FUSION OF THE C-TERMINAL TRIDECAPEPTIDE OF HIRUDIN TO α_1-PI

FUSING THE C-TERMINAL TRIDECAPEPTIDE OF HIRUDIN TO -1- PROTEINASE INHIBITOR M358R ACCELERATES ITS RATE OF THROMBIN INHIBITION

By

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ABSTRACT

The serpin α-1 proteinase inhibitor (API) normally only impacts the coagulation cascade through its ability to inactivate factor XIa. However, the point mutation (Met to Arg) at position 358 results in a potent thrombin inhibitor, API M358R. This mutation also enhances this serpin's ability to inhibit the anticoagulant protein, activated protein C (APC) and hence this property limits its therapeutic potential. As a result, various modifications to this protein have been engineered in order to enhance its specificity towards thrombin. Previously, the Heparin Cofactor II (HCII) N-terminal tail, HCII 1-75, which binds exosite 1 of thrombin, was tethered to the N-terminus of API M358R, creating HAPI M358R. Although this change did not alter anti-APC activity, it did augment the anti-thrombin activity of API M358R. In addition, further changes in the reactive center loop, the region that interacts with the thrombin active site, resulted in a significant reduction in APC activity while maintaining antithrombotic activity similar to HAPI M358R; this variant was termed HAPI RCL5.

Preliminary experiments were performed with the C-terminal tridecapeptide of Hirudin Variant 3 (HV3) to determine its exosite 1 binding capacity compared to HCII 1-75. Three different variants of this peptide were tested: one with a hexahistidine tag (H_6HV3_{54-66}), another that also had a hexa-glycine C-terminal addition ($H_6HV3_{54-66}G_6$) and a third without either addition. All were found to bind exosite 1 with a greater affinity than HCII 1-75. Thus, the $H_6HV3_{54-66}G_6$ peptide was fused to API M358R and API RCL5 in hopes of creating an inhibitor with heightened specificity compared to HAPI M358R and HAPI RCL5, respectively.

HV3API M358R and HV3API RCL5 were expressed in a bacterial system and purified by nickel-chelate and ion exchange chromatography. Second order rate constants for the inhibition of thrombin and APC by the API variants and fusion proteins were determined. The K_2 values for α-thrombin inhibition ranged from 186 $M^{-1}min^{-1}$ to 22 $M^{-1}min^{-1}$ with an order of inhibitory potency observed as follows: HAPI M358R > HAPI RCL5 > HV3API M358R > HV3API RCL5>API RCL5 > API M358R.

The ability of recombinant chimeric serpins to bind thrombin exosite 1 in a manner independent of RCL-thrombin active site interactions was also investigated through competitive inhibition of the binding of active site-inhibited thrombin to immobilized HCII 1-75. It was found that the order of exosite 1 binding affinity was HV3API RCL5 > $H_6HV3_{54-66}G_6$> HCII 1-75 > HAPI RCL5. Our results indicate that fusing the C-terminal tridecapeptide of HV3 to API variants enhanced their ability to inhibit thrombin, but to a lesser extent than fusing the N-terminal 75 residues of HCII. This finding likely reflects a requirement for the exosite 1-binding motif of the fusion protein to bind exosite 1 in a way that allows for subsequent optimal active site attack on the RCL by the serpin moiety of the fusion protein. In general, this work provides a second novel example of how the activity of a thrombin-inhibitory serpin can be enhanced by fusion to an exosite-1 binding motif.

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PREFACE

All of the work presented in this thesis was done independently by me, with the exception of cloning the HV3API variants into the pBAD vector which was performed by Varsha Bhakta and some batches of purified HAPI M358R, HAPI RCL5, API M358R and HCII 1-75 were also produced by Sharon Gataiance.

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α	alpha
β	beta
γ	gamma
μL	microliter(s)
μM	micromolar
°C	degrees Celsius
ADP	adenosine diphosphate
APC	activated protein C
API	α_1-proteinase inhibitor
Arg	arginine
AT	antithrombin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CS	cleaved serpin
C-terminal	carboxy-terminal
DAB	diaminobenzidine tetrahydrochloride hydrate
DCT	double clotting time
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DS	dermatan sulfate
E	enzyme
E. coli	*Escherichia coli*
eNOS	endothelial nitric oxide synthase
EPCR	endothelial protein C receptor

F	Factor
GAG	glycosaminoglycan
Gln	Glutamine
Glu	glutamic acid
Gly	glycine
GP	glycoprotein
h	hour
H_2SO_4	sulfuric acid
HCII	heparin cofactor II
HIT	heparin-induced thrombocytopenia
HRP	horseradish peroxidase
HS	heparan sulfate
HV3	hirudin variant 3
IC50	inhibitor concentration required to reduce activity 50%
ICAM-1	Intercellular adhesion molecule -1
IL	interleukin
k_2	second order rate constant
kb	kilobase(s)
kDa	kiloDalton(s)
Ki	inhibition constant
KK	kallikrein
L	litre(s)
LB	Luria-Bertani broth
Lys	lysine
M	molar
mg	milligram(s)

MGS	methionine, glycine, serine
min	minute(s)
ml	milliliter(s)
mM	millimolar(s)
Met	methionine
MOBIX	Molecular Biology and Biotechnology Institute, McMaster University
N-terminal	amino-terminal
NaCl	sodium chloride
NBT	nitro blue tetrazolium
NFκβ	nuclear factor κβ
Ni-NTA	nickel-nitrilotriacetic acid
ng	nanograms
nm	nanometer
nM	nanomolar
OD	optical density
OPD	o-phenylenediamine dihyrochloride
P_o	initial thrombin activity
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PARs	protease activated receptors
PBS(T)	phosphate-buffered saline (with tween)
PC	protein C
PCR	polymerase chain reaction
Phe	phenylalinine
PPACK	D-phenylalanyl-L-prolyl-arginine chloromethyl ketone
Pro	proline

Pt	thrombin activity at time, t
R^2	regression coefficient
RCL	reactive center loop
S	serpin
S-2238	H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline
S-2366	L-Pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline hydrochloride
S. cerevisiae	*Saccharomyces cerivisiae*
SD	standard deviation
SDS	sodium dodecyl suflate
SEC	serpin-enzyme complex
Ser	serine
SI	stoichiometry of inhibition
TAFI	thrombin activable fibrinolysis inhibitor
TBST	Tris-buffered saline with tween
TCT	thrombin clot time
TEMED	N,N,N',N'-tetramethylethylenediamine hydrochloride
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGFβ	transforming growth factor β
TM	thrombomodulin
TNF-α	tumor necrosis factor-α
tPA	tissue plasminogen activator
TxA_2	thromboxane A_2
Tyr	tyrosine
Val	valine
VSMCs	vascular smooth muscle cells

1. INTRODUCTION

1.1 Overview of Coagulation

1.1.1 The Coagulation Cascade

The mammalian coagulation cascade has evolved to prevent excessive blood loss due to trauma or injury. This cascade is comprised of both extrinsic and intrinsic pathways that coordinate to promote coagulation (Figure 1). The extrinsic pathway is initiated when FVIIa comes in contact with tissue factor (TF) (*1*). TF is found on membranes in the subendothelial space and therefore only associates with circulating FVIIa when the endothelium is injured (*1, 2*). This TF-FVIIa complex is responsible for the cleavage of FX to generate active FXa and the subsequent activation of prothrombin to thrombin on negatively charged phospholipids. Thrombin subsequently converts fibrinogen to fibrin (*3*). Trace amounts of thrombin, initially produced by FVIIa, feed back in the cascade and activate FVIII and FV and this feedback causes amplification of the cascade (*3*). FVIIIa acts as a cofactor for the tenase complex by which, with Factor IXa, calcium and anionic phospholipids, FX is activated. FVa is a cofactor for FXa to cleave prothrombin into thrombin (*3*). Thus, this cascade can continue to progress after the TF-VIIa complex has been inactivated by tissue factor pathway inhibitor (TFPI) (*4*).

Alternatively, coagulation can begin with the intrinsic pathway that converges with the extrinsic pathway at the tenase prothrombinase complex (*1*). This pathway is initiated when FXII is activated on a charged surface. It subsequently cleaves and activates

Figure 1: The coagulation cascade. A schematic diagram illustrating the intrinsic and extrinsic pathway as well as physiological inhibitors of the pathway is shown. Modified from Enzyme Research Laboratories, Inc., 2004. Reprinted with written permission of Enzyme Research Laboratories, Inc., South Bend, IN, provided on June 20, 2012.

