved outcome following thrombolysis (Lansberg et al., 2008).

Other thrombolytics: Desmoteplase

Desmoteplase is vampire bat plasminogen activator; its inherent advantage over rtPA in that does not cause neurotoxicity (Reddrop et al., 2005). Although enthusiastically received as a possible safe thrombolytic agent that improved clinical outcome when administered within nine-hours (Hacke et al., 2005; Furlan et al., 2006), results of the much larger Phase III clinical trial (DIAS-II) declared that use of desmoteplase did not demonstrate a benefit when given 3-9 hours after stroke, even in patients with a clear perfusion/diffusion mismatch (Hacke et al., 2008).

Other thrombolytics: Ancrod

Ancrod is a fibrinogenolytic agent derived from the pit viper snake that was investigated for solitary use in treating acute ischemic stroke. The phase III STAT trial in 2000 demonstrated a barely significant improvement in clinical outcome at three months for patients treated with ancrod, though all other measures were nonsignificant (including the incidence of sICH). However, the ESTAT (European STAT trial) was halted early in 2000 due to increased mortality. Currently, there is one phase III trial being conducted with ancrod--ASP I. This trial differs from the (E)STAT trials in that now, a brief, 2-3 hour infusion of ancrod is administered within six hours as opposed to the previous 72 hour continuous infusion.

Other thrombolytics: Plasminogen activators

Reteplase (r-PA, Retavase) is a second-generation deletion mutant of recombinant tissue-type plasminogen activator that may have a more rapid onset and lower bleeding risk, as well as faster plasma clearance and a shorter half-life (~11-19 minutes) than the first-generation agent alteplase. As reteplase does not bind fibrin as
tightly as native tissue plasminogen activator, it is able to diffuse more freely through the clot rather than binding only to the surface like tissue plasminogen activator. In high concentrations, reteplase does not compete with plasminogen for fibrin-binding sites; this allows plasminogen at the site of the clot to be activated into clot-dissolving plasmin. These two modifications help explain the faster clot resolution seen in patients receiving reteplase as opposed to those receiving alteplase. Currently, the ROSIE trials testing combinations of antithrombotic and thrombolytic agents are using reteplase as their thrombolytic of choice.

Unlike reteplase, steptokinase is not a true protease, as it does not exert proteolytic activity by itself. Instead, streptokinase forms a tight-binding complex with human plasminogen or plasmin, which enhances the specificity of the complex for plasminogen activation. Unfortunately, streptokinase is highly antigenic and frequently results in high levels of antistreptococcal antibodies, as it is produced by beta-hemolytic streptococci. In addition, three clinical trials investigating streptokinase for treatment of stroke (MAST-1, MAST-2 and ASK) were halted prematurely due to an unacceptable increase in sICH (see table 1-1). For these reasons, streptokinase is no longer deemed an option for treatment of AIS.
Table 1-1: Clinical trials using streptokinase for treatment of acute ischemic stroke

<table>
<thead>
<tr>
<th></th>
<th>MAST-I</th>
<th>MAST-E</th>
<th>ASK</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full Name</strong></td>
<td>Multicentre Acute Stroke Trial- Italy</td>
<td>Multicenter Acute Stroke Trial- Europe</td>
<td>Australian Streptokinase Trial</td>
</tr>
<tr>
<td><strong>Purpose</strong></td>
<td>To determine whether together or separately if streptokinase and aspirin have clinical effects in stroke similar to that seen with acute MI</td>
<td>To evaluate the safety and efficacy of streptokinase in pts with AIS</td>
<td>To determine if streptokinase given within 4 hours will reduce morbidity &amp; mortality at 3 months &amp; how this compares to receiving SK within 3 hours</td>
</tr>
<tr>
<td><strong>Design</strong></td>
<td>Randomized, prospective, MC, PC</td>
<td>Randomized, prospective, MC, DB, PC</td>
<td>Randomized, prospective, MC, DB, PC</td>
</tr>
<tr>
<td><strong>Dose/Drug</strong></td>
<td>1.5 MU SK, 300 mg/day aspirin for 10 days</td>
<td>1.5 MU</td>
<td>1.5 MU</td>
</tr>
<tr>
<td><strong>Time Window</strong></td>
<td>0-6 hours</td>
<td>0-6 hours</td>
<td>0-4 hour, 0-3 hours</td>
</tr>
<tr>
<td><strong>Primary Outcome</strong></td>
<td>No significant difference in reduction of fatality/severe disability at 6 months (OR for SK (with or without aspirin) or aspirin= 0.9)</td>
<td>No significant difference in number of patients with target mRS score (79.5 vs. 81.8; p=0.6)</td>
<td>Nonsignificant trend towards improvement if treated in less than 3 h. (OR= 1.08)</td>
</tr>
<tr>
<td><strong>Secondary Outcome/ Other</strong></td>
<td>Significant increase in death at 10 days (OR= 3.5 for SK+aspirin vs. neither)</td>
<td>Significant increase in death at 10 days (from hemorrhagic transformation of the ischemic infarcts)</td>
<td>Nonsignificant increase in death/morbidity with use of SK, especially when greater than 3 hours</td>
</tr>
<tr>
<td><strong>Death (drug vs. placebo)</strong></td>
<td>STAT. SIG. (10 day: 53% vs. 23%; 6 month: 72% vs. 49%)</td>
<td>STAT. SIG. (10 day: 34% vs. 18.2%, p=0.02)</td>
<td>Not significant 19.9% vs. 12.8%</td>
</tr>
<tr>
<td><strong>Symptomatic ICH (drug vs. placebo)</strong></td>
<td>STATISTICALLY SIGNIFICANT (16% vs. 2.6%)</td>
<td>STATISTICALLY SIGNIFICANT (21.2% vs. 4.6%)</td>
<td>STATISTICALLY SIGNIFICANT (12.6% vs. 2.4%)</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>(1995A)</td>
<td>(1996)</td>
<td>(DONNAN ET AL., 1996)</td>
</tr>
</tbody>
</table>

Abbreviations used: AIS: Acute Ischemic Stroke, SK: Streptokinase, MC: Multi-Centered, MU: Million Units, MI: Myocardial Infarction, DB: Double-Blind, PC: Placebo-Controlled, OR: Odds Ratio, stat. sig.: statistically significant, mRS: modified Rankin Scale
Prourokinase is a relatively inactive precursor that must be converted to urokinase before it becomes active in vivo. Because it is inactive in plasma, prourokinase does not bind to or consume circulating inhibitors. As with tissue-type plasminogen activator, prourokinase is somewhat clot specific, as the presence of fibrin enhances the conversion of prourokinase to active urokinase by an unknown mechanism. Prourokinase was used in the PROACT-I and II trials, demonstrating the efficacy of intra-arterial thrombolysis. However, it is not available in the US and as such, urokinase or tPA replaced its use in IA thrombolysis.

Tenecteplase (TNK) is a mutant version of tPA that has a longer plasma half-life, lending itself towards bolus administration and enhanced fibrin specificity, which together decrease the incidence of bleeding. A pilot study using TNK for treatment of acute stroke demonstrated its safety, with three month neurological outcomes similar to that of the NINDS tPA trials (Haley et al., 2005). The Study of Tenecteplase (TNK) in Acute Ischemic Stroke (TNK-S2B) Phase II trial is currently ongoing and is evaluating the administration of three different doses of TNK within 24 hours after symptom onset.

Neuroprotectants

Neuroprotective agents have historically demonstrated positive results in preclinical testing, but have failed to translate to benefits in clinical trials. This may be due to the complex physiology of humans, the metabolism of the drugs themselves, or preclinical testing design. In efforts to diminish the disparity between positive animal studies and failed clinical trials, the Stroke Therapy Academic Industry Roundtable (STAIR) committee published their recommendations as to how therapies for AIS may be advanced from basic science research to preclinical testing, thus becoming positioned for success in clinical trials. These suggestions include the generation of dose-response curves, demonstration of a reasonable window of opportunity for successful
administration of the drug, preclinical testing first in rodents and then larger animals (e.g., cats and primates) in a randomized and blinded manner (preferably in more than one laboratory), monitoring of physiologic parameters throughout the surgery, and measurement of at least two outcomes which should include functional response and infarct volume (evaluated on a short-term and long-term time scale) (1999).

In light of these recommendations, several clinical trials evaluating novel neuroprotectants are currently ongoing; these include the phase III FAST-MAG trial investigating administration of magnesium sulfate within 1-2 hours following symptom onset, the phase III ALIAS trial where albumin is administered with and without tPA, and a phase II trial using ONO 2506, a compound that inhibits astrocyte activation. Trials using caffeinol, a mixture of caffeine and ethanol, are under evaluation in combination with hypothermia, with and without tPA. However, the most promising neuroprotectant agent, NXY-059, was recently shown in an pooled analysis of the two SAINT trials to be ineffective in improving outcome following administration up to 6 hours after initial symptom onset (Diener et al., 2008).

**Risk factors for developing sICH following IV tPA**

The primary concern of thrombolysis is hemorrhagic transformation of the ischemic infarct. In an attempt to avoid hemorrhage, several risk factors have been identified, which include: 1) the severity of the neurological deficit, especially National Institutes of Health Stroke Scale (NIHSS) scores ≥20 (P=0.003) (1997; Cocho et al., 2006); 2) the incidence of protocol violations (Katzan et al., 2000; Lopez-Yunez et al., 2001); 3) elevated glucose levels/diabetes (Demchuk et al., 1999; Barber et al., 2000; Tanne et al., 2002; Hill et al., 2005); 4) the extent of hypoattenuated brain parenchyma on pretreatment CT (1997; Larrue et al., 2001; Tanne et al., 2002; Demchuk et al., 2005); 5) increased fibrinogen degradation products (Trouillas et al., 2004); 6) clot burden scores
measuring the extent of the occluding thrombus (Puetz et al., 2008); and 7) poor pial collateral flow as identified by angiography (Christoforidis et al., 2008).

The concept of old age as a risk factor for hemorrhagic transformation following thrombolysis is currently a topic of debate, as some studies have shown a correlation between advanced age and progression to hemorrhagic transformation following thrombolysis (Larrue et al., 2001; Heuschmann et al., 2004), while other studies have noted that there is not a significant correlation between advanced age and progression to hemorrhagic transformation following thrombolysis, and as such, advanced age of a patient should not preclude thrombolytic treatment (Pundik et al., 2008).

Although these risk factors (and obvious contraindications to thrombolysis as outlined by the NINDS protocol) are used to provide as much information as possible that may direct treatment choices, differences in patient pathophysiology may lend itself towards hemorrhagic transformation, as evidenced by a transformation rate of approximately 9% (Paciaroni et al., 2008). As a result, there remains a medical need for safer treatments of acute ischemic stroke.

1.2.4 Current treatments of intracranial hemorrhage

Intracranial hemorrhage may arise as a result of hemorrhagic transformation of an ischemic infarct, particularly after thrombolytic therapy, or may exist as the primary insult. If ICH is suspected during thrombolytic therapy (as evidenced by headache, an acute deterioration of neurological status or reduced level of consciousness), the thrombolytic infusion is first stopped, followed by immediate non-contrast CT to verify the presence of hemorrhage (Khaja et al., 2007). If confirmed, supportive medical treatment for stabilization and reduction of further injury is commenced. This includes management of the patient’s airway and circulation, as well as maintenance of thermoregulation, normalization of glucose levels, and seizure prophylaxis. In addition,
fresh frozen plasma, cryoprecipitate, and/or platelets are administered to acutely elevate clotting factors and reduce bleeding; also, vitamin K or protamine may be used to reverse any prior anticoagulant therapy. However, if the hematoma has already reached a critical volume and the patient is rapidly deteriorating, surgical evacuation of the hematoma should be conducted such that complications of a mass effect and direct toxicity of the blood on the surrounding tissue may be avoided (Mendelow et al., 2005).

Administration of individual coagulation factors such as recombinant Factor VIIa (rFVIIa) was deemed a viable treatment for intracranial hemorrhage, as results from a Phase IIb clinical trial demonstrated a reduction in hematoma size, with improvements in neurologic function and mortality when rFVIIa was given within 4 hours of symptom onset (Mayer et al., 2005). However, the larger Phase III clinical trial to generate further safety and efficacy data did not demonstrate benefits in mortality or neurological outcome following rFVIIa administration for sICH, though hematoma size was decreased (Mayer et al., 2008). In addition, use of rFVIIa is associated with an increased incidence of thromboembolism, which acutely increases morbidity and mortality (O’Connell et al., 2006; Diringer et al., 2008).

### 1.3 Aptamers and SELEX

#### 1.3.1 Properties of aptamers as inhibitors

Aptamers, from the Latin ‘aptus’ meaning ‘to fit’ and the Greek ‘meros’ meaning ‘part or region’ (Ellington et al., 1990), are single stranded nucleic acid ligands that fold into three-dimensional structures and bind to their protein target with high affinity and specificity. The concept of generating nucleic acids that bind to and subsequently inhibit the function of virtually any protein was purported simultaneously by both Tuerk and Gold, and Ellington and Szostak in 1990 (Ellington et al., 1990; Tuerk et al., 1990). This
The process of affinity purification to produce aptamers is known as SELEX (Systematic Evolution of Ligands by EXponential enrichment). It begins with a library of approximately $10^{14}$ different nucleic acid molecules that are incubated with a protein of interest. The ligands that do not bind to the protein are then partitioned from those that do bind; these molecules are then amplified through PCR (if using DNA) or RT-PCR (if using RNA). The procedure of binding, purification and amplification is known as one “round”. These rounds are then repeated (typically 8-12 times), usually with increasing stringency, until a high affinity pool is generated. “High affinity” is defined as binding to a target with a dissociation constant ($K_d$) in the low nanomolar ($1 \times 10^{-9}$ M) to high picomolar ($1 \times 10^{-12}$ M) range. These pools are then cloned and sequenced to generate individual aptamers (see figure 1-5, (Nimjee et al., 2005)).
Figure 1-5: Schematic of the SELEX process (from Nimjee et al., 2005).
1.3.2 Aptamers as therapeutic compounds

Aptamers are an attractive class of therapeutic molecules for several reasons. First, they demonstrate high affinity binding that rivals that of monoclonal antibodies without initiating an immune response (White et al., 2000; Wlotzka et al., 2002). In addition, given their small size and chemical manufacturing, aptamers exemplify an ease of synthesis not enjoyed by monoclonal antibodies, which must be raised intracellulary. Thirdly, aptamers can undergo chemical modification to tailor their bioavailability for practical use in vivo. The bioavailability of a drug is determined by both its plasma stability and rate of clearance from the body. Because the half-life of RNA aptamers is usually on the order of a few seconds (White et al., 2000), they must be rendered nuclease resistant in order to be used in vivo. This is accomplished through substitution of ribonucleotides for those with 2'-fluoro modifications, which greatly extends the half-life to that of hours (Pieken et al., 1991). Additionally, aptamers are renally cleared within minutes due to their small size; to compliment the enhanced bioavailability due to nuclease resistance, various moieties such as cholesterol and polyethylene glycol (PEG) groups may be annealed to the aptamer to decrease its rate of clearance. For example, conjugation to PEG increased the circulating half life of the ARC83 aptamer to nearly 12 hours (Healy et al., 2004). Finally, aptamers are ideal for therapeutic use because antidotes can be rationally designed that mitigate the function performed by the aptamer (Rusconi et al., 2004). These aptamer-antidote pairs represent a very safe modality for drug delivery.

In 1990, an aptamer against bacteriophage T4 DNA polymerase was generated, representing the first chemically selected ligand of this emerging class of molecules (Tuerk et al., 1990). Since then, there have been aptamers raised against targets that range from reverse transcriptases, to cell adhesion molecules to various growth factors.
Within the Sullenger laboratory, aptamers have been successfully raised against coagulation factors Factor VIIa (Rusconi et al., 2000), IXa (Rusconi et al., 2002), Xa [Layzer et al., in preparation] and thrombin (White et al., 2001), as well as VWF (Oney et al., 2007). In addition to numerous aptamers in pre-clinical studies, there are currently two aptamers in Phase II clinical trials—Antisoma’s AS1411 which binds to nucleolin and shows promise for treatment of advanced cancers (Ng et al., 2006) and Reg1, the anti-factor IXa aptamer/antidote pair developed in the Sullenger laboratory, now undergoing further development by Regado Biosciences. Most encouraging was the 2004 FDA approval of pegaptanib (Macugen), an anti-VEGF aptamer used for treatment of age related macular degeneration (Gragoudas et al., 2004). As the first aptamer to be successfully developed as a therapeutic agent in humans, pegaptanib set the precedent for future widespread use of nucleic acid therapies.

1.3.3 Antidote control of aptamers

Aptamer reversal is accomplished through the use of rationally designed antisense oligonucleotides that specifically inhibit the activity of the aptamer for which it was created. Antisense oligonucleotides, in particular antisense RNA, can be defined as any RNA that interferes with the activity of another RNA (Eguchi et al., 1991). Eguchi et al. first described the concept of an RNA disrupting the activity of another RNA in 1991; they observed that replication of the E. coli plasmid ColE1 was arrested when a small stretch of RNA bound to the plasmid’s precursor RNA. This precursor RNA was required to form a unique structure in order to function and thus allow for replication; with the small RNA bound, it was no longer able to form this structure and thus, its function was inhibited (Eguchi et al., 1991). From this concept, Rusconi et al. hypothesized that oligonucleotides complimentary to RNA aptamers could function in a similar fashion and could thus inhibit the activity of the aptamer (Rusconi et al., 2002).
The Sullenger laboratory demonstrated this successfully with respect to inhibition of factor IXa and VWF; binding of the rationally designed complimentary oligonucleotide to the aptamer caused a conformational change that rendered it inactive and thus reversed its inhibitory activity (see figure 1-6, (from Nimjee et al., 2005)) (Rusconi et al., 2002; Oney et al., 2007). Furthermore, the Sullenger laboratory has translated the concept of using an aptamer-antidote pair *in vivo* through successful demonstration of factor IXa inhibition and reversal in porcine systemic anticoagulation models (Rusconi et al., 2004), murine arterial thrombosis and tail transaction models (Rusconi et al., 2004), and porcine coronary artery bypass models (Nimjee et al., 2006).
Figure 1-6: Antidote control of aptamer activity. From (Nimjee et al., 2005).
1.4 PAI-1

1.4.1 General information

Plasminogen Activator Inhibitor-1 (PAI-1) is a single-chain glycoprotein of 45,000 Da, comprised of 379 amino acids. It is primarily produced by the liver, vascular endothelium, and adipose tissue, and is also released from activated platelets, though this is not a major contributor of the 2 µM plasma level under normal conditions. PAI-1 is an acute phase response gene; as such, gene expression in the liver is upregulated by endotoxin and inflammatory mediators, increasing the circulatory level during surgery/trauma, sepsis, stress, and inflammatory disease (Dellas et al., 2005). In addition, a 4G/5G polymorphism in the promoter region of the gene has an affect on plasma levels as the 4G allele lacks an additional binding site for the transcriptional repressor that is present in the 5G; this explains why the homozygous 4G genotype is associated with 25% greater plasma PAI-1 levels than homozygous 5G (Dawson et al., 1991; Eriksson et al., 1995).

PAI-1 is a member of the serine protease inhibitor (serpin) superfamily, and is formally denoted as SERPINE1. Serpins share several structural similarities that are common to this class of proteins; all contain nine α-helices and three β-sheets with a reactive center loop (RCL) that contains a P1-P1′ mobile bond that acts as bait for its target protease. The protease forms a non-covalent Michaelis-like complex with the P1-P1′ bond in the RCL, then attacks and cleaves the P1-P1′ bond, leading to a covalent ester linkage between Ser-195 of the protease and the backbone carbonyl of the P1 residue. The RCL then inserts into β-sheet A, transporting the covalently bound protease with it. When this occurs, the protease is translocated over 70 Angstroms, distorting its active site; thus both serpin and protease are inactivated.
PAI-1 exists in several structural conformations; first, the active state is characterized by an exposed RCL that is available to bind its target protease. If PAI-1 is not stabilized by vitronectin (VN), an extracellular matrix glycoprotein, within 1-2 hours, it converts to a more energetically favorable, yet inactive, latent form through insertion of its RCL into β-sheet A. This conformation is unable to bind to tPA/uPA or VN. Though irreversible in vivo, the latent conformation can undergo reconversion to its active form in vitro via denaturation with guanidium chloride or interaction with negatively charged phospholipids (Lambers et al., 1987). In addition to the active and latent conformations, a rare non-inhibitory substrate form of PAI-1 also exists; this arises when the target protease cleaves PAI-1, without undergoing inactivation itself.

1.4.2 Significant PAI-1 interactions

uPA/tPA

PAI-1 is the most important physiological inhibitor of tPA and uPA. Because the presence and activity of uPA is primarily limited to the vascular wall in tissues, uPA is most important in generating plasmin for extracellular matrix (ECM) turnover (Horrevoets, 2004). Therefore, PAI-1 inhibition of uPA affects not only ECM degradation, but also binding interactions between cells and the underlying ECM. These interactions have a direct effect on cell migration, which influences angiogenesis, cancer, and atherosclerosis. tPA is present in the circulation and is most important for generation of plasmin for fibrinolysis (Durand et al., 2004). PAI-1’s interaction with tPA therefore leads to a decrease in plasmin generation and as a result, reduced fibrinolysis.
**Heparin**

Unaided, PAI-1 exhibits a low level of thrombin inhibition, which is increased by greater than two orders of magnitude in the presence of heparin as a co-factor (Rezaie, 1999). Heparin enhances PAI-1 inhibition of thrombin via a template mechanism, that is, heparin binds to exosite 2 of thrombin, and also to PAI-1 (Rezaie, 1999). In addition, heparin binding to PAI-1 changes the conformation of the binding site for thrombin and enhances the reactivity of PAI-1 for thrombin (Ehrlich et al., 1992). This PAI-1/heparin complex thus competes with thrombomodulin for binding to thrombin, acting as a local anticoagulant. However, the physiologic importance of this reaction has not yet been fully elucidated, as the inhibition of thrombin by PAI-1 pales in comparison to heparin-mediated thrombin inhibition by antithrombin III.