Protein Concentrations

Component	Molecular Weight	Plasma Concentration µg/ml	Plasma Concentration µM
Fibrinogen (I)	330,000	3000	9.09
Prothrombin (II)	72,000	90	1.388
Factor V	330,000	10	0.03
Factor VII	50,000	0.5	0.01
Factor VIII	330,000	0.1	0.0003
Factor IX	56,000	5	0.08928
Factor X	58,800	8	0.13605
Factor XI	160,000	5	0.031
Factor XII	80,000	30	0.375
Factor XIII	320,000	10	0.03125
Protein C	62,000	4	0.0645
Protein S	69,000	10(free)	0.1449
Protein Z	62,000	2.2	0.0355
Prekallikrein	86,000	50	0.5814
HK	110,000	70	0.6363
Fibronectin	450,000	300	0.6667
Antithrombin III	58,000	140**	2.4**
Plasminogen	90,000	216	2.4
Urokinase	53,000	0.1	0.001887
Heparin Cofactor II	66,000	90	1.3636
$Alpha_2$-Antiplasmin	63,000	60	0.9524
Protein C Inhibitor	57,000	4	0.0702
$Alpha_2$-Macroglobulin	725,000	2100	2.8966

Inhibitors in black
**Thrombophilia gene mutation*
***Corrected value*

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plasma prekallikrein; this active kallikrein (KK) propagates this pathway in an autocatalytic manner where it activates additional FXII as well as more KK (*5*). Activation of FXIIa initiates a chain of reactions following with the activation of FXI and FIX (*1*). FIX can also be activated through the extrinsic pathway by the TF-FVIIa complex (*6*). This cascade concludes with thrombin activation of FXIII which leads to the crosslinking of fibrin and thus strengthening of the clot (*7*). The role of FXII is not completely understood as FXII deficiency does not lead to a reduction in coagulation efficiency, as is the case with the other coagulation factors (*8*). However, this apparent inconsistency can be partly explained by noting that FXI can be activated by thrombin in addition to FXIIa and hence, the extrinsic pathway can still proceed in the absence of FXIIa (*9*). The coagulation cascade is a highly complex process that requires the coordination of both the extrinsic and intrinsic pathways to maintain hemostasis.

1.1.2 The Role of Platelets in Coagulation

Platelet adhesion and aggregation at the site of injury are also essential aspects of coagulation. Upon vessel wall injury platelets begin to form transient interactions between the glycoprotein GPIb-V-IX and von Willebrand factor bound to collagen (*10*). These transient interactions reduce the velocity of platelets travelling through the circulation; the "deaccelerated" platelets, now close to the vessel wall, adhere to the collagen-bound glycoproteins, GPVI and GpIa-IIa, resulting in platelet activation (*11*). These interactions also initiate intracellular signaling leading to the release of secondary platelet agonists such as thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). Together with locally produced thrombin, these agonists contribute to platelet activation

through G-protein coupled receptors (*11*). This combination sets in motion a complex signaling cascade that performs various functions such as promoting coagulant activity by mobilizing anionic phospholipids and activating integrins such as GPIIb-IIIa involved in firm adhesion and platelet aggregation (*12*). Lastly, these aggregates are stabilized through fibrin formation and inter-platelet signaling (*12*). This process of platelet adhesion and aggregation works in conjunction with the coagulation cascade to form a hemostatic plug, preventing excessive blood loss.

1.1.3 Maintaining Hemostatic Balance

To maintain hemostasis this complex coagulation cascade is tightly regulated by various mechanisms. Antithrombin (AT) is the main physiological inhibitor of thrombin, but can also inhibit other coagulation factors such as FIXa and FXa (*13*). The glycosaminoglycan (GAG), heparan sulfate (HS), acts as a cofactor to accelerate AT's ability to inhibit thrombin by 1000 fold (*13*). Heparin cofactor II (HCII) is another endogenous inhibitor of thrombin that also requires a GAG to enhance its antithrombin activity (*14*). Thrombin, when bound to thrombomodulin (TM), can also act as an anticoagulant as it is integral to the activation of protein C. Activated protein C (APC) cleaves FVa and FVIIIa and thus limits the conversion of prothrombin to thrombin as well as the activation of FXa (*15*). In addition, tissue factor pathway inhibitor (TFPI), which inactivates the TF-VIIa complex in a FXa dependent manner, is highly important for regulating coagulation; upon TF-VIIa inactivation further thrombin formation becomes dependent upon FXa and FIXa (*4*). Lastly, fibrinolysis can shift the balance away from a procoagulant state. Tissue plasminogen activator (tPA) and urokinase

promote the conversion of plasminogen into plasmin and thus cause fibrinolysis to occur (*16*). This process may be inhibited by plasminogen activator inhibitor-1 (PAI-1) and α-2 antiplasmin (*16, 17*). Hence, coagulation is tightly controlled by multiple factors to maintain an appropriate balance between the procoagulant and anticoagulant states and to prevent the formation of thrombi, which are pathological intravascular clots.

1.1.4 Coagulation and Inflammation

Coagulation is strongly linked to inflammation as many regulators are common to both pathways. This link can be explained evolutionarily as the primary function of the ancestral clotting cascade was to act as a defense against invading pathogens (*18*). Studies have shown that pro-inflammatory molecules can activate the coagulation cascade and likewise coagulation can modify inflammatory signaling pathways. Invading pathogens activate the innate immune response through intermediaries such as toll-like receptors (*17*). This results in the production of various cytokines, chemokines and other proinflammatory stimuli that promote coagulation. This process is mediated through three main routes: stimulation of procoagulant factors, reduction of the synthesis of anticoagulants and suppression of fibrinolysis (*17*). For instance, the production of IL-6, IL-8 and TNF-α promotes the release of von-Willebrand factor from the endothelium and thus promotes platelet aggregation (*19*). Furthermore, such cytokines can also increase the expression of TF on monocytes and endothelial cells (*20*). Other cytokines, such as TNF-α and IL-1β, decrease the expression of thrombomodulin and endothelial protein C receptor (EPCR), causing a reduction in the formation of APC (*21*) (*22*). PAI-1 expression is also increased in platelets and endothelium due to cytokine release; since

PAI-1 inhibits tPA, clot lysis is reduced (*17*). On the other hand, thrombin has been shown to upregulate the expression of cytokines and chemokines on different cell types. One way this upregulation has been shown to occur is through thrombin's ability to activate protease activated receptors (PARs), specifically PAR-1, 3 and 4 (*20*). APC can also interact with PARs but this signaling has the opposite effect to thrombin as APC-PAR signaling results in an anti-inflammatory response and other cytoprotective reactions (*20*). Hence, inflammation and coagulation are closely entwined resulting in vastly complex pathways.

1.2 Structure and Function of Thrombin

Thrombin is a multifunctional serine protease that is part of the chymotrypsin family (*23*). It has a molecular weight of approximately 37 kDa and is composed of a light A chain containing 36 amino acids that is connected by a disulfide bond to the catalytic heavy B chain (*24*). Thrombin has two insertion loops that flank the active site, known as the 60-loop and the 148-loop (*25*). These loops add a surface for interaction with potential substrates and may sterically limit the access of compounds to the active site. Thus, theoretically, these loops increase the specificity of thrombin as an inhibitor; however the role of these insertion loops on substrate recognition is still not completely understood (*25*). Thrombin also contains two basic exosites that govern its interaction with substrates and cofactors. Exosite 1 is necessary for its recognition of PAR-1, fibrinogen and thrombomodulin, while exosite 2 is the glycosaminoglycan binding domain and it also interacts with FV, FVIII and GPIbα (which is involved in platelet recognition) (*23*). Thrombin also contains a sodium binding site necessary for

procoagulant activity (*26*); when sodium is bound it coordinates Arg221 and Lys224 as well as four water molecules to create the substrate binding pocket that interacts with the P1 residue of its substrates (*23, 27*). The structure of thrombin allows it to be very versatile as reflected in the various roles it plays such as in coagulation, inflammation, vasomotor regulation, as well as platelet activation.

1.2.1 Role in the Coagulation Cascade

Thrombin's role as a procoagulant in the coagulation cascade begins with its conversion from prothrombin to thrombin. Prothrombin is a 70 kDa glycoprotein that is produced in the liver and secreted into the blood (*24*). It is converted to thrombin when it comes in contact with the prothrombinase complex, consisting of FXa and FVa as a cofactor and calcium ions; this reaction occurs on anionic phospholipids on the surface of activated platelets or endothelial cells (*24, 28*). Upon activation, thrombin's exosites become able to interact with various substrates and cofactors, allowing it to play a significant role in the coagulation cascade. Thrombin catalyzes the final step in the coagulation cascade by converting fibrinogen to fibrin, and it also activates FXIII required to strengthen clots via fibrin crosslinking (*7*). Furthermore, thrombin amplifies the cascade by activating factors V and VIII, cofactors involved in the activation of prothrombin and FX, respectively (*3*). Lastly, thrombin is responsible for the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) which inhibits the breakdown of fibrin clots (*23*). Thrombin also acts as an anticoagulant as it is responsible for the activation of protein C. Activation occurs when thrombin associates with membrane bound TM. APC cleaves FVa and FVIIIa and consequently prevents the activation of both Factor Xa and

thrombin (*15*). Hence, thrombin has various procoagulant functions as well as an anticoagulant role within the coagulation cascade.