**Vitronectin**

In a similar manner to heparin, PAI-1 binding to vitronectin also lowers the dissociation constant for the initial interaction between PAI-1 and thrombin, enhancing PAI-1 inhibition of thrombin by two orders of magnitude (Naski et al., 1993). However, there are two additional interactions between vitronectin and PAI-1 that are of great physiological importance; vitronectin’s stabilization of PAI-1 in an active conformation, and PAI-1’s competition with the uPA receptor (uPAR) for binding to vitronectin.

The somatomedin B domain of vitronectin binds across PAI-1’s E and F helices in a 1:1 stoichiometric complex with a $K_d$ of ~1 nM (Zhou et al., 2003). This binding slows the transition to the latent form by blocking the associated sliding movement of strands 1 and 2 of the main $\beta$-sheet into the gap between helices E and F. In this manner, PAI-1 is stabilized in an active conformation. Vitronectin also enhances PAI-1’s specificity for
fibrin binding; this prevents premature fibrinolysis (therefore averting bleeding), but also leads to resistance of thrombolysis.

As stated above, PAI-1 usually binds to vitronectin in a 1:1 complex when PAI-1 is at normal levels. However, when PAI-1 levels are pathologically high (as is the case in many cancers and during acute inflammation), an additional lower-affinity (~25-50 nM as opposed to ~1 nM) PAI-1 binding site on vitronectin becomes occupied. In this case, PAI-1 forms a 2:1 complex, and may form 4:2 complexes (and higher orders); this complex then becomes targeted to the ECM in diseased tissues where it has an effect on tumor growth and angiogenesis (Minor et al., 2005; Schar et al., 2008).

An additional interaction of PAI-1 and vitronectin involves competition binding; the uPA receptor binds to the somatomedin B domain of extracellular matrix-bound vitronectin in order to facilitate cellular adhesion. However, because PAI-1 also binds to vitronectin at the somatomedin B domain, PAI-1 competes with uPAR for vitronectin binding. Therefore, when high concentrations of PAI-1 are present, cellular adhesion to the extracellular matrix via vitronectin is decreased, mediating cellular detachment.

1.4.3 Pathophysiological roles of PAI-1

PAI-1 plays a pathophysiological role in several diseases characterized by resistance to thrombolysis due to inhibition of tPA (e.g., myocardial infarction) and degradation of the extracellular matrix and modulation of cell migration, mediated by its interactions with the uPA system (e.g., fibrosis, cancer).

Resistance to thrombolysis

PAI-1 is released in an active form from platelets (Brogren et al., 2004), thus increasing resistance to thrombolysis via its inhibition of tPA. This may explain why elevated PAI-1 levels are associated with an increased risk of thrombosis (Dellas et al.,
Low fibrinolytic activity is associated with the development of coronary artery disease, and elevated PAI-1 may serve as a biomarker for recurrent MI (Hamsten et al., 1987). In addition, the circadian rhythm of PAI-1 concentration may partially explain why more myocardial infarctions and strokes occur during the morning (Muller et al., 1989; Zhang et al., 2008). Because of this, inhibition of the PAI-1/tPA interaction would be applicable to several thrombotic disorders in addition to myocardial infarction, namely acute ischemic stroke, pulmonary embolism, venous thrombosis, arterial thrombosis and hepatic veno-occlusive disease.

Degradation of the extracellular matrix/modulation of cell migration

Degradation of the extracellular matrix is important in tissue and vascular remodeling. In addition, elevated PAI-1 levels have been associated with glomerulosclerosis, tubulointerstitial fibrosis, and inflammatory lung disease. Each of these diseases is characterized by pathological increases in fibrin deposition due to low local levels of plasmin, which is mediated by PAI-1 inhibition of uPA (Vaughan et al., 2007). Plasmin degradation of the pericellular extracellular matrix intuitively lends itself towards enhanced cellular metastasis, as elevated uPA is associated with a worsened outcome for several cancers (Janicke et al., 2001). It would therefore logically follow that elevated levels of PAI-1 would be beneficial through its inhibition of uPA, thus decreasing plasmin levels. However, this is not the case, as elevated PAI-1 levels have been associated with a poor prognosis for several cancers, particularly breast cancer, pulmonary adenocarcinoma, ovarian cancer, and urinary tract cancer (Dellas et al., 2005). This seeming contradiction is known as the PAI-1 paradox.

PAI-1 increases cancer cell metastasis through primarily two mechanisms--reduction of cell adhesion and mediation of cellular detachment. As mentioned above, PAI-1 competes with uPAR for binding to vitronectin. In addition, the RGD sequence on
vitronectin, required for integrin adhesion, is located near the somatomedin B domain; PAI-1 binding to vitronectin precludes this binding as well, inhibiting cellular attachment (Czekay et al., 2003). Cellular detachment, however, occurs via a VN-independent mechanism. Urokinase-type plasminogen activator bound to uPAR receptors on the cellular surface mediates binding to matrix-bound integrins, forming cellular attachments. However, PAI-1 can bind to uPA, leading to the formation of a PAI-1/uPA/uPAR/integrin complex that is rapidly internalized. When this complex undergoes internalization, the bond to the extracellular matrix is broken, facilitating cellular detachment (Dellas et al., 2005). Together, these interactions lead to cancer cell metastasis. Therefore, agents that disrupt PAI-1 mediated ECM accumulation or cellular detachment may have applications in these diseases. In particular, an agent that inhibits PAI-1’s interaction with vitronectin without disrupting its protease activity would act as an ideal antimetastatic agent, allowing for maintenance of ECM integrity while concomitantly maintaining cellular attachment.
2. Isolation, Characterization and Mapping of Nucleic Acid Aptamers to Plasminogen Activator Inhibitor-1

2.1 Introduction

Stroke is the leading cause of disability and the third leading cause of death in the United States. Over 80% of strokes result from a thrombus in the cerebral circulation which prevents blood from flowing freely to the brain (Bamford et al., 1990). When possible, this thrombus is dissolved through use of a thrombolytic agent, a process called thrombolysis. For thrombolysis to occur, plasmin must degrade the fibrin meshwork that gives structure to the thrombus. However, plasmin generation requires cleavage of its zymogen, plasminogen, by tissue-type plasminogen activator (tPA). Plasminogen activator inhibitor-1 (PAI-1) inhibits this reaction via direct binding to tPA at its reactive center loop (RCL), which causes inactivation of both tPA and PAI-1. Therefore, an increase in PAI-1 leads to a decrease in plasmin levels and thus resistance to thrombolysis. One method of inducing thrombolysis is to exogenously increase the level of tPA present, thus overcoming the resistance rendered by PAI-1. This is the method used by Alteplase (recombinant tissue-type plasminogen activator, rtPA), the only drug FDA approved for lysis of stroke inducing thrombi. Unfortunately thrombolytic therapy is often incomplete and it is not uncommon for treatment to result in hemorrhage. In fact, 70% of patients receiving intravenous thrombolysis for acute ischemic stroke will not achieve or will attain only partial recanalization (Alexandrov et al., 2002). In addition, depending on the type of medical center in which treatment was rendered, up to 15% of patients receiving medical thrombolysis for stroke suffer from symptomatic intracranial hemorrhage (sICH) (Katzan et al., 2000). Finally, about half of all patients that survive the acute stroke event retain some level of disability after six months (Wade et al., 1987).
To decrease the morbidity and mortality that result from stroke and its current therapy, improved treatments must be developed. One response to this initiative is the development of an effective thrombolytic that is paired with its specific reversal agent. This drug-antidote pair would serve to enhance perfusion to the brain while possessing additional safety measures so that should unforeseen circumstances arise, a non-immunogenic, non-toxic compound may be administered to reverse the activity of the thrombolytic agent.

Plasminogen activator inhibitor-1 is the principal negative regulator of tPA and uPA in the fibrinolytic system. It is a 45 kDa single chain glycoprotein belonging to the serine protease inhibitor (serpin) superfamily. PAI-1 consists of 379 amino acids and lacks cysteines; this may account for the biological instability of its active form in that it rapidly converts to an inactive “latent” form if not bound to vitronectin (Declerck et al., 1988). In its active form, PAI-1 binds to tPA and uPA, forming an irreversible, covalent 1:1 stoichiometric complex through its reactive center loop. This complex permanently inhibits plasminogen activator function and results in the cleavage of PAI-1, inactivating it as well (Wilczynska et al., 1995). It is for this reason that PAI-1 is known as a ‘suicide inhibitor’.

PAI-1 is produced in a variety of cells, most notably endothelial cells, smooth muscle cells, macrophages, platelets, and adipocytes. In addition to hemostasis, PAI-1 also plays a role in atherosclerosis, wound healing, insulin resistance syndrome, tumor angiogenesis, and other diseases involved with fibrin deposition (Lijnen, 2004). Of particular interest is PAI-1’s rapid and constitutive secretion after production in all cells except platelets, where it is stored in α-granules. Once these platelets are activated, as is the case in thrombus formation, a large amount of active PAI-1 is released from α-granules (Brogren et al., 2004). This high local concentration of active PAI-1 renders the
clot more resistant to thrombolysis and explains why arterial thrombi are more difficult to lyse than their less platelet dense venous counterparts (Potter van Loon et al., 1992).

For this reason, elevated PAI-1 levels are correlated with thromboembolic disorders (Huber, 2001), thrombolysis failure (Nicholls et al., 2001; El Menyar et al., 2006), and increased risk of primary and recurrent myocardial infarction (Hamsten et al., 1985; Hamsten et al., 1987; Thogersen et al., 1998) making PAI-1 inhibitors an attractive option for use as antithrombotic or thrombolytic agents.

It has been previously demonstrated that inhibition of PAI-1 can improve clot lysis in vitro (Eitzman et al., 1995; Urano et al., 2000; Chavakis et al., 2002; Liang et al., 2005; Rupin et al., 2008) and can enhance thrombolysis while inhibiting thrombus growth in rabbit jugular vein occlusion models (Biemond et al., 1995; Friederich et al., 1997). In rodent and canine models of arterial thrombosis, PAI-1 inhibitors decreased time to clot lysis, facilitated reperfusion, reduced thrombus weight and decreased vessel reocclusion (Biemond et al., 1995; Berry et al., 1998; Hennan et al., 2005; Vaughan et al., 2007; Baxi et al., 2008; Hennan et al., 2008; Suzuki et al., 2008). The type of PAI-1 inhibitor used ranges from monoclonal antibodies to small molecules to peptides, and although they have demonstrated their ability to functionally inhibit PAI-1, they all have serious short comings that impede their clinical development. None of these molecules are reversible, and at high enough doses may cause severe bleeding, undermining patient safety. In addition, antibodies are immunogenic and their bioavailability is difficult to modify, which can further complicate their use as thrombolytic agents.

For these reasons, I exploited the properties of nucleic acid ligands termed aptamers. Aptamers are relatively small (~8-15 kDa) single stranded nucleic acid ligands that bind to their targets with high affinity and specificity. In addition, they are non-toxic, have low to no immunogenicity, can be synthetically manufactured, and have
adjustable bioavailability (EytechStudyGroup, 2002; Nimjee et al., 2005). Therefore, aptamers possess the affinity properties of monoclonal antibodies, while retaining the ease of synthesis of small molecules, making aptamers ideal agents for disrupting protein-protein interactions. I hypothesized that RNA aptamers that target PAI-1 could disrupt the interaction between PAI-1 and its target proteases, uPA and tPA, which may translate to a clinical application in enhancing thrombolysis. To test this hypothesis, I employed combinatorial chemistry to develop aptamers that bind to PAI-1 with high affinity ($K_a < 4$ nM) using SELEX (systematic evolution of ligands by exponential enrichment). Through functional assays including mutagenesis studies, competition binding and chromogenic assays, I determined that aptamers WT-15 and SM-20 do not bind in a region that would disrupt the PAI-1/uPA interaction, for example, the reactive center loop. Because of this, these aptamers would not be effective as thrombolytic agents. However, further investigation revealed that the aptamers bind to PAI-1 in a region that spans the heparin- and vitronectin-binding domains, which could have functional implications in disease processes such as breast cancer metastasis and angiogenesis.
2.2 Materials and Methods

Generation of Aptamers using Systematic Evolution of Ligands by EXponential enrichment (SELEX)

The sequence of the starting RNA library (denoted Sel2 Library) was 5’-GGGAGGACGATGC-GG-N_{40}-CAGACGACTC-CCGATCC-3’, where “N_{40}” denotes a random region 40-nucleotides in length. The RNA consisted of 2’-fluopyrimidines (2’-fluorocytidine triphosphate and 2’-flourouridine triphosphate; Trilink Biotechnologies, San Diego, CA) in order to render the RNA nuclease resistant. Two separate selections against PAI-1 were performed; one selection used human wild-type PAI-1 (hWT PAI-1) protein, while the other used human stable mutant PAI-1 (hSM PAI-1), which contains four amino acid modifications (K154T, Q319L, M354I and N150H) that hold PAI-1 in an active conformation (both kindly supplied by Dr. David Ginsburg, University of Michigan Medical Center) (Berkenpas et al., 1995). For both selections, the RNA library was incubated with its respective protein for 15 minutes at 37°C; unbound RNA was separated from RNA/PAI-1 complexes by passing the mixture over a nitrocellulose membrane. The selection against WT PAI-1 began in BSA binding buffer E (20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and 0.1% bovine serum albumin [BSA]) and continued in this buffer for five rounds. Rounds 6c and 7c were performed in CHAPS binding buffer E (20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and 0.05% CHAPS), and rounds 8c and 9c were performed in CHAPS binding buffer F (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.05% CHAPS). The selection against hSM PAI-1 began in BSA binding buffer E and continued in this buffer for five rounds. Rounds 6c and 7c were performed in CHAPS binding buffer E/F (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM CaCl₂, and 0.05% CHAPS), and rounds 8c and 9c were performed in CHAPS binding buffer F.
Round and clone binding assays.

Affinity constants ($K_d$ values) were determined using nitrocellulose filter binding assays as previously described (Rusconi et al., 2000). Briefly, RNA was first dephosphorylated using bacterial alkaline phosphatase (Gibco BRL, Gaithersburg, MD) and 5’ end labeled with [$\gamma$-$^{32}$P] ATP (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Round pools of RNA were incubated with increasing amounts of their respective PAI-1 protein in BSA binding buffer E, CHAPS binding buffer E, CHAPS binding buffer E/F, or CHAPS binding buffer F, depending on the initial round conditions. Following cloning to yield individual aptamer sequences, all binding was performed in BSA binding buffer F (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl$_2$, and 0.1% bovine serum albumin [BSA]). Radiolabeled RNA was incubated with PAI-1 protein for five minutes at 37°C; the mixture was then passed over a nitrocellulose membrane. The fraction of RNA that bound to the protein was quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Nonspecific binding of the RNA was subtracted out such that only specific binding remained (denoted ‘corrected fraction bound’).

Competition binding assay

To determine if heparin and vitronectin were able to compete with aptamers WT-1, WT-15 and SM-20 for binding to PAI-1, the aptamers were first dephosphorylated and radiolabeled as described above. Briefly, a constant amount of PAI-1 (378 pM for WT-1, 888 pM for WT-15, and 3.4 nM for SM-20 when competing with heparin; 888 pM for WT-15 and 2.2 nM for SM-20 when competing with vitronectin) was incubated with increasing amounts of heparin or vitronectin at 37°C for 5 minutes. The PAI-1/competitor solution was then combined with a trace amount of radiolabeled aptamer
and incubated at 37°C for 5 minutes before passing the mixture over a nitrocellulose membrane. The fraction of RNA bound to the protein was then quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Direct uPA binding assay

Increasing amounts of round nine pools of aptamers selected against wild type PAI-1 (R9chWT) and stable mutant PAI-1 (R9chSM) were heated to 65°C for 5 minutes and allowed to cool to room temperature. Next, increasing amounts of an inhibitory mouse antihuman monoclonal antibody against PAI-1 (MA-33B8; Molecular Innovations, Novi, MI), IgGk1 isotype control mouse antibody (eBioscience, Inc., San Diego, CA) or the cooled aptamer pools were incubated with 50 µL PAI-1 at 10 µg/mL for 5 minutes at 37°C. The mixture was then added to 50 µL uPA at 500 ng/mL and incubated at room temperature for 30 minutes. Following incubation, the solution was transferred to a 96-well microplate and 100 µL of S-2444 at 3 mM (the chromogenic reagent specific for uPA activity; DiaPharma, West Chester, OH) was added. The plate was then immediately placed into a microplate reader (Bio-Tek EL311, BioTek Instruments, Inc., Winooski, VT) and set for kinetic readings every 35 seconds for 15 minutes at an absorbance of 405 nm. All RNAs, antibodies and proteins were diluted in uPA assay buffer (150 mM NaCl, 50 mM Tris pH 7.5, 100 µg/mL BSA).

PAI-1 complex formation

PAI-1 forms a high molecular weight, SDS-stable complex with tPA, uPA, and thrombin/heparin (Ehrlich et al., 1991; Perron et al., 2003) that can be visualized by SDS PAGE. For that reason, we used SDS-PAGE analysis to evaluate the influence of the aptamers on the products generated during the interaction of PAI-1 with its target
proteases. The aptamers (500-1000 nM) were incubated with PAI-1 (500 nM; Molecular Innovations, Novi, MI) at 37°C for 10 minutes in HNPN buffer (20 mM Hepes, 150 mM NaCl, 0.01% PEG, 0.005% sodium azide) containing CaCl$_2$ (2 nM), prior to the addition of proteases (tPA [Molecular Innovations, Novi, MI] or thrombin [Haematologic Technologies, Inc., Essex Junction, VT]). The aptamers were first heated to 65°C for 5 minutes before use. PAI-1/aptamer was then incubated with tPA (400 nM) for 15 minutes or with thrombin/heparin (200 nM thrombin and 10 mg/ml heparin) for 60 minutes at 37°C. The reactions were stopped through the addition of Laemmli buffer containing β-mercaptoethanol and were boiled for 5 minutes. Finally, the reaction products were analyzed by SDS PAGE followed by Coomassie staining to visualize PAI-1, thrombin and tPA bands, as well as PAI-1/tPA and PAI-1/thrombin complexes.
2.3 Results

2.3.1 SELEX yielded aptamers that bind to PAI-1 with high affinity

In order to generate aptamers that bound to the active form of PAI-1 with high affinity, SELEX was performed against two versions of human PAI-1. The native wild-type form of PAI-1 was used (hWT PAI-1), as well as a stable mutant version (hSM PAI-1) that contains the amino acid changes K154T, Q319L, M354I and N150H that stabilize the otherwise labile protein in an active conformation (Berkenpas et al., 1995). First, a starting RNA library modified with 2'fluoropyrimidines was bound with each protein; the RNA that bound to PAI-1 was separated from unbound RNA using a nitrocellulose membrane as described in Materials and Methods. By increasing the stringency in subsequent rounds, the binding affinity of each round increased (see Fig. 2-1 and Fig. 2-2).