1.2.2 Role in Platelet Activation

Platelet activation is crucial to hemostasis. Thrombin is one of the most potent platelet activators and acts by targeting PAR-1 and PAR-4 receptors found on the platelet surface (*29*). Activation occurs when exosite 1 of thrombin binds to the hirudin-like domain found on PAR-1 on platelet surfaces (*30*). Thrombin cleaves PAR-1 between arginine 41 and serine 42. This newly exposed NH_2 terminal sequence resembles a tethered ligand and binds intra-molecularly to the body of the PAR-1 receptor and this interaction leads to platelet activation (*31*). PAR-4 lacks the hirudin-like sequence and thus requires a higher thrombin concentration for activation (*30, 32*). Both of these PARs appear to couple to the same G-proteins and signaling molecules during platelet activation, but the magnitude and duration of their response varies (*29*). PAR-1 triggers a rapid but short-lived increase in intracellular calcium whereas PAR-4 has a slower but longer response. Therefore, it has been proposed that PAR-1 is responsible for the initial platelet aggregation, but PAR-4 is accountable for the continued stability of aggregation (*29, 33*). The activation of these G-protein coupled receptors leads to increased TxA_2 and ADP production, integrin activation, and increased local production of thrombin, all of which lead to platelet adherence and aggregation (*11*). Platelet activation is yet another mechanism by which thrombin exerts a procoagulant response.

1.2.3 Thrombin Inhibitors

Thrombin is an integral component of the coagulation cascade and therefore it has been a drug target for the prevention and treatment of venous and arterial thrombosis. Though there are endogenous inhibitors of thrombin, notably AT and HCII, other agents have been developed to prevent excessive thrombus formation for those at risk when these natural inhibitors are overwhelmed. Indirect thrombin inhibitors, mainly heparins, are used clinically and can inhibit circulating thrombin but are less effective against fibrin-bound thrombin (*34, 35*). Heparins act as cofactors for AT by binding to both AT and exosite 2 of thrombin, thus bridging the two proteins and enhancing AT's ability to inhibit thrombin (*34*). Direct thrombin inhibitors can inhibit both free and fibrin-bound thrombin (*35*). Hirudins, originally isolated from the medicinal leech, bind to both thrombin's active site and exosite 1 with very high affinity and thus prevent thrombin from cleaving fibrinogen (*36*). Currently the recombinant hirudins on the market are lepirudin and desirudin; in addition, a hirudin-related synthetic peptide, bivalirudin, is also used (*36*). Dabigatran etexilate is a new prodrug that is orally administered, unlike heparins and hirudins that are administered intravenously or subcutaneously. Dabigatran is also very promising since it has a predictable pharmacokinetic profile and thus a fixed dosage regime (*24*). However, it still has a similar bleeding profile to other anticoagulants already on the market and there is no antidote to reverse its anticoagulant activity (*24*). The new anticoagulants directed against thrombin have promising features but are still far from ideal.

1.2.4 Cellular Impacts of PAR Activation by Thrombin

Thrombin also plays a key role in inflammation through the activation of PARs, a class of G-protein coupled receptors. PARs 1 through 4 have been identified to date and of these 1, 3 and 4 are activated by thrombin through cleavage of their N-terminal extracellular domains (*30*). PAR-2 is not activated by thrombin but instead by coagulation factors Xa and VIIa and other proteases such as trypsin (*30*). Thrombin elicits proinflammatory responses through the activation of PARs in various cell types including platelets, macrophages, endothelial cells and vascular smooth muscle cells. For instance, on endothelial cells, thrombin can initiate the expression of various proinflammatory molecules such as IL-6, IL-8, TGFβ and ICAM-1, mainly by stimulating the production of the transcription factor NFκB (*37*). Various studies have linked this production of pro-inflammatory molecules to the activation of PARs. For example, PAR-1 -/- mice have shown protection from mortality elicited by severe endotoxemia and this phenotype is replicated in wild type mice that have been administered either the thrombin inhibitor hirudin, that blocks thrombin's PAR-1 binding site, or a PAR-1 antagonist (*38*). Thrombin is integral to the bridging between coagulation and inflammation.

Thrombin also contributes to other diverse cellular processes. For instance, thrombin plays a role in vasoregulation. It has been shown that PARs can regulate endothelial nitric oxide synthase (eNOS) and this compound is responsible for the synthesis of nitric oxide and thus vasorelaxation (*39*); prolonged incubation of PARs with thrombin inhibits eNOS production (*40*). It has also been demonstrated that thrombin promotes angiogenesis by various mechanisms. For example, through PAR-1 activation

thrombin increases the expression of vascular endothelial growth factor and angiopoietin-2, two factors integral to this process (*24*). Through the activation of PARs, thrombin induces the production of various pro-inflammatory mediators in multiple cell types and participates in various cellular processes including proliferation, survival and vasoregulation.

1.3 Activated Protein C

Protein C (PC) is a vitamin K dependent serine protease that has a molecular weight of 62 kDa (*15*). It circulates in the plasma as an inactive zymogen and is activated when residues 158-169, containing the activation peptide domain, are cleaved (*41*). Thrombin performs this cleavage when it is bound to TM, an endothelium-associated transmembrane receptor. This activation process is augmented by up to 20-fold when PC is bound to the endothelial protein C receptor (EPCR) (*41*). PC also contains a γ-carboxyglutamic acid (Gla) domain which is responsible for the ability of protein C and APC to bind to EPCR and its cofactor protein S (*42*). APC levels are regulated by its three inhibitors, α_2-macroglobulin, α_1-antitrypsin and protein C inhibitor, the last two of which are serine protease inhibitors (*15*). Upon activation, APC either dissociates from EPCR and functions as an anticoagulant or it remains bound to EPCR and carries out a cytoprotective role (*43*). Thus, like thrombin, APC is also a multifunctional enzyme.

1.3.1 Role as an Anticoagulant

APC, along with its cofactor protein S, performs its anticoagulant function by cleaving and thereby inactivating cofactors Va and VIIIa. APC, not only has the ability to

target free FVa but also FVa in the prothrombinase complex, although this is at a rate reduced by 2 orders of magnitude (*15*). APC inactivates FVa at positions R306, R506 and R679, the last of which only plays a minor role. Cleavage after R506 is protein S independent, kinetically favoured and results in an intermediate that has reduced cofactor activity for the conversion of FX to FXa (*44*). Cleavage after R306 is slower, requires protein S and results in complete inactivation of FVa (*45*). APC can also cleave FVIIIa at 3 positions (R326, R462, R740) and uses both protein S and FV act as cofactors (*15*) (*46*). Thus, through cleavage and inactivation of integral cofactors to the coagulation cascade, APC performs its anticoagulant function.

APC is a vital component in the maintenance of hemostasis. This is exemplified by individuals who have a heterozygous or homozygous deficiency of APC as they present clinically with increased morbidity and mortality (*47, 48*). Furthermore, a similar phenotype is observed with individuals with FV Leiden, a genetic abnormality that results in a missense mutation at position 506 from Arg to Gln (*15*). With this FV mutation, APC can no longer cleave it at residue 506 and thus inactivation only occurs slowly at R306. Also, APC cannot properly bind to FVa and thus its ability to inhibit the prothrombinase complex is diminished (*49*). Hence, APC is a key component in hemostasis, and if its anticoagulant activity is compromised by mutation or deficiency then hypercoagulability ensues.

1.3.2 Cytoprotective Role of APC

APC also has a cytoprotective function as evidenced through its anti-apoptotic, anti-inflammatory and endothelial stability roles. For instance, APC inhibits caspase activation as well as the transcription of proapoptotic genes such as p53 and Bax while upregulating the gene transcription of anti-apoptotic BCL-2 (*15, 50*). Thus, it is able to normalize the Bax:BCL-2 ratio on mitochondrial membranes and in this way prevent apoptosis. Since apoptosis results in the release of nuclear material such as nucleosomes as well as mitochondrial content that induces inflammation, APC's anti-apoptotic activity is indirectly cytoprotective (*51*).

APC also directly inhibits inflammation through EPCR and PAR-1 signaling. This signaling modulates the transcription of genes involved with inflammation by down regulating the activity of the nuclear transcription factor, NF-κβ (*51*). In addition, APC upregulates sphingosine phosphate I (SIP) and this molecule promotes inhibition of cytokine signaling, reduction in cell surface adhesion molecules and an increase in expression of anti-inflammatory genes such as IL-10 (*15*). Lastly, APC improves endothelial barrier function in conjunction with PAR-1, EPCR and SIP. This is a natural physical barrier that protects tissues from blood borne pathogens by controlling the passage of molecules and leukocytes into or from tissues (*15*). One way APC improves this function is by stimulating the migration of smooth muscle cells (SMCs) into the endothelium. These SMCs form a sheath around the endothelium and reduce permeability (*52*). Therefore, APC performs its cytoprotective role through various mechanisms such as reduction of apoptosis, inflammation and protection from endothelial dysfunction.

Its dual role as both an anticoagulant and anti-inflammatory agent is likely the reason why exogenous APC, and not other anticoagulant protein such as AT and TFPI, have prevented hypercoagulability and death in patents with sepsis or other inflammatory diseases. However, now with advancements in clinical practice concerning the ability to care for septic patients, it has recently been demonstrated in the PROWESS-SHOCK study that APC administration during severe sepsis no longer shows a beneficial effect on 28 day mortality (*53, 54*).

1.4 Overview of Serpins

Serine protease inhibitors, also known as serpins, are the largest family of protease inhibitors and are found throughout all forms of life: archaea, bacteria, eukarya and even viruses. Serpins have low primary sequence identity but are highly conserved in terms of their tertiary structure. They tend to have bundles of 8-9 alpha helices, three β-Sheets (A-C) and a mobile reactive center loop (RCL)(*55*).

1.4.1 Serpin Mechanism of Inhibition

There are multiple aspects of the inhibitory mechanism conserved among this family of proteases. Firstly, inhibition begins when the RCL of the serpin comes in contact with the catalytic active site of the protease and a non-covalent Michaelis complex is formed (*56*). Next the catalytic serine of the protease undergoes nucleophilic attack of the P1-P1' scissile bond of the serpin forming a tetrahedral intermediate and upon cleavage of the P1-P1' bond an acyl-enzyme intermediate is created (*56, 57*). Following the formation of this intermediate, the RCL incorporates itself into Beta-sheet

A and simultaneously, the protease is translocated to the opposite pole of the serpin (*56*). During this complex formation, serpins are driven from their metastable native conformation into a more energetically favorable conformation, resulting in less strain on the molecule and thus enhanced thermostability (*55, 58*). These serpin-enzyme complexes are irreversible and are also SDS-stable. Lastly, this inhibition complex binds to certain lipoprotein scavenger receptors in order to be cleared from circulation. This essentially irreversible inhibition is optimal for tightly regulated processes such as hemostasis, fibrinolysis and thrombosis where serpins are mediators (*55*).

1.4.2 The Importance of the RCL for Protease Inactivation

The RCL is variable among serpins as the RCL sequence and structure correlate with serpin specificity for their target proteases. Cleavage of the RCL occurs at P1-P1'(*55*). Those residues N-terminal to the protease cleavage site in the RCL of serpins are termed P residues (P17-P1) and those C-terminal to the cleavage site are P' residues (P1'-P10') (*59*). The P1 residue is very important for serpin specificity. In fact, changing this residue can cause a serpin to target an alternate protease (*55, 60*). The RCL length also needs to be very specific for a successful inhibitory reaction. The non-prime side is more constrained than the prime side, and usually is composed of 17 amino acids; in very rare cases serpins have been known to have 16 residues on the N-terminal side but that is the extent of variation in length (*56*). Furthermore, there is more flexibility on the non-prime side (P17-P1) than on the prime side (P1'-P10') (*61*). Both of these characteristics reflect the inhibitory mechanism involving the insertion of the RCL into β-sheet A and the

subsequent translocation of the protease to the opposite pole of the serpin. This flexibility is necessary, as if loop insertion is not rapid enough then the serpin becomes a substrate and is cleaved (*62*). Thus, the constrained length on the non-prime side is necessary to avoid steric clashes, while still providing adequate length to reach the bottom of β-sheet A and maintain the link of the carbonyl of PI to the active site serine of the protease (*56*). However, the length of the prime side is less constrained and the limited flexibility of the RCL on the prime side keeps the RCL close to the body of the serpin, consequently requiring the serpin to come close to the protease for inhibition (*61*). In general terms, those serpins with an extension of 3-4 residues on the P' side tend to have multiple physiological targets as they do not need to come into close proximity with the protease and can therefore have an increased range of contacts with various proteases. For instance, AT has extended P' residues in the RCL and can inhibit multiple proteases in the coagulation cascade, whereas, HCII with a smaller RCL only has one physiological target (*61, 63*). Thus, the RCL is a key component of serpin specificity.

1.5 Antithrombin

Antithrombin is a 58 kDa molecule that is synthesized in the liver (*64*). AT is a serpin that is capable of inhibiting all of the activated enzyme factors of the coagulation pathway, but has the highest activity towards thrombin, FXa and FIXa (*13*). It has limited ability to inhibit thrombin and other factors until its activity is enhanced by about 1000-fold by GAGs such as heparan sulfate or heparin (*34*). Its role in the coagulation cascade is vital, as noted when complete AT deficiency is engineered in mice, embryonic lethality ensues at the time that coincides with vascular development; AT deficiency-related

embryonic death is a result of extensive fibrin deposits in the heart and liver (*65*). Therefore, AT is an important regulator of coagulation.

1.5.1 Mechanism of GAG Enhanced Anticoagulant Ability

AT follows the same inhibitory mechanism as other serpins but there are a few variations involving the accessibility of the PI-Arg and the conformational changes induced by the GAG. The PI-Arg and the flanking residues of the RCL act as a substrate recognition sequence to bait the trypsin-like serine proteases of the coagulation cascade (*66*). Furthermore, the GAG activated sites regulate the protease binding to AT. The GAGs that are generally responsible for the enhancement of AT antithrombotic activity are heparan sulfate that lines the surface of blood vessel endothelial cells and, to a lesser extent, heparin released from extravascular mast cells that are closely associated with blood vessels (*66, 67*). Thus, heparin and HS come in contact with AT when a blood vessel is injured and allow AT to quickly inactivate blood clotting factors that escape the site of injury and could possibly cause thrombosis in undesired downstream locations (*66*). Native AT also has the ability to inhibit coagulation factors in the absence of GAGs, but at a much lower rate. The position of the PI-Arg in the RCL is of high importance in the recognition by target proteases in the absence of HS or heparin (*68*). In native AT the P1 residue is not extended away from the serpin and available for protease recognition, but is in fact associated with AT (*68*). The location of this association contributes to the low basal rate of inhibition as the association occurs at the principal exosite for FXa and FIXa recognition. Experimental evidence shows that inhibition is possible in AT's native state because it exists in equilibrium between at least two conformations (*68*). The P1

side chain in the RCL is either available for protease recognition and free from intramolecular interactions or the RCL is anchored by the Arg-393 (P1) residue to the FXa/FIXa recognition exosite (*68*). AT has basal anticoagulant activity but interaction with GAGs results in conformational changes that allow the PI residue to adequately interact with its target proteases.

1.5.2 Importance of GAG length for AT's Target Protease

The mechanism for enhancement of anticoagulant activity by AT differs for thrombin and other coagulation factors such as FXa and FIXa. The heparin pentasaccharide is sufficient to enhance AT's ability to inactivate FXa and FIXa by two orders of magnitude, but only by a two-fold enhancement for inactivation of thrombin (*68*). Full acceleration of thrombin inhibition requires a full-length GAG consisting of at least 26 residues (*64*). The longer GAGs enhance AT activation through a bridging mechanism by binding both AT and thrombin, thereby promoting interaction in a ternary complex. This bridging also enhances AT's ability to inhibit FXa and FIXa, but to a much lesser extent than is seen with thrombin. (*69*). The GAG binding site falls over three distinct regions of AT: the N-terminal region, the N-terminal end of helix A, and all of helix D including its N-terminal loop. The binding of HS or heparin to these sites cause distinct conformational changes that result in the repositioning of basic residues to interact with the pentasaccharide as well as the extension of the RCL away from the serpin (*70, 71*). The extension of the RCL is important in the activation of AT, but is not the main factor, otherwise the pentasaccharide would be sufficient for the enhancement of inhibitory activity against thrombin (*72*). Thus, exosites outside of the RCL are important

in GAG enhancement of AT activity. The strand 3 of sheet C in AT has been substituted with that from API and the resulting chimeric serpin showed a reduction in the ability of heparin pentasaccharide to enhance the activity of AT in the inhibition of FXa and FIXa without affecting thrombin (*73*). Later, it was discovered that the complementary exosites on these proteases involve critical conserved Arg150 that binds to exosites on strand 3C of AT. Thrombin lacks the conserved Arg150 and therefore cannot interact with the AT exosite and consequently requires the bridging mechanism for inactivation (*66, 74*). AT is the main physiological inhibitor of thrombin and also plays a significant role regulating other factors in the coagulation cascade. Heparin or HS is required to enhance AT's anticoagulant ability and the mechanism by which this occurs differs between thrombin and FXa/FIXa.