After nine rounds of SELEX, the affinity of the rounds reached low nanomolar levels. The round nine libraries (R9c) were then cloned and sequenced to generate individual aptamer sequences, as shown in Table 1. The selection against wild-type (WT) PAI-1 produced only three aptamers, one of which, WT-1, dominated the pool (see table 2-1). The SM selection, however, yielded a family of aptamers with similar sequences and several additional aptamers. WT aptamers (WT-1, WT-15 and WT-18), and selected aptamers from the SM selection, were then chosen to undergo further characterization. As seen in figures 2-3a and 2-3b, aptamers generated from both the WT and SM selections bound to WT PAI-1 with high affinity, as evidenced by their low nanomolar/high picomolar affinity constant ($K_d$) values (see table 2-1).
Table 2-1: Aptamers selected against PAI-1

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Variable region sequence</th>
<th>Frequency (%)</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Selected against WT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R9chWT-1</td>
<td>5’-GTCCCTAGCAGACACTCGGCCATCACGCCCATTGGTTTGGCA-3’</td>
<td>89</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>R9chWT-15</td>
<td>5’-ATCAACTCACGTTAGTCTAGTGAGAAGTCTACCCAGTACT-3’</td>
<td>5</td>
<td>177 pM</td>
</tr>
<tr>
<td>R9chWT-18</td>
<td>5’-ACTCCAAGTCCCACGGGACAGGAGTCTCCGGACCAGT-3’</td>
<td>5</td>
<td>35 nM</td>
</tr>
<tr>
<td><strong>Selected against SM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R9chSM-20</td>
<td>5’-AGCGACTGACGACTCTGTTGAGTAACCCGCCTCCAGCCACGCTAGT-3’</td>
<td>15</td>
<td>920 pM</td>
</tr>
<tr>
<td>R9chSM-14</td>
<td>5’-GTCCAGCTAAATCTCTACTGAGCCCAGTTCCCCGTAACCT-3’</td>
<td>10</td>
<td>37 nM</td>
</tr>
<tr>
<td>R9chSM-3</td>
<td>5’-GTCTCAATACGACCCCTGATCCCTGACTGAGCTTGGCTAGC-3’</td>
<td>10</td>
<td>106 nM</td>
</tr>
<tr>
<td>R9chSM-23</td>
<td>5’-ATCTTTTGCCCTCCACAATATCCCCATGGGATCCAG-3’</td>
<td>5</td>
<td>24 nM</td>
</tr>
<tr>
<td>R9chSM-2</td>
<td>5’-ATCCACTAGAAGTGGCGAGTTCCCAAGACACTTGGAGCT-3’</td>
<td>5</td>
<td>3.3 nM</td>
</tr>
<tr>
<td>R9chSM-7</td>
<td>5’-GTCAAGNACGCCGAACCGCCATTTCAAGAACGCGACACCCT-3’</td>
<td>5</td>
<td>120 nM</td>
</tr>
<tr>
<td>R9chSM-24</td>
<td>5’-TATCAACTAAGCTACGCCGATTTCCCCACAAACAGACTGCTCC-3’</td>
<td>5</td>
<td>60 nM</td>
</tr>
<tr>
<td>R9chSM-6</td>
<td>5’-NTCAATACGCACGCTGATTCGCGGAAACCGGACCT-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-16</td>
<td>5’-NTCAATACCGGAAAGGGAGTAGCCCTGACCT-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-8</td>
<td>5’-ATCAGGTCTAGCTAGCATTCCCAGGCTCCAGGAGCTC-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-5</td>
<td>5’-GTCAAGCTCACTACGCCGTAGTCCAGAGCTTCCAGCTTACCTCCAGGACACT-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-9</td>
<td>5’-GTCAGCTGAGCTGATCCGGGAAACCGGAGAGGGTCTCCAGGTCATCT-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-17</td>
<td>5’-GTCAGGATACGCACCCTGGGACTTCCACATTCCAGGACTTCCAGGATC-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-12</td>
<td>5’-GTCAGGCTCGGAGTTCCAGCTCAGGAGGAGTCCCTCCAGGACCTTCCAGGACTTCCAGGATC-3’</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>R9chSM-18</td>
<td>5’-GTCATATACGCACCCTGAGTGATCTCCAGGACCTTCCAGGACTTCCAGGATC-3’</td>
<td>5</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND- Not Determined
Figure 2-1: Successive rounds against hWT PAI-1 increase in affinity. The progress of the selection against the wild type (WT) version of PAI-1 was followed using a nitrocellulose filter binding assay. Inverted triangles (▼) represent the starting RNA library (Sel2). Diamonds (♦) represent round 3, circles (●) represent round 5, triangles (▲) represent round 7, and squares (■) represent round 9. The x-axis shows the human wild-type PAI-1 concentration and the y-axis shows the fraction of RNA bound to the protein.
Figure 2-2: Successive rounds against hSM PAI-1 increase in affinity. The progress of the selection against the stable mutant (SM) version of PAI-1 was followed using a nitrocellulose filter binding assay. Inverted triangles (▼) represent the starting RNA library (Sel2). Diamonds (♦) represent round 3, circles (○) represent round 5, triangles (▲) represent round 7, and squares (■) represent round 9. The x-axis shows the human stable mutant PAI-1 concentration and the y-axis shows the fraction of RNA bound to the protein.
Figure 2-3: Individual aptamers bind to WT PAI-1 with high affinity. a. Binding affinities of aptamers WT-1, WT-15, and WT-18 generated against WT PAI-1 were determined using a nitrocellulose filter binding assay. Squares (■) represent WT-1, triangles (▲) represent WT-15, and inverted triangles (▼) represent WT-18. Each data point was performed in triplicate; error bars represent the standard error of the mean (SEM) of the data. b. Binding affinities of aptamers SM-2, SM-20 and SM-23 generated against SM PAI-1 were determined using a nitrocellulose filter binding assay. Squares (■) represent SM-2, triangles (▲) represent SM-20, and circles (●) represent SM-23. Each data point was performed in triplicate; error bars represent the standard error of the mean (SEM) of the data.
2.3.2 Aptamers do not disrupt the PAI-1/uPA interaction

After noting that the aptamers bind to PAI-1 with high affinity, I determined the binding location of the aptamers. Because PAI-1 inhibits the proteases uPA and tPA, I first tested the aptamers in an assay that measures the ability of PAI-1 to inhibit uPA in solution. If the interaction between PAI-1 and uPA is disrupted, uPA will remain and thus react with its chromogenic reagent, as demonstrated by an increase in absorbance. As seen in figure 2-4a, an inhibitory monoclonal antibody against PAI-1 restores uPA activity by inducing the conversion of PAI-1 to its inactive, latent conformation (MA-33B8; Verhamme et al., 1999)). However, round nine pools of aptamers were unable to restore uPA activity, even when present in nearly a 40-fold excess (Fig. 2-4b), suggesting that the aptamers do not disrupt the PAI-1/uPA interaction.

This was further confirmed when the aptamers were preincubated with PAI-1, followed by incubation with tPA. This reaction was then run on a non-denaturing acrylamide gel. In this assay, aptamer inhibition of the PAI-1/tPA interaction would be noted as a decrease in the complex formed between the two proteins. However, as seen in figure 2-5, no attenuation of band intensity was noted, further suggesting that the aptamers do not disrupt the direct interaction between PAI-1 and the proteases uPA/tPA.
Figure 2-4: Aptamers do not disrupt the PAI-1/uPA interaction in a direct uPA assay. Antibodies/aptamers were preincubated with PAI-1, followed by incubation with uPA and subsequent addition of the chromogenic reagent for uPA, S-2444. a. An inhibitory mouse anti-human monoclonal antibody against PAI-1 restores uPA activity in a dose dependant manner. b. Round nine aptamer pools do not restore uPA activity following inhibition by PAI-1.
2.3.3 Clones bind near the heparin/vitronectin binding site on PAI-1

After demonstrating that the aptamers do not disrupt the interaction between PAI-1 and uPA/tPA, the interaction between PAI-1 and heparin was examined, given its similar charge distribution to RNA, as well as PAI-1 and vitronectin, because of its proximity to the heparin-binding site. This was first undertaken using competition binding experiments. In this assay, a fixed amount of PAI-1 was incubated with increasing amounts of heparin or vitronectin, and was then combined with a trace amount of RNA. Protein competition with RNA for binding to PAI-1 is seen as a decrease in the fraction of RNA bound to PAI-1. As shown in figure 2-6a, increasing amounts of heparin abolished binding of aptamer clones WT-1, WT-15, and SM-20 to PAI-1. In a like manner, competition binding was tested with vitronectin and similar results were attained with WT-15 and SM-20 (Fig. 2-6b). Therefore, heparin and vitronectin compete with aptamers WT-15 and SM-20 for binding to PAI-1 in solution, implying that these aptamers bind to PAI-1’s heparin/vitronectin binding domains.
Figure 2-6: Heparin and vitronectin compete with aptamers for binding to PAI-1 in solution. a. Increasing amounts of heparin compete with aptamers for binding to PAI-1; squares (■) represent competition with clone WT-1 (PAI-1 concentration, 378 pM), triangles (▲) represent competition with WT-15 (PAI-1 concentration, 888 pM), and circles (●) represent competition with SM-20 (PAI-1 concentration, 3.4 nM). The x-axis shows the concentration of heparin, while the y-axis shows the fraction of RNA bound to PAI-1. b. Increasing amounts of vitronectin compete with clones for binding to PAI-1; triangles (▲) represent competition with WT-15 (PAI-1 concentration, 888 pM), while circles (●) represent competition with SM-20 (PAI-1 concentration, 2.2 nM). The x-axis shows the concentration of vitronectin, while the y-axis shows the fraction of RNA bound to PAI-1.
Mutagenesis studies were also performed to further explore the binding location of the aptamers. When WT-15 and SM-20 were incubated with a version of PAI-1 that was mutated (R76E) to abolish binding to heparin (Stefansson et al., 1998), binding was greatly reduced, as seen in figure 2-7. While aptamer binding to wild-type PAI-1 demonstrated picomolar affinities with greater than 80% of the RNA bound (see Fig. 2-3a, Fig. 2-3b, and Table 2-1) the aptamers bound to the mutated PAI-1 with nanomolar to micromolar affinities, with a maximum of approximately 30% of the RNA bound to this protein. Together, these experiments suggest that the aptamers bind to a location on PAI-1 that overlaps with the heparin and vitronectin binding sites.

Figure 2-7: Aptamers demonstrate reduced binding to mutated PAI-1 that does not bind heparin. Nitrocellulose filter binding assay; squares (■) represent WT-15, while triangles (▲) represent SM-20.
2.4 Discussion

PAI-1 is a multifunctional protein that interacts with several macromolecular substrates including plasminogen activators, vitronectin and thrombin. It contains three major domains, the active site region, the vitronectin binding domain, and the lipoprotein-related protein binding domain (where heparin binds). Each of these domains have been shown to contribute to PAI-1 mediated pathogenesis of various diseases, which include thromboembolic disorders and resistance to thrombolysis, a major cause of failed treatment of acute ischemic stroke (Wu et al., 2002). Therefore, I hypothesized that aptamer inhibition of PAI-1 could produce a novel thrombolytic agent.

To investigate this hypothesis, I first used SELEX technology targeting the human wild-type (WT) and constitutively active stable mutant (SM) versions of PAI-1 to better ensure selection of an aptamer that bound the active version of PAI-1 (as opposed to the latent conformation). After nine rounds of SELEX, the RNA pools were deemed enriched given their binding affinities in the low nanomolar range (Fig. 2-1 and 2-2). These rounds were then cloned and sequenced to generate individual aptamer sequences (Table 2-1). The three aptamers that bound to WT or SM PAI-1 with the highest affinities were chosen for further characterization (WT-1, WT-15, and WT-18 from the WT selection, as well as SM-2, SM-20, and SM-23 from the SM selection; see Fig 2-3). Following these assays, the two aptamers possessing binding affinities for WT PAI-1 in the picomolar range, WT-15 and SM-20, were carried forward into all additional assays.

The first step in determining if the aptamers were able to disrupt the interaction between PAI-1 and uPA/tPA was to elucidate their binding location. When the aptamers were added to PAI-1 and uPA in a chromogenic assay used to detect uPA
activity, no restoration of uPA function was found (Fig 2-4b). This is in contrast to a
known inhibitory monoclonal antibody raised against PAI-1 that converts PAI-1 to a
latent conformation; in the same assay, this antibody restored uPA activity (Fig 2-4a). In
addition the aptamers were unable to disrupt the PAI-1/tPA high molecular weight
complex (Fig. 2-5). These observations led to the conclusion that the aptamers were not
binding to PAI-1 in a region (most likely the reactive center loop of PAI-1) that is
important for direct inhibition of its target proteases, tPA and uPA. As such, they would
not be suitable for use as fibrinolytic agents.

I then shifted focus and began to investigate the heparin-binding site of PAI-1, as
aptamers tend to bind to highly positive regions on proteins, particularly heparin
binding sites. For example, the thrombin aptamer (TOG25) binds thrombin’s heparin
binding site and has been shown to eliminate the heparin accelerated inhibition of
thrombin by various serpins (Jeter et al., 2004), and the VEGF aptamer that inhibits
angiogenesis binds to the heparin binding domain of VEGF 165 (Lee et al., 2005).
Considering this, and that PAI-1 is a heparin binding serpin, I suspected that the
aptamers would have a high probability of binding in the vicinity of this site. To test this
hypothesis, I investigated the ability of heparin to compete with aptamer binding to
PAI-1. As seen in figure 2-6a, increasing amounts of heparin prevented aptamer binding
to PAI-1, suggesting that heparin binds in an overlapping site with the aptamers.

Because PAI-1’s vitronectin binding site is located near the heparin binding site, I
hypothesized that if the aptamers bound to the heparin site, they may also have an effect
on vitronectin binding. When this assay was repeated with vitronectin as a competitor,
similar results were achieved (Fig. 2-6b); however, at higher concentrations of
vitronectin, binding somewhat increased. This was most likely due to non-specific RNA
interactions with high concentrations of vitronectin. Recent studies have provided
evidence that there are two vitronectin binding sites on PAI-1, the somatomedin B (SMB) domain and another site outside of the SMB domain (Schar et al., 2008). Consequently, another plausible explanation is that the aptamers may also bind to the second vitronectin binding site on PAI-1, although with reduced affinity. Nevertheless, collectively these data suggest that the aptamers bind to PAI-1 in a region spanning both the heparin and vitronectin binding domains (α-helices D and E, respectively). Binding studies with the PAI-1 R76E mutant (Stefansson et al., 1998) which does not bind heparin, further suggested that the aptamers bind in the heparin binding region, as aptamer binding to this mutant protein was greatly reduced (Fig 2-7).

Aptamers have been shown to be non-toxic, well tolerated molecules that cause low to no immunogenicity, can be synthetically manufactured, and have an adjustable bioavailability (EytechStudyGroup, 2002; Nimjee et al., 2005; Dyke et al., 2006; Chan et al., 2008a; Chan et al., 2008b). Through this work, I have isolated aptamers that bind to PAI-1 with high affinity. Furthermore, I characterized two of these aptamers, mapping their binding location to an area on PAI-1 that spans α-helices D and E. As a result, I have developed the first RNA aptamer that binds PAI-1 and inhibits the binding of PAI-1 to vitronectin. These aptamers are not suitable for use as thrombolytic agents as they do not bind in a region that inhibits PAI-1’s antiprotease activity. However, aptamer prevention of vitronectin binding could potentially have applications to other disease states such as breast cancer metastasis or inhibition of angiogenesis. In these situations, complete inhibition of all of PAI-1’s activities might hinder its ability to regulate fibrinolysis, which could in principle engender bleeding. Therefore, elimination of the pathological functions of PAI-1 without hindering its physiological functions in hemostasis would be beneficial in a variety of disease settings. Here I show that
aptamers WT-15 and SM-20 are able to disrupt vitronectin binding without compromising PAI-1’s other functions, such as the inhibition of plasminogen activators.
3. Functional Analysis of Nucleic Acid Aptamers to Plasminogen Activator Inhibitor-1

3.1 Introduction

In the United States, the most prevalent cancer and second most common cause of death due to cancer in women is breast cancer (Group, 2007). Once detected, staging of the cancer ensues, which determines the therapeutic course of action. In addition to surgery, several classes of therapeutic agents can be utilized, depending on the nature of the cancer. However, many of these therapies, especially chemotherapeutic agents, cause side effects that greatly diminish the patients' quality of life. Moreover, if metastases are discovered, treatment goals are no longer curative but are largely palliative in nature, almost exclusively seeking to improve the patient's comfort as much as possible. Therefore, to prevent progression to this stage of disease, a non-toxic anticancer agent that represses metastasis could serve as an adjuvant to traditional therapeutic agents, possibly reducing the dose necessary for cytotoxicity while lessening the adverse effects associated with such therapies.

The serpin plasminogen activator inhibitor-1 (PAI-1; SERPINE1) is the main physiological inhibitor of the fibrinolysis system, as PAI-1 binds to and inhibits tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). This results in inhibition of the conversion of plasminogen to plasmin. Plasmin cleaves pro-matrix metalloproteases, thereby permitting the invasion and migration of tumor cells through the MMP-mediated proteolytic breakdown of the extracellular matrix proteins (for review see Dano et al., 2005; Beaulieu et al., 2007). Plasmin also directly cleaves components of the extracellular matrix. Plasminogen activation is greatly enhanced when uPA undergoes high affinity binding to the urokinase plasminogen activator receptor (uPAR). Since PAI-1 inhibits uPA activity, one would logically assume that an
increase in PAI-1 would decrease tumor invasiveness. Surprisingly, this is not the case, as increases in expression levels of PAI-1, uPA and uPAR are associated with a poor prognosis for several cancers including breast, brain, lung, bladder, liver and pancreatic cancer (Dano et al., 2005; Lah et al., 2006). Many investigators have focused on blocking the interaction of selected components of the plasminogen activator (PA) system given its role in promoting tumor cell progression (Min et al., 1996; Ploug et al., 2001; Gondi et al., 2003; Pulukuri et al., 2005; Subramanian et al., 2006). For example, direct inhibition of uPA with a low molecular weight inhibitor retards tumor cell growth and metastasis (Zhu et al., 2007). Likewise, low molecular weight inhibitors of PAI-1 have successfully suppressed cancer cell invasion and angiogenesis (Leik et al., 2006). PAI-1 has also been proven responsible for the regulation of cellular adhesion and migration, and has been shown to interact with several extracellular matrix components (Kjoller et al., 1997; Chorostowska-Wynimko et al., 2004; Dellas et al., 2005). Based on these qualities, many consider PAI-1 to be both a key regulator of tumor progression and an ideal target for cancer therapy.

In plasma, PAI-1 exists in two major forms, active and latent. While latent PAI-1 is functionally inactive, active PAI-1 effectively inhibits target proteases but is labile as it spontaneously converts to the latent form (Hekman et al., 1985; Levin et al., 1987; Lindahl et al., 1989). To prevent this conversion, active PAI-1 binds to the extracellular matrix protein, vitronectin, which is unable to bind the inactive PAI-1 or PAI-1 in complex with its target proteases (Declerck et al., 1988; Seiffert et al., 1994; Deng et al., 1996). Vitronectin also facilitates cell adhesion by binding to integrins and to surface bound uPA (Wei et al., 1996; Chapman et al., 2001; Wei et al., 2001). PAI-1 competes with integrins and uPAR for vitronectin binding, resulting in the detachment of cells from the extracellular matrix (Deng et al., 1996; Stefansson et al., 1996a; Kjoller et al., 1997).
Therefore, the binding of PAI-1 to vitronectin prevents integrins from binding to vitronectin and inhibits cell adhesion in some cells, particularly breast cancer cells (Deng et al., 1996; Stefansson et al., 1996a; Kjoller et al., 1997; Redmond et al., 2001; Palmieri et al., 2002). Moreover, the concentration of PAI-1, as well as the presence or absence of vitronectin, plasminogen activators and thrombin all appear to play a role in the PAI-1 mediated effect on cell migration.

To shift the balance of the PA system towards cell adhesion, I exploited the properties of nucleic acid ligands termed aptamers. Aptamers are relatively small (~8-15 kDa) single stranded nucleic acid ligands that bind to their targets with high affinity and specificity. In addition, they are non-toxic, have low to no immunogenicity, can be synthetically manufactured, and have adjustable bioavailability (EyetechStudyGroup, 2002; Nimjee et al., 2005). Therefore, aptamers possess the affinity properties of monoclonal antibodies, while retaining the ease of synthesis of small molecules. For these reasons, aptamers are ideal agents for disrupting protein-protein interactions. Following the 2004 FDA approval of the first aptamer-based therapeutic agent, pegaptanib, a number of aptamers have entered clinical trials; for example, the aptamer targeting the interaction between factor IXa and its substrate factor X is currently in Phase II clinical trials as an antithrombotic agent, with applications for percutaneous coronary intervention and generalized surgical anticoagulation (EyetechStudyGroup, 2003; Dyke et al., 2006; Chan et al., 2008a; Chan et al., 2008b).

After concluding that aptamers WT-15 and SM-20 bound to PAI-1 with high affinity at a region overlapping the heparin and vitronectin binding sites, I characterized the functional applications of this interaction using chromogenic assays, solution-based binding, and cell adhesion experiments. As a result, I have determined that aptamers SM-20 and WT-15 disrupt PAI-1 inhibition of thrombin mediated by vitronectin,
stabilize PAI-1 in an active conformation without affecting its antiprotease activity, and prevent PAI-1 from binding to vitronectin bound to a solid surface. Furthermore, aptamer SM-20 restores breast cancer cell adhesion in a setting of PAI-1 induced detachment. This aptamer molecule has therefore demonstrated potential as a novel antimetastatic therapeutic agent, which upon further development could possibly serve as adjuvant therapy for breast cancer.
3.2 Materials and Methods

Thrombin activity assays

*Heparin-mediated PAI-1 inhibition of thrombin.* Increasing amounts of aptamers WT-15 and SM-20 (as well as the Sel2 library as a negative control) were heated to 65°C for 5 minutes and incubated with 50 nM PAI-1-A (active fraction of wild-type PAI-1; Molecular Innovations, Inc., Novi, MI) for 10 minutes at room temperature. Heparin (Diosynth Oss, the Netherlands; 10 mg/mL) and 1 nM α-thrombin (Haematologic Technologies, Inc., Essex Junction, VT) were then added to the RNA/PAI-1 mixture to bring the final volume to 100 µL and incubated at room temperature for 30 minutes. Residual thrombin activity was determined by cleavage of the chromogenic reagent specific for thrombin activity, S-2238 (DiaPharma, West Chester, OH) (150 µM). The plate was then immediately placed into a microplate reader (Bio-Tek EL311, BioTek Instruments, Inc., Winooski, VT) and set for kinetic readings every 35 seconds for 15 minutes at an absorbance of 405 nm. All RNAs and proteins were diluted in HNPN buffer (20 mM Hepes, 150 mM NaCl, 0.01% PEG, 0.005% sodium azide).

*Heparin-mediated serpin inhibition of thrombin.* To determine if the aptamers were specific for PAI-1, various serine protease inhibitors (serpins) that also inhibit thrombin via the cofactor heparin were examined. In these experiments, 10 nM antithrombin III (ATIII; previously purified according to Pratt et al., 1992) 30 nM heparin cofactor II (HCII; Molecular Innovations, Inc., Novi, MI) or 50 nM protein C inhibitor (PCI; previously purified according to Rehault et al., 2005) were incubated with the aptamers as opposed to PAI-1; the remainder of the protocol remained unchanged.
**Vitronectin-mediated PAI-1 inhibition of thrombin.** When vitronectin was used to mediate PAI-1 inhibition of thrombin, the protocol remained unchanged with the exception that 10 µg/mL vitronectin (Molecular Innovations, Inc., Novi, MI) was used as opposed to heparin.

**Serpin binding assays.**

RNA was first dephosphorylated using bacterial alkaline phosphatase (Gibco BRL, Gatihersburg, MD) and 5’ end labeled with \[\gamma^{32}\text{P}\] ATP (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). All binding was performed in BSA binding buffer F (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl\(_2\), and 0.1% bovine serum albumin [BSA]). Briefly, radiolabeled RNA was incubated with antithrombin III (ATIII; previously purified according to Pratt et al., 1992), heparin cofactor II (HCII; Molecular Innovations, Novi, MI) or protein C inhibitor (PCI; previously purified according to Pratt et al., 1992) for 5 minutes at 37°C; the mixture was then passed over a nitrocellulose membrane. The fraction of RNA that bound to the protein was quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Nonspecific binding of the RNA was subtracted out such that only specific binding remained (denoted ‘corrected fraction bound’).