1.6 Heparin Cofactor II

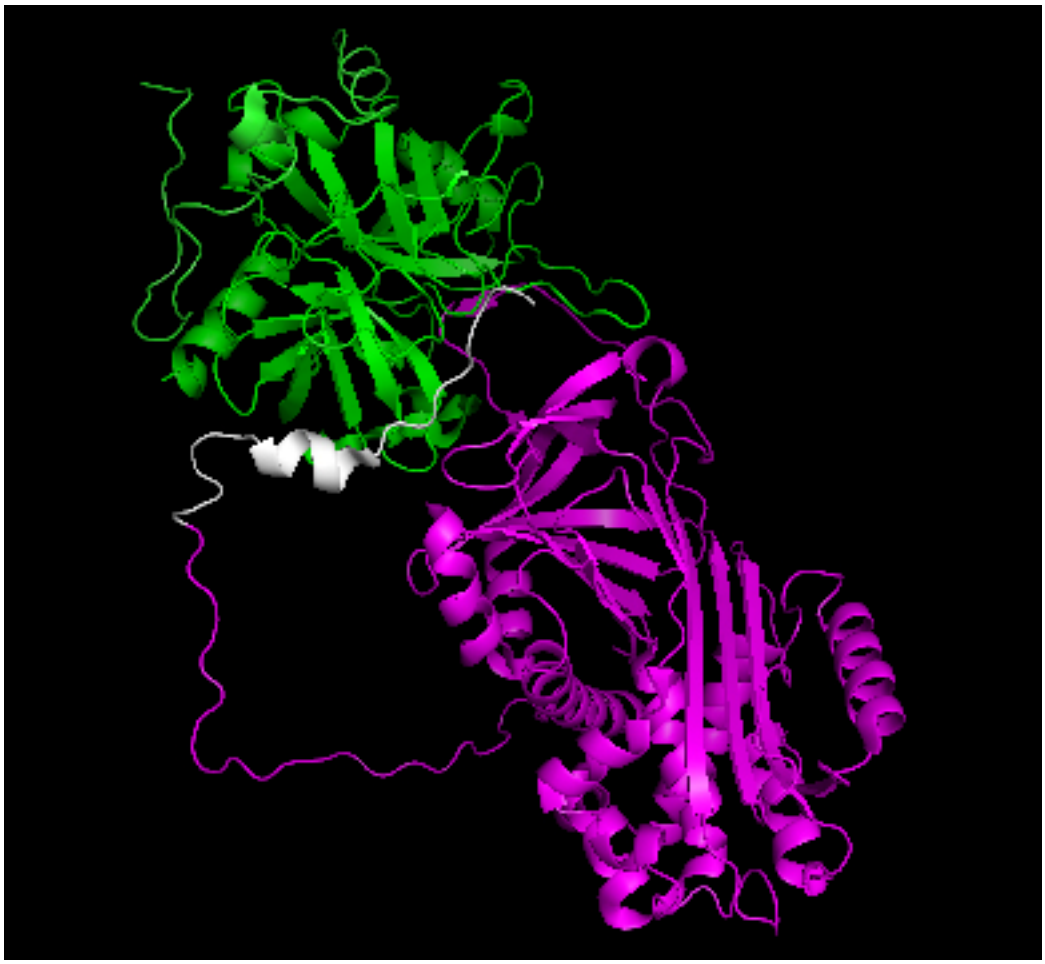
Heparin cofactor II (HCII) is a 65.6 kDa serpin (*75*) that is specific for the procoagulant protease thrombin, although it can also inhibit proteases with which it is highly unlikely to contact physiologically, such as the digestive enzyme chymotrypsin (*13*). It is synthesized by hepatocytes and secreted into the blood stream; it also eventually becomes distributed into the intima and media of atherosclerotic lesions as well as the walls of normal arteries (*75*). Like AT, HCII has a limited ability to inhibit thrombin without the presence of a cofactor, but the presence of a GAG augments its ability to inhibit thrombin by 1000-fold (*76*). While AT's inhibition of thrombin is enhanced only by HS or heparin, HCII's reaction is also enhanced by dermatan sulfate (DS). In fact, DS enhances HCII's ability to inhibit thrombin to a greater extent than other

GAGs (*77*). DS is synthesized and secreted by vascular smooth muscle cells (VSMCs) and fibroblasts and it is deposited in the matrix of vascular intima and media and therefore only comes in contact with HCII during vascular injury (*75*). Furthermore, in contrast to AT, HCII, with DS as a cofactor, is able to inhibit free as well as clot-bound thrombin (*78*). Their presumed physiological roles and their GAG-dependent mechanisms of inhibition also differ.

1.6.1 Mechanism of Thrombin Inhibition by HCII

HCII has a unique structure. It possesses a Leu-Ser P1-P1' bond, unusual in that most thrombin inhibitors require an Arg-Ser bond for inhibition. Furthermore, it has an acidic N-terminal extension, or "tail", that is key for its inhibition of thrombin. The three dimensional location of the tail in native HCII has not yet been determined as the acidic tail is not ordered in the crystal structure. It was hypothesized that the tail was associated with helix D of HCII but no crystal contacts were found on this region (*79*). These GAGs enhance the inhibition of thrombin by HCII likely by expelling the RCL and displacing the N-terminal acidic tail from HCII thereby allowing it to bind to the positively charged exosite 1 of thrombin (*76*); thus thrombin is tethered to HCII and the catalytic serine of thrombin is in optimal orientation to cleave the Leu-Ser bond of HCII (*79*) (Figure 2). If HS or heparin is present then the GAG also causes bridging between HCII and exosite 2 of thrombin. Though AT's ability to inhibit thrombin is also enhanced by HS, it is through a different mechanism, as AT does not bind exosite 1. After cleavage of HCII's scissile bond, just as with other serpins, the RCL is incorporated into β-sheet A and

Figure 2: The three-dimensional structure of heparin cofactor II bound to S195A thrombin. Thrombin is coloured green while HCII is magenta. The white section represents residues 54-75 of HCII that bind to exosite 1 of thrombin. This section of residues also closely resembles the C-terminal tail of hirudin that also binds exosite 1. This figure was generated with PyMOL using the protein data bank file IJMO.



thrombin is flipped to the other pole of HCII. At this point steric clashes disrupt the binding of the acidic tail to exosite 1 and the tail cannot bind back to HCII; it is hypothesized that the tail is cleaved by other endogenous proteases. Lastly, there is an expansion of the β-sheet A causing a reduction in the affinity for the GAG. Thus, the HCII-thrombin complex is released from the cell surface and expelled from circulation while the GAG is recycled (*79*).

1.6.2 The Importance of the N-terminal Tail

The N-terminal tail of HCII is critically important for maximal thrombin inhibition by this serpin. The acidic tail comprises the first 75 amino acids of HCII and includes two acidic repeats that resemble hirudin, the leech anticoagulant protein that is the most potent known natural inhibitor of thrombin (*76*). These acidic repeats occur between residues 55-75. If the first 52 amino acids are deleted there is no significant change in the inhibitory effect on thrombin (*76*). However, if the first acidic repeat is deleted (Δ67) there is a significant reduction in the ability of HCII to inhibit thrombin in the presence of GAG. If both acidic repeats are deleted (Δ74) there is an even greater reduction of inhibitory effect by HCII on thrombin with GAG present (*76*). In addition, since a portion of the tail stretching from Gly54 to Asp75 can be visualized in co-crystallized HCII bound to exosite 1 of active-site mutated thrombin S195A (*79*) (Figure 2), it is clear that the tail plays a major role in the inhibition of thrombin in the presence of a GAG.