**Latency assay**

Aptamers WT-15 and SM-20 (as well as the Sel2 library as a negative control; all RNA at 200 nM) were heated to 65°C for 5 minutes and allowed to cool to room temperature. Following cooling, the RNA was incubated with 40 nM PAI-1-A (Molecular Innovations, Novi, MI) for 4 hours at 37°C. Standard wells contained only PAI-1 (to demonstrate PAI-1’s conversion to its inactive, latent conformation), PAI-1
with 200 nM vitronectin (to demonstrate vitronectin’s ability to stabilize PAI-1 in an active conformation), or uPA alone (10 nM, to show maximal uPA activity). Following incubation, 10 nM uPA (Molecular Innovations, Novi, MI) was added and allowed to incubate at 37°C for an additional 5 minutes. Finally, 100 µL S-2444 (for a final concentration of 300 µM) was added and the mixture was transferred to a 96-well plate, added to a microplate reader (Bio-Tek EL311, BioTek Instruments, Inc., Winooski, VT) and set for kinetic readings every 35 seconds for 15 minutes at an absorbance of 405 nm. Maximal (100%) uPA activity was set as the absorbance measured when only uPA was present and 0% as no detectable absorbance.

**Solid phase vitronectin studies**

Solid phase PAI-1 binding studies were performed according to Lawrence et al., with slight modification (Lawrence et al., 1990). Briefly, 96-well plates used for immunochemistry (Nunc Immuno-module, Nalge Nunc International, Thermo Fisher Scientific, Rochester, NY) were coated with 150 µL of vitronectin (Molecular Innovations, Inc., Novi, MI) at 250 ng/mL and allowed to incubate overnight at 4°C. The wells were then washed three times with 300 µL PBST (phosphate buffered saline [PBS] with 0.01% Tween 20). Following drying at room temperature (RT) for 15 minutes, the plate was blocked with 3% BSA for 1 hour at RT, followed by three washes with PBST. After heating an increasing amount of WT-15 and SM-20 (as well as the Sel2 library as a negative control) to 65°C for 5 minutes and allowing for cooling, the aptamers were added to 200 ng/mL PAI-1-A (active fraction of wild-type PAI-1, Molecular Innovations, Inc., Novi, MI) for a final volume of 200 µL. The RNA/PAI-1 mixture was incubated at RT for 10 minutes prior to its addition to the blocked plate, which then incubated at RT for 30 minutes. The plate was then washed three times with PBST and blotted on a paper
towel, and 150 µL uPA (Molecular Innovations, Inc., Novi, MI) was added to the plate for a final concentration of 1 nM; this was incubated at RT for 30 minutes. Next, 50 µL S-2444 (DiaPharma, West Chester, OH), the chromogenic substrate for uPA activity, was added to the plate, which was then immediately placed into a microplate reader (Bio-Tek EL311, BioTek Instruments, Inc., Winooski, VT) and set for kinetic readings every 35 seconds for 15 minutes at an absorbance of 405 nm. All RNA and protein were diluted in PBS with calcium and magnesium.

**Cell adhesion assay**

All cell adhesion assays were performed in 96-well microtiter plates that were coated with 150 µL vitronectin (Molecular Innovations, Inc., Novi, MI) at 7.5 µg/mL for 1 hour at 37°C. The plates were washed three times with warm PBS, and blocked for 1 hour at 37°C with 1% heat-inactivated BSA. The plates were then washed three times with PBS, air dried, and either used immediately or wrapped in parafilm, and stored at 4°C. Aptamers WT-15 and SM-20 (as well as the Sel2 library as a negative control) (50-1000 nM) were heated to 65°C for 5 minutes prior to adding 50 nM PAI-1-A (Molecular Innovations, Inc., Novi, MI) for a final volume of 50 µL. The RNA/PAI-1 mixture was then incubated at 37°C for 15 minutes. MDA-MB-231 breast cancer cells were seeded at 5 x 10⁴ cells/well in 200 µL serum free medium onto vitronectin coated plates and were allowed to attach for 1 hour at 37°C in the presence of PAI-1 or the RNA/PAI-1 complex. Following incubation, the non-adherent cells were removed with a multi-channel pipette, and adhered cells were washed twice with warm PBS. To determine the quantity of adherent cells an MTT assay was performed. Briefly, 20 µL of MTT (5 mg/mL) in 200 µL of serum-free media was added to each well and incubated for 3 hours at 37°C. Following incubation, the media was removed and 100 µL dimethyl
sulfoxide (DMSO; Sigma, St. Louis, MO) was added to solubulize the formazan crystals for 15-30 minutes at room temperature. The absorbance was then measured at 570 nm in a VersaMax microtiter plate reader (Molecular Devices). The background absorbance was measured at 690 nm, which was subtracted for the absorbance at 570 nm.
3.3 Results

3.3.1 Aptamers WT-15 and SM-20 prevent heparin- and vitronectin-mediated PAI-1 inhibition of thrombin

Previously generated aptamers WT-15 and SM-20 were found to bind to PAI-1 with high affinity in an area that overlaps the heparin and vitronectin binding sites (α-helices D and E, respectively). To functionally characterize this interaction, I first tested the aptamers’ ability to impede thrombin inhibition by PAI-1 that is mediated by the cofactors heparin or vitronectin. In this assay, the RNA/PAI-1 complex was incubated with either heparin or vitronectin prior to addition of thrombin. Residual thrombin activity was determined as described in Materials and Methods. In figure 3-1a, thrombin inhibition by PAI-1/heparin is demonstrated, as evidenced by the decrease in thrombin activity to less than 30% of its original value. On the other hand, when PAI-1/heparin is incubated with 1 µM WT-15 or 1 µM SM-20, thrombin activity is completely restored. This rescue was also found to be dose dependent, as seen in figure 3-1b. Moreover, a decline in maximal restoration was not observed until the concentration of the clones reached below 62.5 nM. Similar results were found when in the presence of vitronectin (figure 3-2a and b). Here, the PAI-1/vitronectin had a more profound effect, decreasing thrombin activity to approximately 15% of its original value. As seen in figure 3-2a, 5 µM WT-15, as well as 5 µM SM-20 restored approximately 65% of thrombin activity. Again, this occurred in a dose dependent manner (figure 3-2b). These data show that aptamers WT-15 and SM-20 were able to prevent a functional reaction that stemmed from PAI-1 binding to heparin or vitronectin.
Figure 3-1: Aptamers prevent heparin-mediated PAI-1 inhibition of thrombin. a. 1 μM SM-20, as well as 1 μM WT-15, restored thrombin activity despite the presence of PAI-1 and heparin, which in combination inhibited thrombin activity. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM). b. SM-20 (represented by squares (■)) and WT-15 (represented by triangles (▲)) restore thrombin activity following PAI-1 and heparin inhibition in a dose-dependent manner. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM).
Figure 3-2: Aptamers prevent vitronectin-mediated PAI-1 inhibition of thrombin. a. 5 μM SM-20, as well as 5 μM WT-15, restored thrombin activity despite the presence of PAI-1 and vitronectin, which in combination inhibited thrombin activity. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM). b. Aptamers SM-20 (represented by squares (■)) and WT-15 (represented by triangles (▲)) restore thrombin activity following PAI-1 and vitronectin inhibition in a dose-dependent manner. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM).
3.3.2 Lead aptamers are specific for PAI-1

After determining that aptamers WT-15 and SM-20 inhibit the reaction between PAI-1 and thrombin when heparin and vitronectin were used as cofactors, I next determined if this effect was specific for PAI-1, as serpins antithrombin III (ATIII), heparin cofactor II (HCII) and protein C inhibitor (PCI) also inhibit thrombin when heparin is used as a cofactor. As seen in figure 3-3a, the aptamers do not appreciably bind to ATIII, as evidenced by a maximum fraction bound of less than 15%, even at relatively large concentrations of protein (> 1 µM). In addition, the aptamers do not functionally cross react with ATIII, as thrombin activity is not restored when WT-15 or SM-20 are combined with ATIII, heparin and thrombin (Fig. 3-3b). In contrast to ATIII, the aptamers are able to bind to HCII and PCI (Fig. 3-4a and 3-5a, respectively). However, this binding is chiefly non-specific, as the binding of WT-15 and SM-20 is not substantially different from that of the Sel2 library. More importantly, the aptamers do not exhibit functional reaction with HCII or PCI above that of control levels (Fig. 3-4b and 3-5b, respectively). When these results are compared to the binding to PAI-1, which exhibits picomolar affinity, and the functional restoration of thrombin activity when PAI-1 is functioning as the protease inhibitor (Fig 3-1a), it is clear that the aptamers are specific for PAI-1.
Figure 3-3: Aptamer interaction with antithrombin III (ATIII). a. Nitrocellulose binding assay with increasing amounts of ATIII. Triangles (▲) represent WT-15, squares (■) represent SM-20, and inverted triangles (▼) represent the starting library (Sel2). b. Aptamers (5 µM) do not restore thrombin activity following heparin-mediated ATIII inhibition. Experimental wells were performed in duplicate; error bars represent the standard error of the mean (SEM).
Figure 3-4: Aptamer interaction with heparin cofactor-II (HCII). a. Nitrocellulose filter binding assay with increasing amounts of HCII. Triangles (▲) represent WT-15, squares (■) represent SM-20, and inverted triangles (▼) represent the starting library (Sel2). b. Aptamers (5 µM) do not restore thrombin activity following heparin-mediated HCII inhibition. Experimental wells were performed in duplicate; error bars represent the standard error of the mean (SEM).
Figure 3-5: Aptamer interaction with protein C inhibitor (PCI). a. Nitrocellulose filter binding assay with increasing amounts of PCI. Triangles (▲) represent WT-15, squares (■) represent SM-20, and inverted triangles (▼) represent the starting library (Sel2). b. Aptamers (5 µM) do not restore thrombin activity following heparin-mediated PCI inhibition. Experimental wells were performed in duplicate; error bars represent the standard error of the mean (SEM).
3.3.3 Aptamers stabilize PAI-1’s antiprotease activity

After demonstrating that the aptamers disrupted the interaction between PAI-1 and heparin/vitronectin in a specific manner, I began to investigate the functional implications of aptamers binding to PAI-1 at the vitronectin binding site. As vitronectin binding engenders PAI-1 stability, it is possible that the aptamers function in a similar manner and can prevent PAI-1 from converting to its inactive, latent form. To test this hypothesis, I incubated PAI-1 with and without vitronectin, as well as PAI-1 with aptamers WT-15 and SM-20, for four hours at 37°C, according to Materials and Methods. As seen in figure 3-6, prolonged incubation at 37°C significantly reduced PAI-1’s ability to inhibit uPA (PAI-1 only group). When vitronectin was added, a portion of PAI-1 remained active, as uPA activity decreased to about half its maximal level (PAI-1 & VN group). However, PAI-1 incubation with WT-15 abolished uPA activity, and the addition of SM-20 to PAI-1 reduced uPA activity to less than 20% of its maximal level, both indicating that PAI-1 remained active to inhibit uPA. Therefore, aptamers WT-15 and SM-20 stabilized PAI-1 in an active conformation despite its tendency to spontaneously convert to a latent form when under similar conditions. Moreover, aptamer binding did not interfere with PAI-1’s antiprotease activity, as evidenced by its ability to inhibit uPA.
Figure 3-6: Aptamers WT-15 and SM-20 stabilize PAI-1 in an active confirmation without affecting the PAI-1/uPA interaction. Aptamers were preincubated with PAI-1, followed by four hours of incubation at 37°C (to allow for PAI-1 conversion to its inactive, latent conformation) and subsequent addition of uPA. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM).
3.3.4 Aptamers WT-15 and SM-20 prevent PAI-1 from binding to solid-phase VN

In vivo, vitronectin, an adhesive glycoprotein, binds to the extracellular matrix (Preissner, 1991). Aptamers WT-15 and SM-20 were previously shown to compete with vitronectin for binding to PAI-1 in solution, and this binding does not interfere with PAI-1’s antiprotease activity (Fig. 3-6). However, to assess the aptamers’ potential as therapeutic agents, assays that better recapitulate the in vivo environment must be examined. For this reason, I next determined if the aptamers could prevent PAI-1 from binding to vitronectin in a solid phase. In this assay, PAI-1 or PAI-1/RNA complex was added to vitronectin-coated plates and incubated as described in Materials and Methods. Following incubation, the plate was washed and incubated with uPA. If PAI-1 retains the ability to bind to vitronectin, it will inhibit uPA activity. As shown in figure 3-7a, when only vitronectin is present, without PAI-1 or aptamers, uPA has maximal activity; the same is true when PAI-1, but no vitronectin, is present. As anticipated, the combination of vitronectin and PAI-1 decreases uPA activity to approximately 60%. Aptamer WT-15 fully restores uPA activity and SM-20 restores uPA activity to greater than 90%, indicating that their binding to PAI-1 precludes binding to vitronectin. Figure 3-7b shows a dose-response curve of aptamer restoration of uPA activity; we see that near maximal levels of uPA activity, and thus inhibition of PAI-1 binding to vitronectin, occurs at concentrations as low as 12.5 nM.
Figure 3-7: Aptamers SM-20 and WT-15 prevent PAI-1 from binding to vitronectin that is bound in a solid phase. a. Both 200 nM SM-20 and 200 nM WT-15 restore uPA activity, indicating prevention of the PAI-1/VN interaction. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM). b. Clones SM-20 (represented by squares (■)) and WT-15 (represented by triangles (▲)) restore uPA activity in a dose dependent manner by preventing PAI-1 from binding to vitronectin and hence inhibiting uPA. Each data point was performed in duplicate in three separate experiments; error bars represent the standard error of the mean (SEM).
3.3.5 Aptamer SM-20 increases breast cancer cell adhesion

Binding to matrix-bound vitronectin via the urokinase-type plasminogen activator receptor (uPAR) is critical for breast cancer cell adhesion (Chapman et al., 2001; Wei et al., 2001). However, PAI-1 binding to vitronectin precludes this reaction, thus leading to cellular detachment (Deng et al., 1996; Stefansson et al., 1996b; Kjoller et al., 1997). Because aptamers WT-15 and SM-20 were able to prevent PAI-1 from binding to solid phase vitronectin, I expanded on these in vitro results and tested the aptamers’ ability to disrupt a clinically relevant reaction, restoration of breast cancer cell adhesion. In this assay, breast cancer cells (MDA-MB-231) were incubated with PAI-1 or PAI-1/aptamer on vitronectin-coated plates. As seen in figure 3-8a, the addition of 50 nM PAI-1 decreases cell adhesion approximately 60%. However, the addition of increasing amounts of clone SM-20, with an optimal concentration at 500 nM, increases the percentage of cells that remain adherent to vitronectin. Although WT-15 demonstrated function in all previous assays, it was unable to increase breast cancer cell adhesion in the presence of PAI-1 at concentrations up to 1000 nM (figure 3-8b).
Figure 3-8: Aptamer SM-20, but not WT-15, restores breast cancer cell adhesion. a. Breast cancer cells (MDA-MB-231) were incubated in the presence of SM-20 and PAI-1 in a vitronectin-coated plate. Experiments with SM-20 were performed in duplicate; error bars represent the standard error of the mean (SEM). b. Breast cancer cells (MDA-MB-231) in the presence of WT-15 and PAI-1 in a vitronectin-coated plate did not show evidence of increased adhesion.
3.4 Discussion

Aptamers WT-15 and SM-20 were previously shown to bind to PAI-1 with high affinity in a region spanning the heparin and vitronectin binding domains. Here, I functionally characterized this binding reaction and found that aptamers SM-20 and WT-15 disrupt the inhibition of thrombin mediated by heparin and vitronectin in a manner specific to PAI-1, stabilize PAI-1 in an active conformation without affecting its antiprotease activity, and prevent PAI-1 from binding to vitronectin bound to a solid surface. Moreover, SM-20 increased breast cancer cell adhesion in an environment of PAI-1 induced detachment.

The interaction between PAI-1 and vitronectin is important in the regulation of cell adhesion, cell migration and fibrinolysis. In addition, vitronectin’s interaction with PAI-1 has been implicated in several disease states (Stefansson S et al., 1996, Dano K, et al., 2005), not only due to its function in keeping PAI-1 in an active conformation, but also due to PAI-1’s competitive inhibition with the uPAR receptor for integrin binding, which disrupts cellular adhesion (Stefansson S, 1996, Kjoller et al., 1997). This interaction is particularly important for breast cancer cells, as increases in PAI-1 have been associated with a worsened prognosis (Beaulieu et al., 2007). Counterintuitively, increased levels of its target protease uPA are also associated with a poor prognosis for cancer patients (Reilly et al., 1992; Janicke et al., 1993). This is primarily because elevated uPA increases plasmin production, leading to increased degradation of the extracellular matrix. Worsened prognosis due to elevation of both uPA and its antagonist PAI-1 is termed the “PAI-1 paradox”. For this reason, an effective antimetastatic agent would ideally address both portions of this ‘paradox’, separating the molecular interactions between PAI-1 and vitronectin from PAI-1’s inhibition of uPA. Specifically, inhibition of PAI-1’s interaction with vitronectin could lead to an increase in cellular adhesion, and
therefore a decrease in cellular detachment and metastasis. In addition, if the antiprotease activity of PAI-1 could remain unaffected, PAI-1 could continue to inhibit uPA, thus leading to a decrease in plasmin generation.

Considering the critical role of the PAI-1/vitronectin interaction in cancer cell progression and metastasis, these data support the development of aptamers as a therapeutic option for cancer management, as aptamers are ideal for disrupting protein-protein interactions. Aptamers have been shown to be non-toxic, well tolerated molecules that cause low to no immunogenicity, can be synthetically manufactured, and have an adjustable bioavailability (EytechStudyGroup, 2002; Nimjee et al., 2005; Dyke et al., 2006; Chan et al., 2008a; Chan et al., 2008b). For these reasons, I hypothesized that if PAI-1 aptamers WT-15 and SM-20 functionally disrupted the interaction between PAI-1 and vitronectin, they would have a therapeutic application in preventing breast cancer metastasis.

To test this hypothesis, I first demonstrated that aptamers WT-15 and SM-20 disrupted the functional interaction between heparin and PAI-1 as it relates to thrombin inhibition (Fig. 3-1). Similar results were found when vitronectin was used as the cofactor (Fig. 3-2), as both heparin and vitronectin accelerate PAI-1’s inactivation of thrombin more than 200-fold, primarily by facilitating the formation of noncovalent complexes (Naski et al., 1993; Rezaie, 1999). In addition, this attenuation of PAI-1/heparin and PAI-1/vitronectin mediated inhibition of thrombin activity occurred in a dose dependent manner. Inhibition was particularly robust for the PAI-1/heparin interaction (Fig. 3-1b), as a decrease in maximal thrombin inhibition was again not apparent until the concentration of the aptamers was less than the concentration of PAI-1, suggesting that the aptamers bind to PAI-1 in a 1:1 manner. On the other hand, the attenuation of the inhibition of PAI-1/vitronectin by the aptamers was not as robust as
seen with heparin. One potential explanation is that at high concentrations of RNA, vitronectin non-specifically binds RNA, reducing the amount available to bind to PAI-1. As vitronectin has heparin binding domains itself that are positively charged, it is not unlikely that RNA would bind to those regions, whereas the negatively charged heparin would repel RNA due to its anionic properties. In spite of non-specific binding, clones WT-15 and SM-20 are both able to attenuate PAI-1/cofactor inhibition of thrombin activity, further suggesting that they bind to a region on PAI-1 that overlaps the heparin and vitronectin binding sites.

As a member of the serpin (serine protease inhibitor) superfamily, PAI-1 shares highly conserved structural similarities to all serpins, i.e., nine α-helices and a β-sandwich consisting of three β-sheets. In addition, all serpins use a reactive center loop to inhibit their target proteases, which is inserted into β-sheet A to form the most energetically stable structure (Rau et al., 2007). In particular, antithrombin III (ATIII), heparin cofactor II (HCII) and protein C inhibitor (PCI) are closer to PAI-1 than other serpins as they, like PAI-1, possess a heparin binding site that is used to inhibit the protease thrombin, using heparin as a cofactor to increase the second-order rate constant by several orders of magnitude (Rezaie, 1999; Rau et al., 2007). Therefore, after determining that the aptamers inhibited heparin-mediated thrombin inhibition, I next investigated the specificity of this reaction for PAI-1. As shown in figures 3-3a, 3-4a and 3-5a, aptamer binding to the serpins was largely nonspecific, as aptamer binding was similar to that of the negative control, the Sel2 starting library. However, there are differences in the fraction of RNA that binds to one protein versus another, as demonstrated by the binding to HCII and PCI vs. ATIII. This could be due to reduced accessibility of the heparin binding site on ATIII as compared to the other proteins, or possibly the presence of additional regions of positive charge on HCII or PCI. In any
case, aptamers WT-15 and SM-20 do not attenuate the inhibition of thrombin by these serpins (Fig. 3-3b, 3-4b, and 3-5b), confirming their specificity for PAI-1.

The somatomedin B domain of vitronectin non-covalently binds to α-helix E on PAI-1, preventing the reactive center loop of PAI-1 from entering into β-sheet A (which converts PAI-1 to its latent, inactive state). Because the aptamers were previously shown to bind in this region, I next hypothesized that aptamers WT-15 and SM-20 would act in a manner similar to vitronectin, stabilizing PAI-1 in an active conformation. This hypothesis was explored by incubating PAI-1 at 37°C for four hours, which allowed PAI-1 to spontaneously convert to its latent conformation (Hekman et al., 1985). Figure 3-6 demonstrates the conversion of PAI-1 to an inactive state following incubation, as evidenced by the preservation of uPA activity. However, when vitronectin was added, PAI-1 activity was not fully lost, as seen in the reduction of uPA activity. Likewise, uPA activity was dramatically reduced when the aptamers were incubated with PAI-1, indicating that the binding of aptamers WT-15 and SM-20 enabled PAI-1 to retain its antiprotease activity. This protection of PAI-1 activity is presumably due to the aptamers’ ability to constrict the shifting of β-sheet A, not unlike vitronectin. However, the addition of WT-15 and SM-20 demonstrated far greater levels of uPA inhibition than vitronectin. This could be due to the differences in affinity for PAI-1 between the aptamers and vitronectin (177 pM for WT-15 and 920 pM for SM-20 vs. ~1 nM for vitronectin; (Andreasen, 2007)). Additionally, vitronectin stabilizes PAI-1 for 4-6 hours (Declerck et al., 1988). With this in mind, the length of incubation chosen for this experiment may have been at the tail end of vitronectin’s protection of PAI-1 activity.