1.6.3 Physiological Role of HCII

The physiological role of HCII is currently unknown. AT is recognized as the main intravascular inhibitor of thrombin. Those who have heterozygous AT deficiency are significantly more likely to show spontaneous thrombosis or disseminated intravascular coagulation, but this does not occur in those who are HCII deficient; hence, there is likely an alternate role for HCII (*80*). One theory is that HCII plays a key part during pregnancy as there is a trace amount of DS found in both the maternal and fetal plasma. Therefore, HCII could be responsible for preventing thrombus formation in the placenta (*81*). AT plays a significant role by inhibiting thrombin activity at the surface of a vascular wall legion, but does not have as large a role in the arteries. HCII has been shown to prevent arterial thrombosis and hence is believed to be an extravascular inhibitor of thrombin (*75*). In addition, DS is normally present in the arterial wall and therefore could come in contact with HCII during a disruption of the endothelium and prevent excessive thrombi formation (*82*). This hypothesis is further supported by the fact that homozygous HCII null mice have been shown to develop carotid arterial thrombi at an increased rate compared to wild type mice, following oxidative damage to the epithelium (*80*). In addition when a different research group attempted to create HCII null mice using different strains of mice to the first group of investigators, only heterozygotes could be obtained, due to the embryonic lethal phenotype of these homozygous HCII null mice; at least in this genetic background, this finding suggested a critical function of HCII(*83*). Furthermore, thrombin has extravascular roles that contribute to atherosclerosis; HCII has been shown to inhibit neointima formation, a sign of the

progression of atherosclerosis. Neointima results from expansion of the intima due to the proliferation and migration of various cells such as SMCs; it is believed that neointima formation is caused by PAR signaling by thrombin and therefore HCII may be inhibiting this extravascular function of thrombin (*75, 80, 84*). Moreover, HCII activity has been shown to be inversely proportional to the severity of carotid atherosclerosis, the prevalence of peripheral arterial disease and endothelium dysfunction (*75*). Therefore, HCII may play a role during pregnancy and while AT is the main intravascular inhibitor of thrombin, HCII may be the main extravascular inhibitor of thrombin.

1.7 α_1 Proteinase Inhibitor

α_1 Proteinase Inhibitor (API) is a serpin whose prime physiological target is neutrophil elastase, which degrades elastin and the extracellular matrix (*82*), although it is capable of inhibition of multiple target proteases, such as the digestive enzyme trypsin, and coagulation Factor XIa. This 52 kDa glycoprotein has an arguably minimal role in regulating coagulation (*56*). However, if the Met-Ser PI-PI' bond is mutated to Arg-Ser at residue 358 (API M358R) this causes API, normally a poor inhibitor of thrombin, to gain the ability to inhibit thrombin effectively. In the absence of GAGs inhibition by API M358R occurs 17-fold more rapidly than pentasaccharide activated AT, the main physiological thrombin inhibitor (*85*). This is known as the Pittsburgh mutation and was first encountered in a patient with a fatal bleeding disorder (*60*). This patient reached the age of 14 before he died of hemorrhage; API is an acute phase reactant and therefore the concentration of this mutant protein increased by several fold after the patient experienced inflammation/trauma. This sudden spike in the levels of API M358R has

been suggested to be the root cause of the fatal hemorrhage in the index case (*60*). Furthermore, about a decade later another patient was found to have the same API mutation (*86*). This time it was discovered that this patient also had a diminished plasma protein C concentration; it is believed that API M358R may impact the metabolism of this anticoagulant (*86*). In addition, APC was essentially absent from the patient's plasma. Firstly, the M358R mutation enhanced API's ability to inhibit APC by about 4400 fold, and thus much of the APC produced was inactivated. Secondly, since API M358R also has a high affinity for thrombin the activation of protein C was reduced (*86*). It is hypothesized that this protein C deficiency contributed to the maintenance of a normal hemostatic balance in this patient; while thrombin might have been inhibited to a greater extent in a procoagulant sense, APC was also inhibited, removing down-regulation of cofactors V and VIII from the new equilibrium. As a result, it was not until this patient was 17 that he experienced a major bleeding episode upon experiencing a trauma that resulted in a profound increase in the acute phase reactant API M358R (*86*). Therefore, this mutation appears to be lethal only when inflammation or tissue damage causes an increase in the mutant acute phase reactant, API M358R.

Inhibition of thrombin by API M358R does not involve binding to exosite 1, as is the case with GAG-activated HCII (*85*). In fact, minimal conformation changes occur in the RCL during inhibition, with the most significant difference noted in the P5-P5' region. Furthermore, the P2 proline is important for thrombin specificity; if this residue is mutated to a glycine, a 20-fold reduction in its antithrombotic activity is noted (*85, 87*). API M358R, unlike HCII or AT, does not require a GAG as a cofactor for thrombin

inhibition and thus for these reasons it has been considered for development as an anticoagulant and/or antithrombotic protein (*85*). However, this Pittsburgh mutation also increased API's ability to inhibit other proteases such as APC, which regulates FVa and FVIIIa by specific proteolysis (*85, 88*). Consequently, the ability of API M358R to inhibit APC partly counteracts its antithrombotic effects. To make API M358R a more favorable anticoagulant for potential clinical use, it needs to be mutated in a manner to increase its potency as a specific thrombin inhibitor while decreasing its ability to inhibit APC.

1.7.1 Enhancing Specificity of API as a Thrombin Inhibitor

In previous work from the Sheffield laboratory, it has been found that the addition of the N-terminal tail of HCII, consisting of residues 1-75, to API M358R (HAPI M358R) increased the rate of inhibition of α-thrombin by 21-fold compared to API M358R (*89*). However, when γ-thrombin, which has an incomplete exosite 1 (*90, 91*), was used there was only a 9-fold increase in inhibition, thus showing that this increase in antithrombotic activity was due to HAPI M358R's ability to bind directly to thrombin (*89*). Furthermore, the mutation of certain residues (P4-P7/P2') in the RCL to those found in AT resulted in a major decrease (108 fold) in HAPI M358R's anti- APC activity while only minimally decreasing its antithrombin activity (approximately 2 fold lower). This construct was titled HAPI RCL5 (*91*). Thus, increasing affinity to thrombin exosite 1 or further mutations in the RCL of API may lead to further specificity for thrombin over APC.

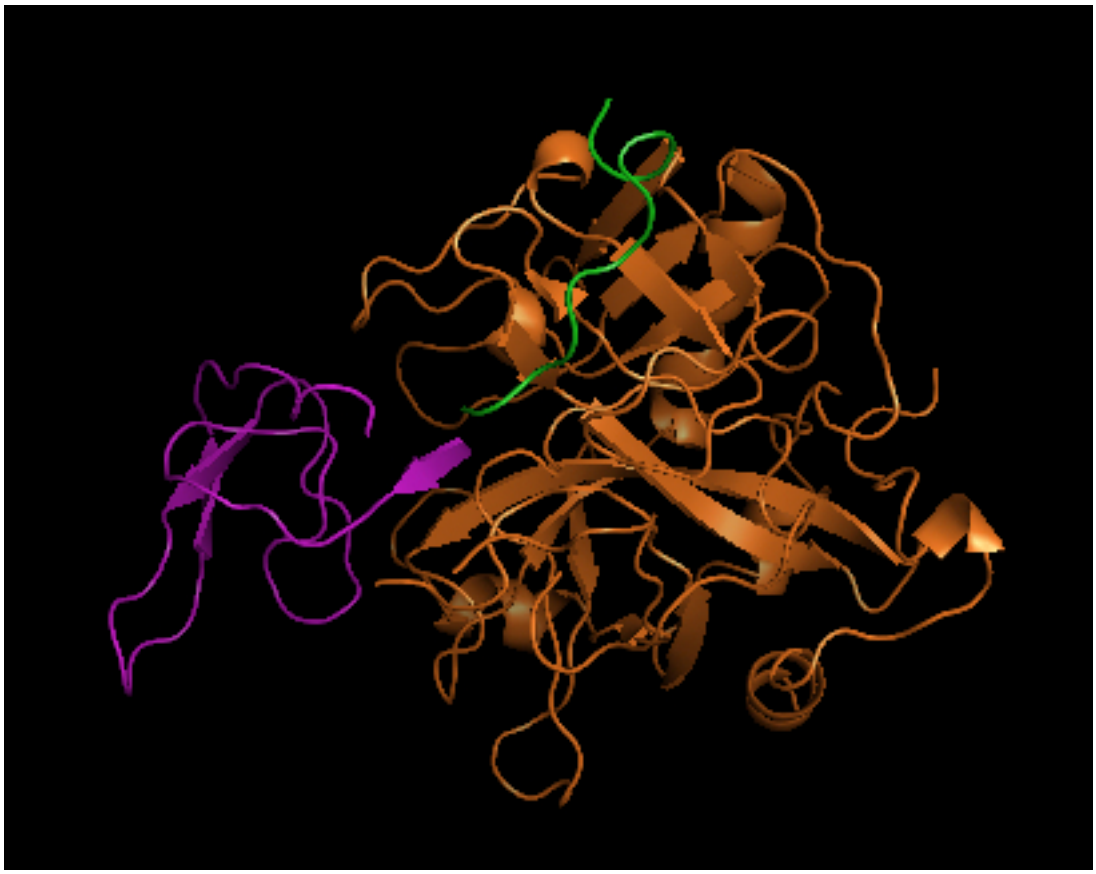
1.8 Hirudin and its Variants

The saliva of leeches has been known to contain anticoagulant properties for over 100 years. In 1957 the active anticoagulant component, hirudin, was isolated from the salivary glands of the medicinal leech, *Hirudo medicinalis*, by Markwardt (*92, 93*). However, it was not until 30 years later, when recombinant hirudin was first expressed, that large enough quantities of hirudin were able to be generated for therapeutic uses (*92, 94*). Furthermore, isolation from leeches resulted in a heterogeneous mixture of hirudin-like molecules and other protease inhibitors, also rendering this method not ideal for treatment (*94*). Presently hirudins are still considered the most potent natural protein-based thrombin inhibitors. They interact with thrombin directly without the need of a cofactor. This bivalent inhibitor forms a 1:1 stoichometric complex with thrombin by binding to both the active site, by the N-terminal region, as well as exosite 1 with its C-terminal region (*95*) (Figure 3). The ability to bind to both these regions of thrombin results in hirudin's ability to inhibit all proteolytic functions of thrombin including activation of FV, FVIII and FXIII and protein C, clotting of fibrinogen and platelet activation (*92*). Since it directly interacts with thrombin it can also inhibit fibrin-bound thrombin, unlike other inhibitors such as AT. It is hirudin's ability to bind to both these regions so tightly that makes it such a potent thrombin inhibitor.