In vivo, vitronectin is bound to the extracellular matrix. For this reason, I performed an assay to determine if the clones were able to prevent PAI-1 from binding to vitronectin bound in a solid phase. As seen in figure 3-7a, both aptamers decreased
PAI-1 inhibition of uPA (indicating that PAI-1 was not bound to vitronectin) in the presence of bound vitronectin. Additionally, aptamer WT-15 fully attenuated PAI-1 inhibition of uPA, and such inhibition decreased only when its concentration became less than the concentration of PAI-1 in the assay (4.54 nM; Fig. 3-7b). This observation further suggests that aptamer WT-15 binds to PAI-1 in a 1:1 stoichiometric ratio.

Because the aptamers demonstrated the ability to functionally inhibit the interaction between PAI-1 and vitronectin while retaining PAI-1’s antiprotease activity, I explored the potential of aptamers WT-15 and SM-20 in preventing breast cancer metastasis. This was achieved by determining if the aptamers could increase breast cancer cell adhesion in the setting of PAI-1-induced detachment. As seen in Figure 3-8, SM-20, but not WT-15, is able to restore cellular adhesion to approximately 90% its maximal value, calculated as the amount of adhesion noted in the absence of PAI-1. It is interesting that both aptamers SM-20 and WT-15 appear to bind to the overlapping heparin/vitronectin binding site on PAI-1, but only clone SM-20 is able to increase cellular adhesion in the presence of PAI-1. Differences between the sequences of the two aptamers likely impart distinct secondary and tertiary structures upon these RNAs; these structural variances, and how they alter PAI-1 binding, may be the basis behind the selectivity of one aptamer over the other in this instance.

I have previously developed aptamers that bind to PAI-1 with high affinity in a region that spans the heparin and vitronectin binding sites. Here, I have functionally characterized aptamers WT-15 and SM-20, demonstrating that aptamer binding precludes heparin and VN binding to PAI-1 in a specific manner. Furthermore, I have shown that aptamers WT-15 and SM-20 inhibit the PAI-1/vitronectin interaction without disrupting PAI-1’s antiprotease activity, addressing both portions of the “PAI-1 paradox”. Furthermore, aptamer SM-20 increased breast cancer cell adhesion, rendering
it as a potential antimetastatic agent and an attractive agent to serve as an adjunct to traditional breast cancer therapy.
4. Factor IXa Aptamer Administration Following Murine Middle Cerebral Artery Occlusion

4.1 Introduction

Stroke is a significant cause of mortality in the United States, where a fatal stroke is estimated to occur every three to four minutes (Rosamond et al., 2008). Additionally, over half of the patients who survive an acute stroke are left with residual disability at six months, making stroke the leading cause of morbidity in the US (Rosamond et al., 2008). Currently, the only pharmacologic agent approved by the FDA for treatment of acute stroke is tissue-type plasminogen activator (tPA), which promotes reperfusion by enhancing the dissolution of intravascular clots. Unfortunately, administration of tPA is not often utilized, with recent reports suggesting that less than 3% of strokes are treated with tPA (1995b). In response to this clinical need for improved treatments to promote reperfusion in acute stroke, many therapeutic strategies utilizing combinations of fibrinolytic, antiplatelet, and antithrombotic agents are currently undergoing evaluation in preclinical and clinical trials (Benarroch, 2007).

Here, I describe the use of an established antithrombotic aptamer-based therapy in a novel application of stroke. Aptamers are nucleic acid ligands that bind to their target with high affinity and specificity and have been shown to have low to no immunogenicity, adjustable bioavailability, and are well tolerated, thus exerting the affinity properties of monoclonal antibodies with the ease of synthesis of small molecules (Nimjee et al., 2005). Moreover, their activity can be reversed using complementary antidote oligonucleotides (Rusconi et al., 2002).

The aptamer used in this study exerts its antithrombotic actions via specific binding to and inactivation of factor IXa. According to the cell-based model of hemostasis, activated factor IX joins with factor VIIIa on the surface of platelets during
the propagation phase of coagulation, forming the tenase complex (Hoffman et al., 2001). This tenase complex then activates factor X, which combines with factor Va already present on the activated platelet surface. Together, factor Xa and Va form the prothrombinase complex that directly converts prothrombin to thrombin. Therefore, I hypothesized that aptamer inhibition of factor IXa would directly decrease thrombin generation, which is known to increase in the setting of ischemia (Karabiyikoglu et al., 2004). Thrombin is a powerful activator of the inflammatory cascade and as such leads to the upregulation of inflammatory cytokines, chemoattractant molecules and vasoactive substances, which then promote neutrophil adhesion and macrophage activation, in addition to a host of procoagulatory effects (Edmunds et al., 2006). Due to the increased presence of inflammatory cells and activation of the coagulation cascade, the end result of increased thrombin levels is decreased reperfusion. The factor IXa aptamer should therefore enhance reperfusion by its inhibition of thrombin generation.

It has been previously shown that inhibition of factor IXa using an active-site blocked competitor (FIXai) and a humanized inhibitory antibody (SB 249417) improved outcome following rodent models of cerebral ischemia (Choudhri et al., 1999; Toomey et al., 2002). Given the properties of aptamers that render them ideal disrupters of protein-protein interactions, I used the factor IXa aptamer, which has demonstrated efficacy as an antithrombotic agent in mice, pigs and humans, in a murine model of transient middle cerebral artery occlusion (Rusconi et al., 2004; Dyke et al., 2006; Nimjee et al., 2006). I found that administration of the aptamer following stroke in mice reduced thrombin generation, decreased inflammation and improved neurological function in the treated animals, demonstrating its efficacy as a potential therapeutic agent for stroke.
4.2 Materials and Methods

Animal Surgical Studies

All animals received humane treatment in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, as approved by the Duke University Animal Care and Use Committee. Prior to induction of ischemia or hemorrhage, wild type male C57Bl/6J mice ages 10-12 weeks (Jackson Laboratory, Bar Harbor, ME, USA) were injected with 2 mg/kg of the cholesterol modified aptamer generated against factor IXa, Ch-9.3t, intravenously via tail vein. After waiting 10 minutes for the aptamer to circulate, 200 µL of blood was removed via cardiac puncture. Whole blood aPTT and ACT values were then measured using the Hemachron Jr. Signature Microcoagulation System (ITC, Edison NJ, USA). All measurements were performed in duplicate. For experiments involving antidote reversal, 2 mg/kg of Ch-9.3t was administered intravenously, and following 10 minutes of circulation, a 50-fold molar excess of 5-2C antidote (46.8 mg/kg) was given via tail vein injection. After waiting an additional 10 minutes, 200 µL of blood was removed via cardiac puncture and aPTT/ACT values were determined in duplicate as before.

For induction of ischemia, mice were anesthetized with 5% isoflurane in a 30% O2:70% N2 mixture. The trachea was then intubated with a 20-gauge intravenous catheter (Insyte-W, Becton Dickinson). Next, the inspired isoflurane concentration was decreased to 1.5% and the lungs were mechanically ventilated at a rate of 130 breaths per minute at a tidal volume of 0.7 mL. Rectal temperature was monitored and servoregulated at 37°C via surface heating and cooling. A midline neck incision was made and the right common carotid artery was isolated. Following isolation and ligation of the right external carotid artery with temporary occlusion of the right common carotid artery, a silicon-coated 6-0 nylon monofilament was inserted into the right
external carotid artery and advanced via the internal carotid artery to the bifurcation of the middle cerebral artery (MCA) and the anterior cerebral artery (ACA). The filament remained in this position for 60 minutes, occluding the origin of the MCA in order to produce an infarct in its region of perfusion. The filament was then withdrawn and the midline incision was closed via interrupted skin sutures.

Assessment of Neurological Function

The neurological status of the mice post 60-minutes of ischemia and reperfusion was determined using a Rotarod test of vestibulomotor function (Ugo Basile, Comerio, Italy) (Hamm et al., 1994). In this assay, mice are placed on a rotating rod that accelerates in speed from 4 to 40 rpm. The length of time the mouse is able to remain on the rod without falling (latency period) is recorded, with a maximum time of 300 seconds. All trials were performed in triplicate and the mean latency was calculated. Prior to assessing neurological status post-stroke, mice were tested and their baseline values were recorded. Mice with a preinjury latency period less than 180 seconds were excluded from the study, whereas dead mice were assigned the lowest latency score (0).

Determination of Thrombin Levels

Thrombin levels were indirectly measured via quantification of TAT (thrombin-antithrombin) complex levels. A commercially available Dade Behring Enzygnost® ELISA kit for TAT complexes was purchased from Siemens Healthcare Diagnostics (Deerfield, IL). For sample preparation, approximately 900 µL of blood was removed from the mice via cardiac puncture and immediately mixed with 100 µL 0.11 M sodium citrate. The blood was then centrifuged at 1500 x g for 10 minutes and the plasma
supernatant was removed, which was used to determine TAT levels; samples were run in duplicate.

**Histology and Determination of Infarct Volume**

A separate cohort of mice was used for histological determination of infarct volumes. Following 60-minute middle cerebral artery occlusion (MCAO), mice received intravenous administration of 2 mg/kg Ch-9.3t aptamer or 2 mg/kg mutant aptamer via tail vein. Forty-eight hours following MCAO, the mice were anesthetized with isoflurane and decapitated. The brain was then removed and frozen at -20°C. Using a freezing microtome, 20-µm thick coronal sections were cut in 440-µm intervals over the rostral-caudal extent of the infarct and mounted on charged slides. Following drying overnight, the slides were stained with hematoxylin and eosin. Infarct volume was measured by converting the slides to a digital image using a Photometrics CoolSnap cf digital imager (Roper Scientific, Inc., Pleasanton, CA) equipped with a micro-nikkor 55 mm lens (Nikon Corporation, Tokyo, Japan), utilizing MCID Elite 7.0 image analyzer software (Imaging Research, Inc., Victoria, Australia). An observer blinded to the experimental conditions then outlined the infarct boarders with an operator-controlled cursor. The area (mm²) of the infarct was determined automatically by counting the number of pixels contained in the delineated region, whereas infarct volume (mm³) was calculated by multiplying the infarct area by the interval between sections (440 µm).

**Aptamers and Antidote**

Aptamer Ch-9.3t is a 35-nucleotide length RNA with sequence 5’-AUG GGG ACU AUA CCG CGU AAU UGC UGC CUC CCC AU-3’ that was modified with 2’-fluoropyrimidines and a 3’ inverted thymidine cap to render nuclease resistance, as well as a 5’ cholesterol moiety to increase its circulating half-life. The mutant aptamer Ch-
9.3tM, 5'-AUG GGG ACU GUG CCG CGU AAU UGC UGC CUC CCC AU-3', is of the same composition as Ch-9.3t with exception of two nucleotides that were changed (denoted in bold) which rendered the aptamer unable to bind to factor IXa and hence inactive as an antithrombotic agent. Antidote oligonucleotide 5-2C is composed of 2'-O-methyl RNA and has a sequence of 5'-CGC GGU AUA GUC CCC AU-3'. Upon binding to Ch-9.3t, 5-2C causes unfolding of the three-dimensional structure of the aptamer and diminishes binding to factor IXa, thus reversing aptamer activity.

**Determination of Inflammatory Markers**

Plasma protein levels of IL-1β and MMP-9 following 60-minute ischemia were determined commercially by Thermo Fisher using Pierce SearchLight proteome array technology (Boston, MA). Mice underwent MCAO and were allowed to survive for 180 minutes (time measured from filament insertion), followed by cardiac puncture to remove approximately 900 µL of blood. The collected blood was immediately mixed with 100 µL 0.11 M sodium citrate and centrifuged at 1500 x g for 10 minutes. Next, the plasma supernatant was removed and stored at -80°C until all samples were collected. The samples were then shipped to Thermo Fisher on dry ice for evaluation of inflammatory marker protein levels.

**Statistical Analyses**

Data are expressed as mean ± SEM unless otherwise noted. Fisher’s exact test was used for mortality levels, while repeated-measures ANOVA was used for analysis of neurofunctional rotarod results. Inflammatory protein levels were analyzed using a two-tailed t-test. TAT complex levels, aPTT and ACT values were compared using one-
way ANOVA with Fisher’s PLSD or Tukey’s post-hoc tests when significant. P < 0.05 was used as an indication of statistical significance.
4.3 Results

4.3.1 The aptamer selected against human FIXa anticoagulates mice

To confirm that the aptamer selected against human factor IXa was able to inhibit the murine factor IXa protein and effectively anticoagulate mice, I performed cross-reactive activated partial thromboplastin time (aPTT) and activated clotting time (ACT) coagulation studies (Rusconi et al., 2002; Rusconi et al., 2004). The aPTT assay measures deficiencies in the intrinsic as well as common pathways of coagulation, while the ACT assay reveals deficiencies in coagulation factors involved in the intrinsic pathway only. Mice were injected with the factor IXa aptamer, Ch-9.3t (2 mg/kg), via tail vein, and after allowing for 10 minutes of circulation, blood was removed via cardiac puncture (Rusconi et al., 2002). aPTT and ACT values were then determined immediately from the whole blood sample. As shown in figure 4-1a, aptamer administration increased the clotting time of the blood as measured by aPTT to 79 ± 3 s. from 27 ± 3 s., the value noted in the control group (P< 0.001). In addition, when the antidote (50x molar excess; 46.9 mg/kg) was given 10 minutes after the aptamer, the clotting time decreased to 31 ± 1 s., indicating that the antidote was able to fully reverse the activity of the aptamer in a rapid manner. ACT values shown in figure 4-1b confirm the ability of the factor IXa aptamer to anticoagulate murine blood; aptamer administration increased the clotting time to 175 ± 1 s. from 105 ± 11 s. (control group; one-way ANOVA, P= 0.0132). Antidote administration reversed anticoagulant activity as evidenced by a clotting time of 102 ± 13 s.
Figure 4-1: Factor IXa aptamer (Ch-9.3t) effectively anticoagulates mice; antidote oligonucleotide (5-2C) use reverses aptamer activity. Mice were injected via tail vein with the FIXa aptamer (2 mg/kg) or the equivalent amount of a non-active, mutant aptamer. After ten minutes, blood was removed via cardiac puncture. Whole blood coagulation measurements were determined using a Hemachron Jr. apparatus. For aptamer/antidote mice, mice were administered the aptamer, 10 minutes passed, and the antidote was then given. After another 10 min, blood was removed and tested in a like manner. a. aPTT measurements; n=3 for aptamer/antidote group; n=2 for all other groups. Means ± SEM are shown; one-way ANOVA; P< 0.0001. **P < 0.01 for aptamer group vs. all other groups. b. ACT measurements; n=2 per group. Means ± SEM are shown; one-way ANOVA; P = 0.0132. *P< 0.05 for aptamer vs. all other groups.
4.3.2 Aptamer use reduces neurological deficit following middle cerebral artery occlusion without modifying infarct volume, rates of intracranial hemorrhage or mortality

In order to determine the effect of the aptamer on mortality, infarct volume and neurological function following stroke, mice were first randomized to receive either the factor IXa aptamer, Ch-9.3t (2 mg/kg, n= 15), or a non-functional mutant version of the aptamer, Ch-9.3tM (2 mg/kg; n= 15), immediately following reperfusion of the 60-minute middle cerebral artery occlusion. Forty-eight hours after induction of ischemia, infarct volumes were determined histologically according to Materials and Methods. Figure 4-2 shows the results of this analysis; mice that received the aptamer demonstrated an infarct volume of 81.42 ± 19.11 mm³, while the mutant aptamer group’s infarct volume was 86.42 ± 12.40 mm³ (P= 0.78). During analysis of the infarcts, rates of intracranial hemorrhage were also noted (3/15 for both aptamer and mutant aptamer groups). In addition, administration of the aptamer did not significantly change mortality levels (as assessed at 48 hours, immediately before determination of infarct volumes), as 5/15 and 3/15 mice died in the aptamer and mutant aptamer groups, respectively (P= 0.682).

In a separate cohort (Ch-9.3t, 2 mg/kg, n= 12; Ch-9.3tM, 2 mg/kg; n= 15), vestibulomotor function was assessed by measuring Rotarod latencies daily as depicted in the experimental timeline of figure 4-3. As shown in figure 4-4, aptamer administration was associated with a durable improvement in neurological function as demonstrated by an increase in Rotarod latency (81 ± 30 s., 152 ± 38 s., and 164 ± 37 s. on day 1, 2, and 3 post stroke, respectively). In contrast, mice that received the mutant aptamer had Rotarod latencies of 43 ± 19 s., 71 ± 27 s., and 87 ± 31 s. on day 1, 2, and 3, respectively; P= 0.0153 vs. aptamer.
Figure 4-2: FIXa aptamer administration does not reduce infarct volume. A separate cohort of mice underwent 60-minute MCAO, followed by intravenous administration of the aptamer (n=10) or mutant aptamer (n=12) (both 2 mg/kg). Forty-eight hours after induction of ischemia, the mice were sacrificed for histological determination of infarct volume. There was no statistical difference between the infarct volumes of mice that received the aptamer and those that received the non-functional mutant aptamer. Means ± SEM is shown. T-test, P=0.78.
Figure 4-3: Experimental timeline for neurological evaluation of mice following 60-minute MCAO. Prior to surgery, mice were evaluated for the length of time they were able to run on a rotating rod without falling (Rotarod latency); mice with a latency less than 180 seconds were excluded. The next day, mice underwent the surgical procedure (lasts ~40 min.) to induce 60-min MCAO. Following surgery, 2 mg/kg Ch-9.3t aptamer or a non-functional mutant aptamer was delivered, followed by ~10 min of recovery. Neurofunction was assessed via Rotarod latency daily for three days, followed by euthanasia.
Figure 4-4: Aptamer administration following MCAO reduces neurological deficit. Mice received either 2 mg/kg of the Ch-9.3t aptamer (n=12) or 2 mg/kg mutant aptamer (n=15) following 60-minute filamentous MCAO with reperfusion. Vestibulomotor function was determined by the Rotarod latency period. Data was collected before stroke was induced (Day 0) and every 24 hours post-stroke, for three days (Day 1-3). Aptamer use resulted in prolonged Rotarod latencies, indicative of superior neurological function over the mutant aptamer control. Means ± SEM are shown. Repeated measures ANOVA, P= 0.0153, Fisher’s PLSD post hoc test, P= 0.0067. *P<0.05 for aptamer vs. mutant aptamer over three days.
4.3.3 Aptamer reduces thrombin activity associated with stroke

After demonstrating that the aptamer improved neurological function, without modulating mortality or infarct volume, I then explored the underlying mechanism behind the aptamer’s efficacy. Thrombin levels were therefore quantified via measurement of thrombin-antithrombin (TAT) complex levels. As demonstrated in figure 4-5a, mice that did not undergo MCA occlusion (sham group) had a TAT complex level of 52.1 ± 24.2 µg/L. Three hours after stroke, the levels increased to 664.6 ± 97.5 µg/L in the mutant aptamer group, but were only slightly increased to 113.3 ± 58.24 µg/L in the aptamer group (P = 0.0001, one-way ANOVA). As seen in figure 4-5b, TAT complex levels in the mutant aptamer and aptamer groups were not significantly different from sham mice 48-hours after stroke (34.8 ± 14.7 µg/L and 28.4 ± 11.9 µg/L vs. 52.1 ± 24.2 µg/L, respectively; P= 0.6, one-way ANOVA). Therefore, aptamer administration decreased thrombin levels that were acutely elevated due to stroke whereas thrombin levels were no longer increased after 48-hours.
Figure 4-5: Aptamer use mitigates MCAO-associated thrombin generation. TAT complex levels were quantified by ELISA using human TAT complexes to generate a standard curve. a. Mice received either the Ch-9.3t aptamer (2 mg/kg; n=3) or mutant aptamer (2 mg/kg; n=3) following 60-minute filamentous MCAO with reperfusion. Sham mice were untreated and did not undergo MCAO (n=5). Three hours after induction of ischemia, whole blood was removed via cardiac puncture and used to prepare plasma. Administration of the aptamer mitigated the associated increase in thrombin levels associated with MCA occlusion. Means ± SEM are shown. One-way ANOVA; P= 0.0001. **P< 0.01 for mutant aptamer vs. all other groups. b. Mice received either the Ch-9.3t aptamer (2 mg/kg; n=7) or mutant aptamer (2 mg/kg; n=6) following 60-minute filamentous MCAO with reperfusion. Sham mice were untreated and did not undergo MCAO (n=5). Forty-eight hours after induction of ischemia, whole blood was removed via cardiac puncture and used to prepare plasma. Thrombin levels were not increased 48 hours after MCAO. Means ± SEM are shown. One-way ANOVA; P= 0.6.
4.3.4 Aptamer administration following MCAO reduces inflammatory protein levels

Thrombin has a potent effect on inflammation, leading to the activation of many inflammatory mediators. Therefore, because aptamer use decreased thrombin levels, I next determined if aptamer administration reduces systemic inflammation in animals that undergo stroke. To this end, plasma levels of IL-1β and MMP-9 were quantified. As seen in figure 4-6a, mice that underwent MCAO and received the mutant aptamer had a mean IL-1β level of 75.33 ± 5.59 pg/mL. However, aptamer administration lowered IL-1β to 52.90 ± 3.25 pg/mL (P= 0.0256, two-tailed t-test). A similar pattern was noted during investigation of MMP-9; figure 4-6b shows mutant aptamer and aptamer groups with MMP-9 levels of 27.64 ± 5436 ng/mL and 8.30 ± 1.57 ng/mL, respectively (P= 0.0268, two-tailed t-test). Therefore, inhibition of factor IXa by the aptamer reduces inflammation following cerebral ischemia and reperfusion.
a. Mutant Aptamer

b. Mutant Aptamer

Figure 4-6: Use of Ch-9.3t aptamer following ischemia decreases inflammation. Mice received either the Ch-9.3t aptamer (2 mg/kg; n=3) or mutant aptamer (2 mg/kg; n=3) following 60-minute filamentous MCAO with reperfusion. Three hours following induction of MCAO, blood was removed via cardiac puncture and used to prepare plasma. a. Aptamer administration mitigates ischemia-induced increases in IL-1β levels; means ± SEM are shown. Two-tailed t-test, P=0.0256 *P< 0.05 for mutant aptamer group vs. aptamer group. b. Aptamer administration mitigates ischemia-induced increases in MMP-9 levels; means ± SEM are shown. Two-tailed t-test, P=0.0268; *P< 0.05 for mutant aptamer group vs. aptamer group.
4.4 Discussion

In this study, I demonstrated the feasibility of using an aptamer-based strategy to inhibit factor IXa and improve neurofunctional outcome, as well as reduce thrombin generation and inflammation, in a murine model of focal cerebral ischemia and reperfusion. As a class of therapeutic agents, aptamers have been shown to have minimal toxicity, low to no immunogenicity, and an adjustable bioavailability, all of which confer additional measures of safety (Nimjee et al., 2005). For example, pegaptanib, an aptamer that antagonizes VEGF-mediated vascularization, has been successfully treating macular degeneration since its FDA approval in 2004 (Gragoudas et al., 2004). Moreover, several aptamer-based treatments are currently in clinical trials, including a modified version of the factor IXa aptamer used in this study (Chan et al., 2008a; Chan et al., 2008b).

Because aptamer Ch-9.3t was originally developed to target human factor IXa (Rusconi et al., 2002), I first confirmed its ability to cross-react with the murine protein and function as an effective anticoagulant. I have shown that the aptamer is able to prolong ACT time and increase aPTT values nearly three-fold, reaching clotting times seen in factor IXa deficient mice (Kung et al., 1998). In addition, antidote 5-2C is able to fully reverse the activity of the aptamer within the chosen time frame of 10 minutes (Figure 4-1). After demonstrating efficacy of the aptamer in mice, I determined if aptamer inhibition of factor IXa activity could improve neurological function if initiated following stroke. Over three days of testing using an objective assay of vestibulomotor function, I saw that aptamer administration significantly reduced the amount of neurological deficit associated with induction of stroke (Fig. 4-4). Although mice that died received the lowest neurofunctional score, this did not confound the results, as there was no significant difference in mortality between the aptamer and mutant
aptamer groups. Upon further investigation I found that the functional improvements were not associated with a decrease in infarct volumes, which were similar between aptamer and control mutant aptamer groups (Fig. 4-2). Given the particular model used to investigate aptamer activity in stroke, these results were not altogether unexpected. For example, it has been shown that tPA has a much more robust effect on decreasing infarct volumes if a thromboembolic model of stroke is used to incite ischemia as opposed to a filamentous occlusion model (Klein et al., 1999; Orset et al., 2007). In addition, the reproducibility of each model varies, which increases error, thereby decreasing statistical significance and confounding interpretation of results. Therefore, filamentous occlusion of the MCA may not be the ideal model for investigation of factor IXa inhibition in treatment of stroke. Also, there are other inciting factors that affect neurological function other than gross infarct volume. For example, in addition to enhanced excitotoxic glutamate release leading to secondary hypoxic-ischemic neuronal injury, formation of microthrombi due to disruption of endothelial integrity and the resulting influx of inflammatory cells can also lead to deficits in neurological function. For these reasons, thrombin generation as measured by TAT complex levels was also investigated. I demonstrated an acute increase in thrombin generation following MCAO, which was significantly reduced when the aptamer was administered (Fig. 4-5a). In contrast, thrombin levels were no longer elevated 48 hours following ischemia, as shown in the mutant aptamer group (Fig. 4-5b).

In addition to inciting thrombus formation by cleaving fibrinogen, thrombin is a powerful activator of the inflammatory cascade. As such, it leads to the upregulation of inflammatory cytokines, chemoattractant molecules and vasoactive substances, which then promote neutrophil adhesion and macrophage activation, in addition to a host of procoagulatory effects, all of which produce vascular thrombosis and is known as the
“no-reflow phenomenon” (Edmunds et al., 2006). Nimjee et al. previously demonstrated the systemic anti-inflammatory properties of this aptamer in a porcine cardiopulmonary bypass model (Nimjee et al., 2006). Because of this, I investigated systemic pro-inflammatory protein levels following stroke and subsequent aptamer treatment. IL-1β was chosen as it has been implicated in stimulation of T-cell proliferation, while increases in MMP-9 are associated with an enhanced risk of hemorrhagic transformation (Feghali et al., 1997; Scalia et al., 1999; Zhao et al., 2004). Here I show that both IL-1β and MMP-9 levels are reduced with aptamer administration (Fig. 4-6). Although I have yet to fully elucidate the mechanism behind improved neurological function with aptamer use, one explanation is that decreased inflammation and thrombin levels lead to reductions in microvascular thrombosis and thus allow for enhanced sparing of individual neurons, leading to improvements in function that may not necessarily translate to gross changes in tissue. Moreover, clinically relevant advances in neurological function must be demonstrated before a novel compound could be translated to use in humans, underscoring the importance of functional improvements over histological results (Fisher et al., 2005).

Inhibition of factor IXa has been previously investigated for treatment of stroke in preclinical animal models. For example, Choudhri et al. utilized an active-site blocked competitive antagonist, FIXai, in a murine model of MCAO (Choudhri et al., 1999). They concluded that inhibition of FIXa decreases microvascular thrombosis as evidenced by decreased fibrin deposition and platelet accumulation; this led to decreases in infarct size and enhanced cerebral perfusion (Choudhri et al., 1999). Toomey et al. expanded on these results, using a humanized murine monoclonal antibody directed against the Gla domain of FIXa in a rat thromboembolic model of stroke; in addition to noting a decrease in infarct volume, they observed reductions in neurological deficits (Toomey et
al., 2002). However, they did not find a correlation between reductions in infarct volume and neurological improvement in the tPA group, nor did they see an improvement in mortality levels, similar to my results. The mechanism behind improvements with FXa inhibition therefore remains somewhat speculative; I did not find a correlation between infarct volume and neurological deficit reduction, but rather show that a reduction in inflammation may play a role in improving neurological function. In addition, neither Choudhri et al., Toomey et al., nor myself observed an increase in the incidence of intracranial hemorrhage associated with inhibition of factor IXa in stroke models. This adds to the inherent safety of inhibition of factor IXa for treatment of stroke.

Here I describe the first investigation of an aptamer therapeutic for treatment of stroke. I found that in a murine model of middle cerebral artery occlusion, administration of the factor IXa aptamer post ischemia improves neurological function while decreasing thrombin levels and systemic inflammation. Moreover, aptamer use did not increase the incidence of intracranial hemorrhage or increase mortality levels. For these reasons, the factor IXa aptamer demonstrates potential as a novel reperfusion agent for treatment of acute ischemic stroke.
5. Antidote Reversal of Aptamer Activity Following Subarachnoid Hemorrhage

5.1 Introduction

Stroke is the leading cause of morbidity in the United States and is the third leading cause of mortality, following heart disease and cancer. Specifically, 8-12% of middle aged adults (45-64 years old) will die within 30 days of having an ischemic stroke; this number increases to ~38% if the stroke is hemorrhagic in nature (Lloyd-Jones et al., 2008). In addition, mortality due to hemorrhagic stroke increases to nearly 45% for adults ≥ 65 years old (Lloyd-Jones et al., 2008).

Aside from the compressed time frame for administration following symptom onset and tPA-associated neurotoxicity, the most common reason for physician reluctance to use tPA is the risk that it may engender uncontrollable intracranial hemorrhage (Brown et al., 2005; Benarroch, 2007). For example, although the original NINDS clinical trial demonstrated a rate of symptomatic intracranial hemorrhage (sICH) following tPA use at 6.4% (1995b), actual practice rates may reach as high as 15% (Katzan et al., 2000). Mechanistically, tPA may induce hemorrhage by directly damaging the basal lamina of blood vessels, leading to edema, disruption of the blood-brain barrier (BBB), and hemorrhage via extravasation of blood into the cerebral parenchyma (Adibhatla et al., 2008). In addition, tPA directly activates matrix metalloproteases (esp. MMP-9) that degrade the extracellular membrane and vascular basement membrane, also leading to hemorrhage (Benarroch, 2007).

Unfortunately, no intervention exists that can reverse the effects of tPA in the setting of acute hemorrhage. In addition, treatment of the hemorrhage itself is less than optimal. Although supportive care with fresh frozen plasma and platelets, as well as surgical evacuation of the hemorrhage may be undertaken, each has its own risks. In
response to this paucity of treatments, recombinant factor VIIa (rFVIIa), which is FDA approved to mitigate hemorrhage in FVIIa-deficient hemophiliacs, was administered to the general population for off-label treatment of intracranial hemorrhage (Mayer et al., 2005). However, it was recently noted that although use of rFVIIa reduced the size of the hematoma, it did not improve survival or functional outcome (Mayer et al., 2008). As a result, thrombolysis-related sICH remains associated with an overall mortality rate of 59.7% (Sloan et al., 1998).

For these reasons, immediate reversibility of the interventional agent in the setting of intracranial hemorrhage would introduce a substantial increase in safety. Thus, a therapeutic strategy that both facilitates reperfusion and also has the potential for immediate reversal would represent a clear advance for stroke therapy. One method of promoting reperfusion in the setting of acute stroke that has the potential for rapid reversibility is the use of aptamer-based therapeutics. Because aptamers are nucleic acid ligands, complimentary oligonucleotides can be designed to bind to the aptamers, which alter their conformation and thus render them inactive (Rusconi et al., 2002). Therefore, these rationally designed “antidotes” can be administered as reversal agents.

The Sullenger laboratory demonstrated this successfully with respect to inhibition of factor IXa; binding of the rationally designed complimentary oligonucleotide to the aptamer caused a conformational change that rendered it inactive and thus reversed its inhibitory activity (Rusconi et al., 2002; Nimjee et al., 2005). Furthermore, the Sullenger laboratory has translated the concept of using an aptamer-antidote pair in vivo through successful demonstration of factor IXa inhibition and reversal in porcine systemic anticoagulation models (Rusconi et al., 2004), murine arterial thrombosis and tail transaction models (Rusconi et al., 2004), and porcine
coronary artery bypass models (Nimjee et al., 2006). Currently, this factor IXa aptamer/antidote pair is in Phase II clinical trials.

It was previously shown that administration of the factor IXa aptamer following 60-minute middle cerebral artery occlusion in mice improved neurological function, reduced thrombin generation and decreased systemic inflammation. Given the need for reversible therapeutics for stroke should hemorrhage arise, I tested the FIXa aptamer/antidote in a murine subarachnoid hemorrhage model to determine the antidote’s ability to improve outcome following aptamer-exacerbated hemorrhage. I found that antidote administration not only reduced the size of the hematoma formed, but also improved survival. Therefore, the ability to rapidly reverse an agent administered for treatment of stroke should hemorrhage arise represents a novel and potentially safer approach to enhance reperfusion in the setting of acute stroke.
5.2 Materials and Methods

Induction of Subarachnoid Hemorrhage

All animals received humane treatment in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, as approved by the Duke University Animal Care and Use Committee. Prior to induction of hemorrhage, wild type male C57Bl/6J mice ages 10-12 weeks (Jackson Laboratory, Bar Harbor, ME, USA) were injected with 2 mg/kg of the cholesterol modified aptamer generated against factor IXa, Ch-9.3t, or an equivalent amount of the non-functional mutant aptamer, Ch-9.3tM, intravenously via tail vein. For induction of hemorrhage, mice were anesthetized with 5% isoflurane in a 30% O₂:70% N₂ mixture. The trachea was then intubated with a 20-gauge intravenous catheter (Insyte-W, Becton Dickinson). Next, the inspired isoflurane concentration was decreased to 1.5% and the lungs were mechanically ventilated at a rate of 130 breaths per minute at a tidal volume of 0.7 mL. Rectal temperature was monitored and servoregulated at 37°C via surface heating and cooling. A midline neck incision was made and the right common carotid artery was isolated. Following isolation and ligation of the right external carotid artery with temporary occlusion of the right common carotid artery, a silicon-coated 6-0 nylon monofilament was inserted into the right external carotid artery and advanced via the internal carotid artery to the bifurcation of the middle cerebral artery (MCA) and the anterior cerebral artery (ACA). Next, the filament was advanced further to puncture the MCA/ACA bifurcation in order to allow for extravasation of blood into the subarachnoid space and was immediately withdrawn; the midline incision was then closed via interrupted skin sutures.
Aptamers and Antidote:

Aptamer Ch-9.3t is a 35-nucleotide length RNA with sequence 5’-AUG GGG ACU AUA CCG CGU AAU UGC UGC CUC CCC AU-3’ that was modified with 2’-fluoropyrimidines and a 3’ inverted thymidine cap to render nuclease resistance, as well as a 5’ cholesterol moiety to increase its circulating half-life. The mutant aptamer Ch-9.3tM, 5’-AUG GGG ACU GUG CCG CGU AAU UGC UGC CUC CCC AU-3’, is of the same composition as Ch-9.3t with exception of two nucleotides that were changed (denoted in bold) which rendered the aptamer unable to bind to factor IXa and hence inactive as an antithrombotic agent. Antidote oligonucleotide 5-2C is composed of 2’-O-methyl RNA and has a sequence of 5’-CGC GGU AUA GUC CCC AU-3’. Upon binding to Ch-9.3t, 5-2C causes unfolding of the three-dimensional structure of the aptamer and diminishes binding to factor IXa, thus reversing aptamer activity.

Mortality Levels:

Mortality levels were recorded as the number of mice that died within 90-minutes of hemorrhage induction; at that time point, all living mice were sacrificed to determine subarachnoid hemorrhage grade.

Assessment of subarachnoid hemorrhage grade:

Mice subjected to subarachnoid hemorrhage (SAH) were sacrificed via decapitation 90-minutes post induction of hemorrhage. Brains were then dissected and analyzed for determination of SAH grade by an observer blinded to the treatment group. Briefly, the hemorrhage grade was assessed and scored according to the following scale: 0- absence of hematoma, 1- hematoma with a diameter < 1 mm, 2-
hematoma diameter measured 1-2 mm, 3- hematoma diameter measured 2-3 mm, 4-
diameter measured 3-4 mm, 5- diameter measured > 4 mm.

Statistical Analyses:

Data are expressed as mean ± SEM unless otherwise noted. Mortality was
analyzed using Chi-squared analysis. Non-parametric hemorrhage grade values were
analyzed using the Kruskal-Wallis test. P < 0.05 was used as an indication of statistical
significance.
5.3 Results

5.3.1 Antidote reversal decreases hemorrhage grade

The aptamer generated against factor IXa was previously shown to improve neurological function while reducing thrombin generation and systemic inflammation when administered following stroke. To expand upon these results, the aptamer was administered followed by induction of subarachnoid hemorrhage in order to recapitulate a situation where the aptamer was used to treat stroke and bleeding ensued. The antidote oligonucleotide (5-2C) was then given to determine if reversal of aptamer activity would improve outcome in the setting of active bleeding. As depicted in the experimental timeline in figure 5-1, mice received either aptamer Ch-9.3t (2 mg/kg) or the non-functional mutant aptamer (2 mg/kg) via tail vein. Following induction of subarachnoid hemorrhage, mice that were given the aptamer received either PBS (aptamer group) or antidote 5-2C (50x molar excess; 46.8 mg/kg) to reverse the activity of the aptamer (aptamer/antidote group); mice given the mutant aptamer received PBS post SAH (mutant aptamer group). Ninety minutes following induction of SAH, the hemorrhage grade of each mouse was assessed. Figure 5-2 shows the aptamer group with a median hemorrhage grade of 4 as opposed to the mutant aptamer group that had a median hemorrhage grade of 2 (P= 0.001, Kruskal-Wallis test). However, antidote administration following aptamer use and induction of hemorrhage reduced the median hemorrhage grade to 3 and was no longer statistically different from the mutant aptamer group (P> 0.05). Hemorrhages are also seen in Figure 5-3 as representative hematomas for each group.
Figure 5-1: Murine subarachnoid hemorrhage experimental timeline. The FIXa aptamer (Ch-9.3t) or a non-active, mutant aptamer was administered to wild-type C57Bl/6 mice via tail vein injection. Following induction of hemorrhage, the mice were then given the antidote (5-2C) or PBS. Hemorrhage grades and mortality levels were noted at 90-minutes post hemorrhage induction.
Figure 5-2: Antidote administration following aptamer use and subsequent SAH decreases hemorrhage grade. Following sacrifice, subarachnoid hemorrhage grade (range, 1-5) was assessed 90 minutes post hemorrhage induction. Mice that were given the Ch-9.3t aptamer (2 mg/kg) before hemorrhage, but following hemorrhage were administered the antidote (50x molar excess; 46.8 mg/kg), had significantly smaller hemorrhages. Circles (○), triangles (▲), and squares (■) represent the hemorrhage grades of mice that received the mutant aptamer, aptamer, or aptamer and antidote, respectively. n= 7, aptamer group; n=6 for aptamer/antidote and mutant aptamer groups; means ± SEM are shown. Kruskal-Wallis test, P= 0.001; **P< 0.01 for aptamer vs. mutant aptamer.
Figure 5-3: Antidote administration mitigates the increase in hematoma size following aptamer use and subsequent SAH. Representative pictures of murine brains with hematomas for the respective treatment groups. a. mutant aptamer group, b. aptamer group, c. aptamer/antidote group.
5.3.2 Antidote reversal improves survival

In addition, mortality was assessed 90-minutes post induction of hemorrhage. As seen in Table 5-1, similar to other reperfusion techniques, the factor IXa aptamer-based anticoagulant worsened mortality in the setting of active intracranial bleeding; however administration of a specific antidote was associated with rapid reversal of the anticoagulated state, which resulted in a subsequent improvement in survival ($P=0.0283$, Chi-squared test).

Table 5-1: Antidote administration mitigates increase in mortality following aptamer-exacerbated hemorrhage

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>90-min mortality</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aptamer</td>
<td>7</td>
<td>5/7</td>
<td>71%</td>
</tr>
<tr>
<td>Aptamer/Antidote</td>
<td>6</td>
<td>2/6</td>
<td>33%</td>
</tr>
<tr>
<td>Mutant Aptamer</td>
<td>6</td>
<td>0/6</td>
<td>0%</td>
</tr>
</tbody>
</table>
5.4 Discussion

I have previously demonstrated the ability of the factor IXa aptamer to improve neurological function and reduce inflammation in a murine model of stroke. Here, I expanded on those results and demonstrated that the aptamer can be rapidly deactivated should hemorrhage arise. As a result, morbidity and mortality due to anticoagulant-exacerbated intracranial hemorrhage is reduced. A modified version of aptamer Ch-9.3t has successfully demonstrated the ability to anticoagulate humans without adverse effects, and use of its antidote fully reverses aptamer activity within 1-5 minutes (Dyke et al., 2006). In addition, multiple consecutive factor IXa aptamer-antidote injections can be administered without major bleeding or other serious adverse events, demonstrating the ability to anticoagulate, reverse, and anticoagulate again if needed (Chan et al., 2008b). Moreover, this aptamer/antidote pair was deemed safe and effective for use in patients with stable coronary artery disease concomitantly using antiplatelet agents (Chan et al., 2008a).

The enhanced safety afforded by the ability to reverse the activity of the aptamer is one of the most compelling features of this therapeutic strategy. When placed in an experimental model of subarachnoid hemorrhage, antidote reversal of aptamer activity reduced the size of the hematoma as evidenced by decreased hemorrhage grades (figure 5-2). Another treatment used clinically to decrease the size of hematomas, given the lack of effective treatment of intracranial hemorrhage, is the off-label administration of exogenous clotting factors. For example, recombinant Factor VIIa (rFVIIa) was used to mitigate intracranial bleeding in patients other than FVIIa-deficient hemophiliacs. Although exogenous clotting factors may be applied to mitigate any hemorrhage, as opposed to those exacerbated from previous aptamer administration, differences in the
mechanism of action of clotting factors versus oligonucleotide antidotes confer antidotes greater safety while maintaining efficacy.

Antidote oligonucleotides are small (~15 nucleotide) nucleic acids that bind solely to the aptamer against which it was designed, altering the three dimensional structure of the aptamer, thus rendering it inactive (Rusconi et al., 2002). In the case of the factor IXa aptamer, thrombin generation was shown to decrease, allowing the subject to enter into a state of anticoagulation. Upon addition of the antidote, only the previously administered aptamer is inhibited, freeing endogenous factor IXa to generate thrombin at the site of injury. For this reason, coagulation parameters are restored to their “pre-aptamer” state and allowed to function in a manner appropriate to the insult. In contrast, addition of an exogenous clotting factor causes systemic coagulation, mitigating bleeding through the generation of supraphysiologic levels of thrombin. Both pathways lead to the formation of a fibrin meshwork used to effectively “patch” the area of denuded endothelium thereby preventing the extravasation of blood. However, given this mechanism of action, it is possible to ‘overshoot’ the level of coagulation needed to reduce bleeding, thus provoking rebound thrombosis. Unfortunately, this is not uncommon as administration of rFVIIa is associated with increased venous and arterial thromboembolism (O’Connell et al., 2006; Diringer et al., 2008). For these reasons, use of the aptamer and antidote would pose a safety advantage over clotting factor administration.

In the 2005 Phase II clinical trial, use of rFVIIa was associated with a decrease in hematoma volume, similar to that noted upon antidote administration (Mayer et al., 2005). However, a larger Phase III clinical trial that expanded the time frame for monitoring survival and functional outcome from 30 days to 90 days demonstrated that although use of rFVIIa again reduced hematoma volume, this was not associated with a
decrease in mortality or improvement in functional outcome (Mayer et al., 2008). As noted in Table 5-1, administration of the antidote translated to a significant decrease in mortality.