1.8.1 Hirudin Derivatives

Hirudin exists naturally in multiple isoforms. For instance hirudin variants 1 through 3 have been isolated from leeches (*96, 97*) and such variants share 85-90% sequence homology (*98*) with hirudin variant 3 being the most potent thrombin inhibitor

Figure 3: Three-dimensional structure of thrombin complexed to sulfated hirudin variant 1. Thrombin is coloured orange while hirudin residues 1-53 are in magenta. The green section represents residues 54-65 from hirudin that bind to exosite 1 of thrombin. This figure was generated with PyMOL using the protein data bank file 2PW8.



(*99*). The application of recombinant DNA technology has resulted in the production of additional hirudin derivatives. Recombinant hirudin does not have a sulfated tyrosine 63 since the *E. coli* or *S. cerevisiae* that are used for protein production are not able to perform that post-translational modification (*93*). However, this lack of sulfation only results in a 10-fold reduction in affinity of the C-terminal tail for exosite 1. This decrease is the result of the sulfated tyrosine being able to stabilize interactions with thrombin through a complex hydrogen bonding network (*93*). Other hirudin derivatives have been produced through peptide synthesis such as hirugen and hirulog (also known as bivalirudin). (*92*). Hirugen is the synthetic dodecapeptide that is comprised of the C-terminal residues 53-64. This peptide has a reduced ability to inhibit thrombin since it only binds exosite 1, and the overall affinity of hirugen for thrombin is substantially reduced compared to full-length hirudin. Therefore it can only compete for binding to thrombin with fibrinogen and does not affect the proteolytic activity of thrombin, such as with the activation of FV, FVIII or FXIII (*92*). However, the addition of D-Phe-Pro-Arg-Pro-(Gly), a sequence that resembles the thrombin active site inhibitor PPACK, to the N-terminus converts hirugen to hirulog, a much more potent thrombin inhibitor as it now has the added ability to bind to the active site of thrombin. Unlike hirudin, the catalytic site inhibition is transient since thrombin slowly cleaves the Pro-Arg bond and therefore, hirulog inhibition is reversible (*92*).

1.8.2 Interactions of Hirudin with Thrombin

Hirudin has high affinity for thrombin because it has multiple contacts with the protease, not only at the active site and exosite 1 but also at points in between (*100*). The

orientation of hirudin is opposite to other inhibitors of serine proteases that have a polypeptide chain that runs N-terminal to the C-terminal into the active site and forms an anti-parallel β-sheet with residues 214-218 of thrombin. On the other hand, hirudin runs N-terminal to C-terminal out of the active site and forms a parallel β-sheet with the same residues of thrombin (*93*). Most of the N-terminal domain is not in contact with the thrombin surface, but there are still multiple interactions at the interface of the two molecules: some form contacts in the active site cleft, most importantly, Val-1 and Tyr-3 (*93*), others, such as Asp-5 and Ser-19 form hydrogen bonds or salt bridges to thrombin and Lys-24 and Asn-20 interact with thrombin through water mediated contacts (*101*). The Lys-47 of hirudin is generally viewed as the equivalent of the P1 residue found in serpins. Though it does not occupy the specific active site pocket of thrombin, it still seems to be integral to the stabilization of hirudin's interaction within the active site cleft (*100, 101*). Differing from the N-terminal section, the C-terminal tail has a long extended conformation and is visualized in the co-crystal structure as two stretches of amino acids with a bend at Asp-55. Due to this extended conformation the C-terminal tail can form multiple interactions with exosite 1. There are various acidic residues found in the tail to complement the basicity of the thrombin exosite. The most important appear to be Glu-57 and Glu-58 but all negatively charged residues contribute to the binding energy (*93*). The composition of this tail closely resembles that of HCII which also binds exosite 1 during inhibition. In fact, both interact strongly with Arg67 and Arg73 of thrombin (*102*). A surprising amount of the interactions between exosite 1 and the C-terminal tail are hydrophobic; in the terminal end of the tail 5 of the 11 residues are hydrophobic or

aromatic and they all participate in nonpolar interactions with thrombin. It is also believed that the binding of the C-terminal tail to thrombin results in a conformational change that then allows the N-terminal portion of hirudin to access the active site (*101*). The extensive contacts and interactions between hirudin and the thrombin active site and exosite 1 account for the profound anticoagulant and antithrombotic activity of hirudin.

1.8.3 Clinical Use of Hirudin

Though hirudins and their derivatives are extremely potent thrombin inhibitors there are disadvantages to their clinical use. Unlike heparin, the anticoagulant effect of hirudin cannot be reversed. Thus, it would be very problematic if someone had a life threatening bleeding episode while taking this drug. However, hirudin does have a short half-life of 0.6 to 2 hours (*92, 103*) which makes such an event unlikely. Various early animal models have demonstrated that hirudin is highly effective at eliminating thrombus formation. For instance, in a model of arterial damage in pigs, hirudin completely prevented thrombus formation and reduced platelet incorporation by about 80% (*94, 104*). Such successes promoted human clinical trials which also looked very promising in various hypercoagulable diseases. For instance, one clinical study for the prevention of venuous thromboembolism demonstrated that the rates of total and proximal vein thrombosis were significantly lower in patients treated with 15-20 mg of hirudin (*105*). However, the risk of hemorrhage can also be quite high with the use of hirudins (*106*) and thus derivations of hirudin such as lepirudin have been clinically approved in certain situations such as for patients that have heparin-induced thrombocytopenia (HIT) (*107*). HIT is an immune disorder that patients administered heparin may acquire. Antibodies

are produced that bind to complexes between heparin and platelet factor 4. These immune complexes adhere to and activate platelets, resulting in an increase in procoagulant activity and this counteracts heparins' anticoagulant ability (*107*). In such cases as well as those of heparin resistance (*105*), hirudins are commonly used as a replacement anticoagulant. Overall, hirudins do have advantages such as a more predictable anticoagulant response and specific mechanism of action compared to heparin. Though hirudins and their derivatives, such as lepirudin and desirudin, are extremely potent thrombin inhibitors, they have a high risk of bleeding (*108*) and thus, are only used clinically on rare occasions such as when patients are experiencing HIT (*109*).

1.9 Rationale for Study

The maintenance of hemostasis is integral for survival and if certain pathologies manifest themselves, such as atherothrombosis, cancer, or trauma, causing this balance to tip towards coagulation, then the administration of anticoagulant and/or antithrombotic agents is required. There are various anticoagulants on the market but they are not ideal since they tend to lack certain characteristics such as a broad therapeutic index, not interacting with food or drugs, and being orally available. Therefore, my rationale for creating the HV3API M358R and HV3API RCL5 fusion proteins was to design an effective and safe novel anticoagulant that might be beneficial clinically for such diseases as VTE. Though these proteins would not be orally active, they would likely not be immunogenic since they are based on endogenous proteins; they also ideally would not interact with food

or drugs and would have a broad therapeutic index, potentially resulting in a low bleeding tendency. The other aspect of this project was to investigate the structure and function relationships of the API M358R variants. In this way it can be seen how far the structure of the serpin can be manipulated to alter its function and specificity towards thrombin. Previously, it was shown that the function of the HCII acidic tail could be transferred to API M358R and that the structure of the RCL plays a critical role in serpin specificity as the RCL5 variant resulted in a greater specificity for thrombin over APC. This project investigated whether the exosite 1 binding function of the C-terminal structural domain of HV3 could also be transferred to API M358R and how this change in structure altered the potency of the serpin as a thrombin inhibitor. Such manipulations can help determine what structural alterations are required to make this serpin as specific of a thrombin inhibitor as possible.

1.10 Hypotheses

1. The peptides MGSHV3$_{54-66}$, MGSH$_6$HV3$_{54-66}$ and MGSH$_6$HV3$_{54-66}$G$_6$ will have a similar affinity for exosite 1 of thrombin and all three will have a greater affinity for exosite 1 than HCII 1-75.