This is the first application of a reversible, aptamer-based therapeutic strategy for treatment of acute stroke. I have shown that in addition to the aptamer’s ability to improve outcome following stroke, should bleeding occur, administration of its antidote not only decreases the hemorrhage grade, but also reduces mortality levels. The ability to rapidly reverse treatment without risk of rebound thrombosis improves the safety profile of the drug and renders aptamer based-therapeutics as an attractive approach for treatment of stroke.
6. Conclusions and Perspectives

6.1 Aptamer Inhibition of PAI-1

By utilizing the SELEX process, I have developed aptamers that bind to PAI-1 with high affinity and specificity in a region that spans the heparin and vitronectin (VN) binding sites. Moreover, this binding precludes heparin and vitronectin binding to PAI-1. The presence of the aptamers at the vitronectin-binding site acts somewhat similar to vitronectin in that they decrease the ability of PAI-1’s reactive center loop to enter β-sheet A. Because of this, PAI-1 cannot take on its latent conformation and remains active. In addition, the aptamers did not affect PAI-1’s ability to directly inhibit uPA. Therefore, PAI-1’s antiprotease activity remains intact while the aptamers prevent PAI-1 from binding to vitronectin.

Disruption of the PAI-1/VN interaction then translated to an increase in breast cancer cell adhesion in an environment of PAI-1 mediated cellular detachment. This may be due to the cells binding to vitronectin via their uPAR receptors, which was previously hindered because PAI-1 was binding to vitronectin, sterically blocking uPAR access to vitronectin. Additionally, PAI-1 is free to inhibit uPA, possibly decreasing plasmin and matrix metalloprotease production, which reduces the degradation of the extracellular matrix. This keeps the extracellular environment in a structure less amenable to cellular metastasis. In this manner, administration of aptamer SM-20 addresses both sides of the PAI-1 paradox, that is, both elevated uPA and PAI-1 as poor prognostic indicators for breast cancer, by inhibiting the pathologic effects of excess uPA while increasing cellular uPAR access to extracellular matrix-bound vitronectin.

Another plausible explanation is that the aptamers also bind to the second vitronectin binding site on PAI-1, although with reduced affinity (Schar et al., 2008). This
would be in addition to binding the primary vitronectin binding domain (α-helix E), as the aptamers affect PAI-1’s interaction with both heparin and vitronectin, whose binding domains are juxtaposed (α-helices D and E). To determine if the aptamers bind to more than one vitronectin binding site, a binding assay using a mutant version of PAI-1 that has diminished binding to vitronectin across its somatomedin B domain, as well as reduced heparin binding could be performed.

With the method of SELEX utilized in these experiments, it is difficult to predict an aptamer’s binding location. It was somewhat serendipitous that the aptamer’s bound in a location where it would interrupt a protein-protein interaction, though heparin-binding sites (and regions of positive charge in general) tend to be “aptogenic” in nature, attracting aptamer binding to those regions. In addition to being unable to predict with assurance the binding location of an aptamer, binding, even with high affinity, does not necessarily translate to function. As was seen with several aptamers selected against PAI-1 that bound with high affinity, not all were able to disrupt the PAI-1/vitronectin interaction. Moreover, WT-15, which was completely active in inhibiting the interaction between PAI-1 and vitronectin in several assays, was unable to restore breast cancer cell adhesion. This disconnection between binding and function is partially a result of the screening process used to generate the aptamers, as binding is the partitioning mechanism used to separate RNA. One possible means to increase the number of functional aptamers is to use a functional assay as the partitioning mechanism. In this situation, the ideal assay would retain all RNA that increased cell adhesion, while discarding those that did not.

Although binding affinity was used to select these aptamers to PAI-1, one molecule was shown to increase breast cancer cell adhesion in an environment of PAI-1 mediated cell detachment. Therefore, these developmental steps could lead to a novel antimetastatic agent that has low to no toxicity and is non-immunogenic, making it an
attractive agent to serve as an adjuvant to traditional breast cancer therapy. Moreover, there may be application to additional cancers such as pulmonary adenocarcinoma, ovarian cancer, and urinary tract cancer, where elevated PAI-1 and uPA also serve as poor prognostic indicators. However, before the claim that aptamer SM-20 can translate to an antimetastatic agent for breast cancer can be valid, several additional assays must be performed. First, only one breast cancer cell line (MDA-MB-231) was used. Additional cell lines such as MCF-7, T-47D, BT-474, SK-BR-3, and Hs578T would be viable options for additional assessment of aptamer-mediated breast cancer cell adhesion. Moreover, SM-20 is stated to have the potential to decrease metastasis, but that is thus far based on the inference that an increase in adhesion translates to a decrease in metastasis. Although logical, PAI-1 plays a direct role in cell detachment as well as decreasing adhesion. Therefore, cell migration assays that measure the degree to which cells translocate from their original positions should also be performed. Moreover, the aptamer should demonstrate efficacy in ideally more than one in vivo model of breast cancer cell metastasis.

In preparation for in vivo experimentation, aptamer SM-20 would need to undergo optimization through truncation to allow for greater ease of commercial synthesis, and chemical modification (cholesterol addition or pegylation, capping the ends of the oligonucleotide, etc.) to increase the circulating half-life of the aptamer. After these modifications are performed, the aptamer should be re-tested in the cell adhesion assays to assure function before proceeding with in vivo examination. Unfortunately, only SM-20 was functional; had there been additional functional aptamers, truncation could proceed in a more rational manner, examining which regions were conserved across all functional aptamers. These additional aptamers possibly would have been identified if more aptamers were screened during the cloning process or if more aptamers were assayed for functionality. Although further cloning may generate new
sequences that may be functional, a better approach is to perform a “doped selection”, using the sequence of SM-20 as a template. Clones should then be screened immediately for activity in a breast cancer cell adhesion assay.

Although not imperative for producing an effective antimetastatic agent, in order to uncover the mechanism of aptamer-mediated increases in cell adhesion, co-crystallization of the aptamers with PAI-1 to confirm binding locations, electron micrograph evidence of intact extracellular matrices surrounding the tumor due to decreased plasmin degradation, and confirmation of enhanced uPAR adhesion to vitronectin via immunohistochemical analysis would be areas of further investigation.

In addition to a clinical application, this research has implications in advancing basic science as well. As the first agent that disrupts the PAI-1/VN interaction without compromising protease activity, this aptamer could serve as an invaluable in vitro research tool. As such, further characterization of the interaction between the aptamer and PAI-1, and how vitronectin affects that interaction, should be performed. For example, although we know that the aptamers have a higher affinity for PAI-1 than vitronectin (mid pM vs. 1 nM), what are the binding rates of the aptamers vs. those of VN? If both the aptamers and VN were present in equimolar amounts, what binding would prevail based on those binding rates and affinity constants?

The first topic of my thesis work, generating an aptamer against PAI-1, has produced agents that disrupt the interaction between PAI-1 and VN, while retaining PAI-1’s protease activity. Although further research and development is necessary, these aptamers may translate to novel in vitro research tools and safer antimetastatic agents to supplement current chemotherapy.
6.2 Antidote-Controlled Aptamer Treatment of Stroke

In the second portion of the thesis, I have demonstrated that administration of the factor IXa aptamer post ischemia improves neurological function while decreasing thrombin levels and systemic inflammation in a murine model of middle cerebral artery occlusion (MCAO). Moreover, aptamer use did not increase the incidence of intracranial hemorrhage or increase mortality levels. In addition, should bleeding occur, administration of its antidote not only decreases hemorrhage grade, but also reduces mortality levels.

Although aptamer administration following MCAO engendered an improvement in neurological function, it did not result from a reduction in infarct volume. There are several plausible explanations for this phenomenon, all leading to additional experimentation for verification. First, as an antithrombotic agent, inhibition of factor IXa may function through decreasing microthrombi formation in small blood vessels and capillaries, thereby acting as a reperfusion agent. If so, it is possible that the model used would not allow for uncovering smaller changes in vessel perfusion that may affect patches of neurons distal to the core, as opposed to the area immediately affected by filamentous occlusion. Staining for fibrin deposition would allow for discrimination of microthrombi, and the groups that received the aptamer versus the non-functional mutant aptamer could be compared.

In addition, reductions in inflammation were noted, though the actual mechanism linking improvement in neurofunction with reduced inflammatory markers was not fully elucidated. It is possible that a decrease in inflammation translated to a diminution of the “no-reflow phenomenon”, allowing for greater reperfusion. In conjunction, a decline in the presence of inflammatory cells would lessen the amount of
free radicals and reactive oxygen species (ROS) present, thus sparing neurons from damage and death. One method of measuring ROS is to use a luminol-dependent chemiluminescence assay (Kobayashi et al., 2001) while contrast-enhanced ultrasound using microbubbles can measure flow within the microcirculation, serving as a means to measure the “no-reflow” phenomenon (Rim et al., 2001).

In regards to the subarachnoid hemorrhage findings of reduced mortality with antidote administration, one could assume that survival was improved as a direct result of mitigating hematoma growth. Although directly related, the two are not necessarily linked in a causative manner, as Mayer et al. have reported significant reductions in hematoma volume following hemorrhage that did not translate to improvements in survival (Mayer et al., 2008). As thrombin generation has been responsible for early brain edema formation due to a direct effect of opening the blood-brain barrier (Hua et al., 2007), it is possible that prior use of the aptamer reduced thrombin generation such that there was less edema following hemorrhage, and in conjunction with decreasing hematoma size from use of the antidote, survival improved. To investigate edema formation surrounding the hemorrhage, MRI could be performed to quantitate the amount of edema, comparing the aptamer to mutant aptamer groups.

One limitation of the study concerns the design of the neurofunctional assay. In this study, mice that died prior to completing the three-day evaluation received the lowest score. This was done to decrease the amount of error and bias that would have been present if the sickest mice that eventually died were excluded from the evaluation. Because these mice were included, neurofunction results are inextricably linked to mortality levels. For example, rotarod results for mice subjected to SAH would not have been valid as more than 70% of the mice in one group died; had they all been given the lowest possible score, those results would add no additional information concerning differences in neurological function. However, because no difference in mortality levels
existed in the mice that underwent MCAO, their neurofunction results can be said to truly represent a difference between the treatment groups. Ideally, one would use a functional assay that is sensitive enough to detect small changes in neurofunction and could be administered early. One crude assay used to judge stroke failure, evidence of hemiparesis that causes the animal to walk in a circle, is noticeable as soon as the mice recover from anesthesia. As such, noticeable differences in neurofunction are present early, and possibly a quantitative assay could be designed that may specifically assess these neuronal changes.

Although an acute assessment of neurological function would address biases and limitations concerning the incorporation of mortality data, a longer term, longitudinal assay should also be performed to determine the long-term effects of the intervention. While this is obviously subjected to the abovementioned conundrum of mortality, it is possible that with a finer-tuned assay, a lesser number of animals would be required and more subtle changes would be uncovered. As mentioned above, MRI could be used to determine edema formation, infarct resolution, and hemorrhagic transformation in mice over several days to weeks. These parameters would therefore not only be measured and compared in a static manner, as was done in this study, but an additional dimension of time could be added. This would allow for a greater understanding of not only the spatial aspects of improvement (i.e., infarct volume), but also their temporal characteristics. For example, although no difference in infarct volume was noted at 48-hours, it is possible that the aptamer slowed initial progression to infarct development which could allow for additional intervention to spare the penumbra. Another possibility is that edema formation was reduced or delayed during a critical period of neuronal vulnerability, or possibly the infarct would regress faster due to aptamer administration—these underlying mechanisms of action and differences between
treatment groups would only be uncovered if a temporal aspect was also integrated into the assay.

According to the Stroke Therapy Academic Industry Roundtable (STAIR) committee, experimental treatments of stroke should be tested in more than one animal model in order to increase its likelihood for positive translation to clinical trials (Roundtable, 1999). As an \textit{in situ} thrombus more closely mimics the pathophysiology of clinical stroke and would be more amenable to vascular recanalization than an occlusive filament, future directions may include a thromboembolic model of stroke for further investigation of not only the factor IXa aptamer, but also combinations of antithrombotic aptamers and antiplatelet agents. In addition, although preclinical models of SAH recapitulate large vessel bleeding, true hemorrhagic conversion of an infarct leads to intracranial hemorrhage. Some have modeled ICH with intracranial injection of blood, but that does not speak to the mechanism of active bleeding and mitigation of thrombus growth. Use of collagenase to destroy blood vessels and thus induce ICH also does not recapitulate reversible bleeding, as the capillaries and vessels in the target area are irreversibly damaged. An ideal hemorrhage model would therefore allow for correct placement (and progression of the hematoma if inciting factors such as anticoagulation were present), as well as a hemostatic vascular response to mitigate bleeding.

In addition to acute ischemic stroke and reversing activity in the setting of hemorrhage, this aptamer/antidote pair could potentially be used to replace anticoagulation with heparin for treatment or prevention of other cerebrovascular diseases in which anticoagulation is indicated (Albers et al., 2008; Redekop, 2008). In general, heparin has been shown to have some beneficial effects in the abovementioned circumstances by reducing the recurrence of thromboembolic complications, but these effects are often offset by increased rates of hemorrhage (Lyrer et al., 2003; Albers et al., 2008). Although the anticoagulant effect of heparin can be abated with use of protamine,
in practice this is rarely performed due to the risk of severe complications; in addition to allergic reactions and hemodynamic compromise, protamine is associated with increased pulmonary artery pressure and decreased systolic and diastolic blood pressure, as well as decreases in myocardial oxygen consumption, cardiac output, heart rate, and systemic vascular resistance (Carr et al., 1999; Nimjee et al., 2006). Thus in addition to avoiding these adverse events, an aptamer-based strategy may also reduce the risk of other complications associated with the use of heparin, such as thrombocytopenia.

This is the first application of a reversible, aptamer-based therapeutic strategy for treatment of acute stroke. Given its ability to improve neurological function while decreasing inflammation, the factor IXa aptamer demonstrates potential as a novel reperfusion agent for acute ischemic stroke. However in general, anticoagulation strategies have not demonstrated benefit in the setting of acute stroke (Adams et al., 2007). In part, the failure of anticoagulation and antiplatelet strategies in the treatment of stroke are a result of the associated risk of intracranial hemorrhage. In response, the ability to rapidly reverse this treatment without risk of rebound thrombosis improves the safety profile of the drug and renders aptamer based-therapeutics as an attractive approach for treatment of stroke.
6.3 Summary

Aptamer-based therapeutics is an emerging field that allows for a variety of protein targets and increased safety through antidote reversal. My thesis project has focused on generating aptamers to PAI-1 and advancement of the factor IXa aptamer and antidote in murine models of middle cerebral artery occlusion and subarachnoid hemorrhage. As a result of these studies, an aptamer has been generated against PAI-1 that shows potential as an antimetastatic agent for breast cancer, and the factor IXa aptamer and antidote demonstrate the ability to not only improve outcome following stroke, but also mitigate subsequent bleeding should it arise. With additional preclinical development, the results of my thesis work have the ability to positively impact not only oncology and neurology, but also medicine in general, as advances in aptamer-based therapeutics could lead to a safer method of treating a multitude of diseases, with targets limited only by the imagination of the investigator.
Appendix A (GPVI SELEX)

Introduction

Thrombosis is the most common cause of mortality in the Western world, as it is responsible for 20-25% of deaths. Although there is no shortage of antithrombotic agents, almost all are limited by their most common adverse event—uncontrolled bleeding. Therefore, novel antithrombotic agents must be not only efficacious, but also safer than what is currently available. One method to increase safety is to regulate a drug’s activity, particularly to reverse its action should an adverse event arise. For this reason, antidote-controlled antithrombotic agents may fulfill this unmet clinical need.

Once vascular injury occurs, subendothelial components such as collagen and tissue factor activate platelets and coagulation, mediating thrombus formation. As collagen is the most thrombogenic component of the subendothelium, inhibition of thrombus formation due to collagen exposure is of great interest.

Glycoprotein (GP) VI is a platelet surface receptor that is 62 kDa when fully glycosylated, as approximately 45% of its mass is comprised of carbohydrate residues (Moroi et al., 2004). It is the major activating receptor for collagen, inducing collagen-mediated platelet adhesion, spreading and activation (Inoue et al., 2006). For functionality, although not necessary for expression, GPVI must form a complex with the immunoreceptor tyrosine-based activation motif (ITAM)-containing subunit, the FcRγ-chain (Moroi et al., 2004). In addition, GPVI forms a back-to-back dimer in order to exhibit high affinity binding to collagen (Horii et al., 2006). Once bound, the platelet becomes activated through an outside-in tyrosine-kinase mediated signaling cascade.
GPVI-null mice demonstrate no-to-moderate increases in bleeding time; however under flow conditions, platelets neither form aggregates nor spread, and exhibit reduced adhesion (Kato et al., 2003). All humans with GPVI deficiencies demonstrate reduced platelet aggregation in response to collagen with some degree of a bleeding diathesis. There are several mechanisms by which platelets may become non-responsive to collagen through GPVI, as some patients demonstrate idiopathic thrombocytopenic purpura due to autoantibodies that clear the GPVI/FcRγ complex from the surface of proteins (Boylan et al., 2004), while others express GPVI and FCRγ, but defective GPVI-mediated signal transduction does not propagate the effects of GPVI binding to collagen (Dunkley et al., 2007).

Monoclonal antibodies or Fab fragments of antibodies have mediated most inhibition of GPVI. Most widely studied is JAQ1, which has been thoroughly tested in several in vitro, ex vivo and in vivo models of thrombosis and cerebral ischemia/reperfusion (Nieswandt et al., 2000; Nieswandt et al., 2001a; Nieswandt et al., 2001b; Schulte et al., 2001; Snell et al., 2002; Massberg et al., 2003; Schulte et al., 2003; Schulte et al., 2006; Kleinschnitz et al., 2007; Wong et al., 2008). The Fab fragment of OM4, another monoclonal antibody raised against GPVI, has also shown to be a viable option as it has reduced arterial thrombosis in rats without increasing bleeding (Li et al., 2007), while another Fab fragment inhibits the functions of GPVI in vitro and ex vivo in non-human primates (Ohlmann et al., 2008).

While antibodies certainly prevail as the most common means of disrupting the function of GPVI, other interesting compounds, such as anopheline antiplatelet protein (AAPP), a protein found in the saliva of malaria-vector mosquitoes, inhibits platelet aggregation in CIPA as it binds to collagen, precluding GPVI binding (Yoshida et al., 2008). Utilizing a different pathway, G6b-B is an immunoglobulin receptor that was
found to inhibit the constitutive signaling of GPVI through Src and Syk pathways (Mori et al., 2008).

Given the role of GPVI in platelet adhesion and activation, and the results of its inhibition in several models, GPVI may be an ideal target for prevention of vascular thrombosis. However, GPVI/FcRγ-null mice did not exhibit protection against thrombosis in three different murine models of deep vascular injury, though thrombus size was reduced by 30% when injuries were more superficial in nature (Mangin et al., 2006). This suggests that inhibition of GPVI may be best served for less severe vascular injury (i.e., as a chronic, prophylactic therapeutic agent) as opposed to applications with deep vascular injury (i.e., surgical insults), which may require additional antithrombotic agents to completely prevent thrombus formation.

Aptamers, single stranded nucleic acid ligands that bind to their targets with high affinity and specificity, may be ideal for GPVI inhibition as they possess the affinity properties of monoclonal antibodies without having to be raised intracellularly. In addition, their bioavailability can be adjusted and they are non-immunogenic. Moreover, as aptamers are primarily negatively charged due to their phosphate groups, they may tend to bind to positively charged amino acids K41, R60, R166, K59 and R117 that mediate the binding between GPVI and collagen, CRP, and calmodulin (O'Connor et al., 2006). This location happens to lie on the apical portion of the protein, rendering it accessible to aptamer binding. In addition, as aptamer activity can be reversed with a paired antidote or possibly a universal antidote (Oney et al., submitted), aptamer inhibition of GPVI may produce a novel, efficacious antiplatelet agent with enhanced safety.
Materials and Methods

Generation of Aptamers using Systematic Evolution of Ligands by Exponential enrichment (SELEX)

The sequence of the starting RNA library (denoted Sel2 Library) was 5'-GGGAGGACGATGCGG-N_{40}-CAGACGACTCGCTGAGGATCC-3', where “N_{40}” denotes a random region 40-nucleotides in length. The RNA consisted of 2'-fluoropyrimidines (2'-fluorocytidine triphosphate and 2'-fluorouridine triphosphate; Trilink Biotechnologies, San Diego, CA) in order to render the RNA nuclease resistant. Selection began against purified, non-glycosylated GPVI (denoted cGPVI; kindly provided by Dr. Andrew Herr, University of Cincinnati; Horii et al., 2006). Following round three, the selection diverged, with one arm continuing selection against cGPVI (cR#), and the other arm (tR#) using commercially available histidine-tagged, partially glycosylated GPVI (denoted hGPVI; R&D Systems Inc., Minneapolis, MN) for round four only; all subsequent rounds used cGPVI (see Table 1). For both selections, the RNA library was incubated with its respective protein for 15 minutes at 37°C; unbound RNA was separated from RNA/GPVI complexes by passing the mixture over a nitrocellulose membrane. All rounds proceeded in BSA binding buffer E (20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM CaCl_2, and 0.1% bovine serum albumin [BSA]).

Binding assays.

Progression in binding was determined using nitrocellulose filter binding assays as previously described (Rusconi et al., 2000). Briefly, RNA was first dephosphorylated using bacterial alkaline phosphatase (Gibco BRL, Gathiernburg, MD) and 5’ end labeled with [γ-32P] ATP (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) using
T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Round pools of radiolabeled RNA were incubated with increasing amounts of cGPVI protein in BSA binding buffer E for 5 minutes at 37°C; the mixture was then passed over a nitrocellulose membrane. The fraction of RNA that bound to the protein was quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Nonspecific binding of the RNA was subtracted out such that only specific binding remained (denoted ‘corrected fraction bound’).

Collagen-induced platelet aggregation (CIPA) assay

Collagen-induced platelet aggregation was performed using platelet rich plasma (PRP) from healthy volunteers. Round pools cR6 and tR6 (1 μM) were mixed with 400 μL of PRP in a flat bottom glass tube and allowed to incubate for five minutes. Collagen was then added to a final concentration of 2 μg/mL. The PRP was stirred using a steel stir bar at 37°C and turbidity was monitored as percent light transmitted for 10 minutes.
Results

Successive rounds induce shifts in binding to GPVI

In order to generate aptamers that bound to GPVI with high affinity, two forms of SELEX (traditional and toggle) was performed against the human protein. The first three rounds used solely the human, non-glycosylated form of GPVI (cGPVI). After round three, the selection divided into two; the first continuing with cGPVI (cR#), and the second using a partially glycosylated, histidine-tagged form of the protein, hGPVI (tR#). All subsequent rounds were performed with cGPVI (see table A-1). First, a starting RNA library modified with 2'fluoropyrimidines was bound to cGPVI; the RNA that bound to the protein was separated from unbound RNA using a nitrocellulose membrane as described in Materials and Methods. By increasing the stringency in subsequent rounds, the binding affinity of each round increased (see Fig. A-1).

Figure A-1: Round binding of GPVI SELEX. The progress of the selection against GPVI was followed using a nitrocellulose filter binding assay. Diamonds (♦) represent the starting RNA library (Sel2), inverted triangles (▼) represent round three (cR3), triangles (▲) represent toggled round 5 (tR5), and squares (■) represent round five (cR5). The x-axis shows the cGPVI concentration and the y-axis shows the fraction of RNA bound to the protein.
Rounds cR1b-3 proceeded without incident. At round four, the selection diverged into two, as cR4 proceeded as before with cGPVI, and tR4 used hGPVI. Following that round, the background binding level of tR5 reached levels greater than 10%, demonstrated unacceptably high background binding levels.

<table>
<thead>
<tr>
<th>Round</th>
<th>Protein used</th>
<th>Target binding</th>
<th>[Protein] (μM)</th>
<th>Buffer</th>
<th>Background binding (avg)</th>
<th>RNA used for round</th>
</tr>
</thead>
<tbody>
<tr>
<td>cR1b</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.25</td>
<td>E</td>
<td>2%</td>
<td>Sel2 library</td>
</tr>
<tr>
<td>cR2</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.35</td>
<td>E</td>
<td>3%</td>
<td>cR1</td>
</tr>
<tr>
<td>cR3</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.3</td>
<td>E</td>
<td>2%</td>
<td>cR2</td>
</tr>
<tr>
<td>cR4</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.25</td>
<td>E</td>
<td>2%</td>
<td>cR3</td>
</tr>
<tr>
<td>tR4</td>
<td>hGPVI</td>
<td>20%</td>
<td>0.05</td>
<td>E</td>
<td>4%</td>
<td>cR3</td>
</tr>
<tr>
<td>cR5</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.15</td>
<td>E</td>
<td>6%</td>
<td>cR4</td>
</tr>
<tr>
<td>tR5</td>
<td>cGPVI</td>
<td>20%</td>
<td>0.15</td>
<td>E</td>
<td>14%</td>
<td>tR4</td>
</tr>
<tr>
<td>cR6</td>
<td>cGPVI</td>
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<td>0.07</td>
<td>E</td>
<td>12%</td>
<td>cR5</td>
</tr>
<tr>
<td>tR6</td>
<td>cGPVI</td>
<td>15%</td>
<td>0.07</td>
<td>E</td>
<td>21%</td>
<td>tR5</td>
</tr>
<tr>
<td>cR6.3</td>
<td>cGPVI</td>
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<td>0.07</td>
<td>E</td>
<td>20%</td>
<td>cR5</td>
</tr>
<tr>
<td>cR7</td>
<td>cGPVI</td>
<td>15%</td>
<td>0.04</td>
<td>E</td>
<td>30%</td>
<td>cR6</td>
</tr>
</tbody>
</table>
cR6 reduces collagen-mediated platelet aggregation

Following six rounds of SELEX, pools cR6 and tR6 were tested for their ability to inhibit collagen-induced platelet aggregation. As seen in figure A-2, cR6 was able to decrease platelet aggregation by approximately 50%, whereas tR6 was non-functional.

Figure A-2: Round cR6 reduces platelet aggregation in the collagen-induced platelet aggregation (CIPA) assay. Platelet rich plasma was combined with 1 μM of round cR6 or tR6 and allowed to incubate for five minutes. Collagen was then added to induce platelet aggregation. Data points were performed in duplicate.
Selection against GPVI was performed in two manners (traditional SELEX using cGPVI and a toggle SELEX, that is, selection between two different proteins [cGPVI and hGPVI] in attempts to select an aptamer that binds to both forms) (White et al., 2001). Toggle SELEX, therefore, was performed in order to increase the odds of selecting an aptamer that would bind to and inhibit the native glycoprotein present on the surface of platelets, as hGPVI is partially glycosylated, and cGPVI is without the aptamer-diverting histidine tag. In this selection, the first three rounds were performed against cGPVI; the selection then diverged, where round four (tR4) was selected against hGPVI, with subsequent rounds against cGPVI). This toggle SELEX proceeded for an additional two rounds (to tR6); at this juncture, the RNA pool (tR6) was found to be non-functional in CIPA, as well as having unacceptably high levels of non-specific binding. For these reasons, that portion of the selection was terminated (indicated by a red “X”; see Fig. A-3). Aptamer pools tR5.2 and tR6.2 were attempts at lowering non-specific binding by re-transcribing the round RNA (italicized; see Fig. A-3). However, background binding levels remained high, therefore those arms were also terminated. Traditional selection against cGPVI proceeded in a manner somewhat similar to tR6, with the exception of cR6 demonstrating functionality in the CIPA assay (see Fig. A-3; denoted by blue rectangle). However, attempts to lower non-specific binding through re-transcription (cR5.2, cR6.2) were unsuccessful. As opposed to re-transcribing from cR6 DNA to lower non-specific binding, round 6 was repeated; this was denoted cR6.3. Before testing the binding of cR6.3, round 7 (cR7) was performed; this round was found to have an extremely high level of non-specific binding (30%), as was cR6.3 (20%) (see table A-1).
The primary limitation of this project was non-specific binding that decreased the amount of RNA available to bind to GPVI. Although cR6 displayed background binding levels slightly over the arbitrary threshold of “acceptable” (< 10%), it was able to inhibit platelet aggregation. Unfortunately, attempts to reduce the background binding of cR6 resulted in even higher background levels (cR6.3 at 20%), which were not uncovered until a subsequent round had already been performed (cR7). At that time, non-specific binding reached such a high level (30%) such that the next logical step would be to return to round 5 (denoted by green arrow, Fig. A-3) and perform the proceeding round in a slightly different manner. Possibilities include performing the round in a modified buffer that reduces non-specific binding; one could replace BSA in the binding buffer with CHAPS, a detergent. This method was successful in reducing
the background levels during the PAI-1 selection (see chapter 2). Another option is to perform additional “pre-clear” reactions. Before partitioning of RNA that binds to the protein from RNA that does not bind, a “pre-clear” reaction is completed in which the RNA is incubated with binding buffer and nitrocellulose in the attempt to remove any RNA that binds to the nitrocellulose. Yet another option may be to combine a preclear reaction with a binding reaction. Because there is such a vast difference between the background binding levels using binding buffer versus wash buffer (data not shown), and the only difference between the two buffers is the presence of BSA, active removal of RNA that binds BSA could be performed. In this experiment, the RNA would be incubated with binding buffer containing a high amount of BSA, which was then passed over a nitrocellulose membrane. The RNA that does not bind should be captured and then combined with the protein of interest. This should actively remove RNA that binds to BSA. Another, much simpler option, would be to completely remove BSA from the binding buffer, as aptamers have been selected without its presence.

After returning to cR5, the resulting round (and all subsequent rounds) should be tested in the CIPA assay. Screening for functionality in addition to binding is ideal, as the possibility of missing functional aptamer clones is decreased. For example, a selection may proceed such that binding affinity steadily increases due to decreased diversity of the pool, leaning towards a few aptamers that bind with high affinity. However, it is possible to select an aptamer that binds with high affinity that is non-functional. To avoid missing functional aptamers, rounds should be tested in a functional assay; if with subsequent rounds high affinity binding is reached that does not align with functionality, a previous functional round can be cloned to identify individual aptamer sequences that are functional.

Once aptamers have been identified as functional in a CIPA assay, their specificity for disrupting the collagen-GPVI interaction should be determined by
assaying platelet adhesion mediated by ristocetin, ADP, and thrombin receptor-agonist peptide. Additional functional assays may include using fibrillar collagen/convulxin as platelet aggregation agonists in CIPA; a collagen-related protein (CRP) binding assay (Horii et al., 2006); and fibrillar collagen plate binding. Following determination of specificity and further investigation, the aptamers should undergo antidote design (and associated testing) and optimization so that in vivo studies may be performed; this includes truncation to ease commercial synthesis as well as chemical modification to increase the circulating half-life of the aptamers. When these steps are complete, the aptamers should be re-tested in vitro before proceeding to an in vivo study such as an arterial-injury model of thrombosis.

Although toggle SELEX was originally initiated to increase the likelihood of selecting an aptamer that inhibits the interaction between glycosylated GPVI and collagen, the area where GPVI interacts with collagen is not glycosylated. This is the most likely area of aptamer binding given the tendency of aptamers to bind to regions of positive charge. However, aptamers may also bind to other areas of the protein and still elicit antiplatelet properties. For example, it is possible that aptamer binding induces shedding of the ectodomain, similar to the monoclonal antibody JAQ1 (Nieswandt et al., 2001b), or it may preclude protein dimerization by binding to the posterior portion of domain two, hence decreasing the affinity of GPVI for collagen (Horii et al., 2006). Therefore, continuing with traditional SELEX using cGPVI, while taking measures to reduce non-specific binding, could possibly yield a novel, reversible antiplatelet agent that inhibits platelet adhesion, aggregation and activation. This would have increased safety over currently available antiplatelet agents due to its antidote reversal, which allows for fast mitigation of activity should adverse events arise.
Appendix B (Antiplasmin SELEX)

Introduction

Pharmacologic thrombolysis is the most common method of treating thrombotic disorders. Induction of thrombus dissolution primarily occurs by increasing the levels of plasmin available to cleave fibrin, the structural component of thrombi. Antiplasmin is the most important inhibitor of plasmin, placing it in a key position in the fibrinolytic pathway. This is evident as antiplasmin-deficient patients have a severe hemorrhagic tendency resulting from late bleeding (Aoki et al., 1977; Favier et al., 2001). Antiplasmin is synthesized mainly by the liver and contains 464 amino acids as secreted in the amino-terminal Met form. Approximately 60-70% of the circulating antiplasmin then loses 12 amino acids from the amino-terminal and is converted to the more inhibitory Asn-form (Sumi et al., 1989; Koyama et al., 1994). This cleavage is conducted by antiplasmin-cleaving enzyme (APCE), which has been shown to be a soluble form of fibroblast activation protein (Lee et al., 2006).

Antiplasmin (also known as $\alpha_2$-AP and $\alpha_2$-plasmin inhibitor) is a single chain glycoprotein, a member of the serpin superfamily, and forms an irreversible covalent complex with its substrate. Antiplasmin inhibits fibrinolysis by three distinct mechanisms owing to structural features that distinguish it from other serpins. In addition to the aforementioned covalent binding to plasmin through its reactive center loop which is common to all serpins, antiplasmin is able to interfere with the binding of plasminogen to fibrin via a high affinity plasminogen binding site located within its last 20 carboxy-terminal amino acids (Aoki et al., 1977). The covalent binding to plasmin reduces the amount of plasmin that is available to degrade fibrin, and antiplasmin’s interaction with plasminogen blocks its binding to fibrin, which is necessary for enhanced plasmin generation (Aoki et al., 1977).
Whereas these first two mechanisms serve to inhibit fibrinolysis through decreasing the amount of plasmin available, the last method inhibits fibrinolysis by means of clot stabilization. The cross-linking of antiplasmin to fibrin by Factor XIIIa occurs most notably at the amino-terminal via Gln-14 (Tamaki et al., 1982). This corresponds to the second residue from the amino terminus of the Asn-form (which becomes crosslinked to fibrin 13 times faster than the Met-form (Lee et al., 2004)). Crosslinking at a location distinct from plasmin(ogen) binding is responsible for the antifibrinolytic activity noted for the partially degraded, non-plasminogen binding form that comprises a minor portion of the circulating antiplasmin (Kluft et al., 1986). Thus, antiplasmin has three different functional domains that work in diverse mechanisms to accomplish the same goal— inhibition of fibrinolysis and thus thrombus stabilization.

As antiplasmin plays such a large role in modulation of fibrinolysis, it is rational to look towards its inhibition as a possible therapeutic target. Inhibitors of antiplasmin have demonstrated that through effective blocking of any one of its three functional domains, clot lysis can be enhanced in vitro. A monoclonal antibody generated against the reactive center loop greatly enhanced tPA mediated clot lysis (Sakata et al., 1989), and synthetic peptides as well as antibodies against the N- and C-terminal extensions increased fibrinolysis substantially (Kimura et al., 1985; Lee et al., 2002; Sazonova et al., 2007). Furthermore, in vivo antibody inhibition of antiplasmin in a ferret model of induced pulmonary embolism demonstrated increased thrombolysis (Butte et al., 1997).

In an attempt to isolate molecules that bind to and inhibit antiplasmin with high affinity and specificity, I employed the SELEX strategy. To prevent immunologic reactions secondary to antibody-based therapeutics, I used aptamers, which in contrast to antibodies have been shown to be lowly immunogenic and nontoxic (White et al., 2000; Wlotzka et al., 2002), and may have their activity reversed using a complimentary oligonucleotide antidote (Rusconi et al., 2002) or possibly a universal antidote (Oney et
These aptamers would have the potential to serve as novel antidote-controlled thrombolytic agents that could be used to treat thrombotic disorders such as ischemic stroke, acute myocardial infarction, and pulmonary embolism.
Materials and Methods

Generation of Aptamers using Systematic Evolution of Ligands by EXponential enrichment (SELEX)

The sequence of the starting RNA library (denoted Sel2 Library) was 5'-GGGAGGACGATGC GG-N_{40}-CAGACGACTCGCTGAGGATCC-3', where "N_{40}" denotes a random region 40-nucleotides in length. The RNA consisted of 2'-fluropyrimidines (2'-fluorocytidine triphosphate and 2'-flurouridine triphosphate; Trilink Biotechnologies, San Diego, CA) in order to render the RNA nuclease resistant. Selection began against human α2-antiplasmin (AP) purchased from Haematologic Technologies (Essex Junction, VT). The RNA library was incubated with its respective protein for 15 minutes at 37°C; unbound RNA was separated from RNA/AP complexes by passing the mixture over a nitrocellulose membrane. Rounds one through six were performed in BSA binding buffer E (BBBE: 20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM CaCl_{2}, and 0.1% bovine serum albumin [BSA]). Round seven and eight were performed in CHAPS binding buffer E (CBBE: 20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM CaCl_{2}, and 0.05% CHAPS), while rounds nine and ten were performed CHAPS binding buffer D (CBBD: 20 mM Hepes, pH 7.4, 25 mM NaCl, 2 mM CaCl_{2}, and 0.05% CHAPS).

Binding assays

Progression in binding was determined using nitrocellulose filter binding assays as previously described (Rusconi et al., 2000). Briefly, RNA was first dephosphorylated using bacterial alkaline phosphatase (Gibco BRL, Gathersburg, MD) and 5’ end labeled with [γ-^{32}P] ATP (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Round pools of radiolabeled RNA were incubated with increasing amounts of antiplasmin protein in
their respective binding buffers for 5 minutes at 37°C; the mixture was then passed over a nitrocellulose membrane. The fraction of RNA that bound to the protein was quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Nonspecific binding of the RNA was subtracted out such that only specific binding remained (denoted ‘corrected fraction bound’).
Results

In total, ten rounds of SELEX were performed against antiplasmin. The first six rounds were performed in a BSA-based binding buffer with 50 mM NaCl; here, binding progressed, demonstrating “shifts” that indicate increases in binding affinity and the fraction of RNA bound to the protein (see Fig. B-1). Rounds seven and eight used a CHAPS-based binding buffer to decrease the amount of non-specific binding to nitrocellulose; no shift occurred as binding stringency was not significantly increased. Finally, rounds nine and ten were performed in CHAPS-binding buffer with a lower salt concentration (25 mM) in an attempt to reduce the stringency of the selection and therefore increase the amount of RNA that binds to the protein. However, round ten demonstrated even lower binding parameters than the previous four rounds (Fig. B-1).

Figure B-1: Round binding of antiplasmin SELEX. The progress of the selection against antiplasmin was followed using a nitrocellulose filter binding assay. Squares (■) represent round ten (R10cAP), triangles (▲) represent round eight (R8cAP), inverted triangles (▼) represent round six (R6AP), Diamonds (♦) represent round four (R4AP), circles (●) represent round 2 (R2AP), and open squares (□) represent the starting RNA library (Sel2). The x-axis shows the antiplasmin concentration and the y-axis shows the fraction of RNA bound to the protein. Error bars for rounds six and eight (two separate experiments) represent the standard error of the mean.
**Discussion and Conclusion**

The objective of this project was to isolate aptamers that bound to antiplasmin with high affinity that interrupted at least one of antiplasmin’s three physiological interactions: 1) antiplasmin and plasmin, 2) antiplasmin’s crosslinking of fibrin with Factor XIIIa, or 3) antiplasmin with plasminogen. In order to accomplish this goal, a total of ten rounds of traditional SELEX was performed against antiplasmin. By round three, non-specific binding levels reached unacceptable levels, however, when the binding buffer was replaced with buffer made with new reagents, the background binding level decreased to an acceptable level (see Table 1).

| Table B-1: Antiplasmin SELEX Round Parameters |
|---|---|---|---|---|
| Round | Buffer | [NaCl] (mM) | [Protein] (uM) | Background binding (in BBB) | Background binding (in CBB) |
| R1AP | BBBE | 50 | 3 | - | - |
| R2AP | BBBE | 50 | 3 | 8% | 4% |
| R3AP | BBBE | 50 | 2 | 12% | 3% |
| R4AP | BBBE | 50 | 0.75 | 13%→9% | 5% |
| R5AP | BBBE | 50 | 0.375 | - | - |
| R6AP | BBBE | 50 | 0.25 | 18% | 3% |
| R7cAP | CBBE | 50 | 0.25 | - | 2% |
| R8cAP | CBBE | 50 | 0.20 | - | 2% |
| R9cAP | CBBD | 25 | 0.3 | - | 1% |
| R10cAP | CBBD | 25 | 0.25 | - | 2% |

However, by round six, these levels had risen again, reaching 18%. Because of this, CHAPS, a detergent, replaced BSA in all binding buffers. This change in buffer reagents greatly reduced non-specific interactions as seen in background binding levels consistently less than 4%. However, all RNA/protein interactions decreased as well, as
evidenced by decreases in binding ($B_{\text{max}}$). To compensate for reduced binding, the concentration of protein added during the binding process of the round was increased, and the salt concentration of the buffer was decreased (see table B-1; changes to increase binding are noted in blue). Unfortunately, the result of these modifications was a reduction in binding stringency; this may explain why the binding of the rounds decreased (Fig. B-1).

One alternative method of decreasing “background levels” is to perform a negative selection using BSA. Here, the RNA would be bound with BSA, the RNA that binds would be partitioned from RNA that does not bind, and the RNA that does not bind to BSA would be recovered and used in subsequent experiments. This may function to decrease the amount of RNA binding to BSA as well as nitrocellulose, as those RNA molecules would remain on the nitrocellulose and would be discarded.

In addition, the stringency of a selection should not be decreased; instead, if caution is warranted, small increases to increase binding stringency should be employed. This is because decreases in stringency would essentially allow for RNA that does not bind well to be increased in the pool through amplification at the worst case, which may have occurred in this project, and no increase in binding would be achieved in the best case scenario.

If this project is to continue, aptamer inhibition of the antiplasmin/plasmin interaction should be taken into consideration, as it might be less likely to occur than inhibition of the other two functional areas. This is primarily because the binding of plasmin to antiplasmin results in a covalent bond; therefore, when the aptamer releases from antiplasmin given its fast on/off rate, it would be unable to bind again and displace plasmin due to the recently formed covalent bond. Also, the selection would need to be repeated, preferably addressing increased levels of background binding through a round of negative selection to BSA. Alternatively, a CHAPS-based buffer, or a
complete removal of BSA from the binding buffer, could be used at the commencement of the selection.
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BIOGRAPHY

Charlene Marie Blake

Born May 20, 1981 in Washington, DC, USA

EDUCATION

2002-2010 M.D. (2010), Duke University School of Medicine, Durham, NC
2004-2009 Ph.D. (2009), University Program in Genetics and Genomics, Duke University Graduate School, Durham, NC
1998-2000 Biochemistry Major, University of Kansas, Lawrence, KS

MANUSCRIPTS AND PUBLICATIONS

2. Charlene M. Blake, Bruce A. Sullenger, Daniel A. Lawrence, Yolanda M. Fortenberry. Antimetastatic potential of PAI-1 specific RNA aptamers. [In Press]
3. Charlene M. Blake, Haichen Wang, Daniel T. Laskowitz, Bruce A. Sullenger. A reversible aptamer-based reperfusion strategy improves outcome following stroke. [Submitted]

AWARDS AND HONORS

2008 UNCF/Merck Graduate Science Research Dissertation Fellowship
2006 Ruth L. Kirschstein National Research Service Award, Individual Fellowship
2005 Top Oral Presentation, First Year Category, UPGG

PROFESSIONAL SOCIETIES

International Society on Thrombosis and Haemostasis
American Heart Association/American Stroke Association
American Association for the Advancement of Science
American College of Physicians
Student National Medical Association