2. The HV3$_{54-66}$API M358R protein will inhibit thrombin more effectively than HAPI M358R and likewise HV3$_{54-66}$API RCL5 will inhibit thrombin more effectively than the HAPI RCL5 protein.

3. The rate of APC inhibition of HV3$_{54-66}$API M358R and HV3$_{54-66}$API RCL5 will be no greater than that of HAPI M358R and HAPI RCL5 respectively.

4. The enhanced inhibition of thrombin by both HV3$_{54-66}$API M358R and HV3$_{54-66}$API RCL5 will correlate with their ability to bind exosite 1.

1. 11 Specific Objectives

The overall aim of this project was to enhance the antithrombin ability of HAPI M358R through the replacement of the N-terminal acidic extension of HCII for the C-terminal tridecapeptide of HV3 in the fusion to API M358R. Towards this aim my specific objectives were:

1. To characterize the HV3 peptides ($HV3_{54-66}$, H_6HV3_{54-66} and $H6HV3_{54-66}G_6$) in terms of their efficacy as thrombin inhibitors and their ability to bind exosite 1 through thrombin clot time assays and determination of IC50 through the use of competitive binding assays.

2. To express and purify $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5 and to assess their efficacy as thrombin and APC inhibitors by determining second order rate constants and stoichiometries of inhibition in comparison to HAPI M358R, HAPI RCL5, α_1PI M358R and α_1PI RCL5.

3. To determine if thrombin inhibition by $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5 correlates to binding of exosite 1 by determining second order rate constants for inhibition of γ_T-thrombin and through the use of competitive binding assays with PPACK-thrombin.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Source of Chemicals and Reagents

Chemicals were purchased from the following suppliers: Thermo Scientific (Burlington, ON), restriction and DNA modifying enzymes (Nco1, EcoRI), Thermo Pageruler prestained protein ladder, T4 DNA polymerase, Coomassie Plus protein assay reagent; Invitrogen (La Jolla, CA), Benchmark unstained 1Kb DNA ladder, *E. coli* Top10 competent cells; New England Biolabs (Pickering, ON), Phusion DNA polymerase; GE Health Care (Baie d'Urfe, QC), mouse anti-his antibody, diethylaminoethyl (DEAE) sepharose; Promega (Madison, WI), anti-mouse AP conjugated antibody; Affinity Biologicals (Ancaster, ON), sheep anti-human α_1-PI HRP conjugated antibody, sheep anti-human thrombin HRP conjugated antibody; Enzyme Research Laboratories (South Bend, IN), α-thrombin; Haematologic Technologies (Essex Junction, VT), γ_T-thrombin; Sigma Aldrich (Mississauga, ON), APC, o-phenylenediamine dihydrochloride (OPD) tablets, diaminobenzidine tetrahydrochloride hydrate (DAB), arabinose; Stago (Asnieres sur Seine, France), Thrombin 10; Advanced Protein Technology Center at the Hospital for Sick Children (Toronto, ON), HV3 peptides; Diapharma (West Chester, OH), chromogenic substrates S2238 (H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline-dihydrochloride), S2366 (L-Pyroglutamy-L-prolyl-L-arginine-p-nitroaniline hydrochloride); Roche (Mississauga, ON) nitro blue tetrazolium/5-bromo-4chloro-3-indolyl phosphate (NBT/BCIP); Bioshop (Burlington, ON), ampicillin, bovine serum

albumin (BSA), electrophoresis grade agarose; Qiagen (Carlsbad, CA), nickel-nitrilotriacetic acid (Ni-NTA) resin, plasmid DNA isolation and purification kits; Calbiochem (La Jolla, CA), phenylalanyl-L-prolyl-arginine chloromethyl ketone (PPACK); Millipore (Billerica, MA), Amicon Ultra-15 Centrifugal Filter Unit; Biorad (Hercules, CA) N,N,N',N'-tetramethylethylenediamine hydrochloride (TEMED). All other reagents and chemicals not specifically listed above were of the highest quality and grade available.

2.1.2 Oligonucleotides

The oligonucleotides listed in Table 1 were synthesized at the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University (Hamilton, ON).

2.2 Methods

2.2.1 HV3 Peptide and HCII 1-75 Analysis

2.2.1.1 TCT Analysis

Thrombin 10 containing freeze-dried human thrombin with calcium at 1.5 NIH/unit, was reconstituted with 10 mL ddH_2O followed by a 30 minute room temperature incubation (as instructed in manual). Human plasma (25 µL) and inhibitor peptides diluted in 75 µL veronal buffer (sodium acetate trihydrate 7.14 mM/ sodium diethyl barbiturate 7.4 mM/ NaCl 0.131M pH7.4) were added to wells containing magnetic mixing beads (Stago) in fibrin timer (Stago) at 37°C. The timer was started and

Table 1: The oligonucleotides used over the course of the project are listed. The forward HV3API primer and the reverse API primer were used for creating HV3API M358R and HV3API RCL5 fusions, while the sequencing primers were used to confirm proper insertion of these fusions into pBAD.

Oligonucleotide Sequence	Description
5′- CACCATGGGGTCTCATCATCATCATCATCATGGAGACTTTGA GCCTATCCCTGAGGATGCCTATGATGAGGGTGGAGGTGGAG GTGGAGAGGATCCCCAG-3’	HV3API Forward primer
5’-CCGGAATTCTTATTTTTG GGTGGGATTCAC-3’	API Reverse primer
5’-AAATTCTGTTTTATCAGACC -3’	Reverse pBAD sequencing primer
5’-ATGCCATAGCATTTTTATCC-3’	Forward pBAD sequencing primer

100 µL of thrombin 10 was added to each well. The time to clot is measured by the fibrin timer. HV3 peptides ($MGSHV3_{54-66}$, $MGSH_6HV3_{54-66}$, $MGSH_6HV3_{54-66}G_6$) tested in this experiment (as well as the assay below) were synthesized by the Hospital for Sick Children. HCII 1-75 was cloned into a pBAD vector and expressed and purified in the Sheffield lab, as described as follows, in Amanda Boyle's MSc thesis (2010). *E. coli* TOP10 cells transformed to ampicillin resistance with pBAD-H_6-$HCII_{1-75}$ were grown in LB/ampicillin with shaking at 37°C to an OD_{600} of 0.5 prior to induction with arabinose to 0.002% (w/vol). Following an additional 3.5 hours of growth, cells were harvested by centrifugation and cell pellets disrupted by sonication in equilibration buffer (EB; 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole), then made 1% (vol/vol) in Triton X-100. The clarified lysate was applied to Ni-NTA agarose resin, washed, and eluted with EB containing 250 mM imidazole. Peak fractions were dialyzed versus 20 mM Tris-Cl pH7.4, 200 mM NaCl, and then chromatographed on Q-Sepharose. Proteins remaining bound after washes with 20 mM Tris-Cl pH7.4, 300 mM NaCl were eluted with 20 mM Tris-Cl pH7.4, 350 mM NaCl and concentrated with an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane.

2.2.1.2 Competitive Binding Assay

Wells were coated with 100 µL of 1µM HCII 1-75, diluted in PBS and incubated overnight at 4°C. Coating solution was removed and 200 µL of blocking solution (5% BSA in PBS-T) was added to each well and incubated at room temperature for 1 hour. Wells were washed twice with PBS-T. Competitor diluted in blocking solution was added

(50 µL) to wells in triplicate and 100 µL of blocking solution was added as blanks. Lastly, 50 µL of 10 nM thrombin was added to each well simultaneously. None was added to the blanks. The plate was covered and incubated at 37°C for 30 minutes. Wells were washed 4-5 times with PBS-T. Sheep anti-human thrombin-HRP conjugated antibody was diluted 1/10 000 in blocking solution (5% BSA in PBS-T) and 100 µL was added to each well. This was incubated at room temperature for 1 hour. The wells were then washed 4-5 times with PBS-T. HRP developing solution (OPD tablet in 12 mL substrate buffer – 100 mM Na_2HPO_4/ 20 mM citric acid pH5; 12 µL of H_2O_2 added just before use) was added to each well (100 µL) and incubated at room temperature. Colour developed slowly over 10 minutes. The reaction was stopped with 50 µL/well of stopping buffer (2.5 M H_2SO_4). The plate was read at 490 nm using the ELx808 plate reader. Note, when this assay was performed with full-length serpins, PPACK-thrombin was used instead of thrombin on its own. A 10:1 molar excess of PPACK to thrombin was utilized to ensure complete active site inhibition. Excess PPACK was not removed prior to experimentation.

2.2.2 Cloning of $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5 into pBADMyc-HisB Vector

A PCR was performed with Phusion DNA polymerase and using previously made pBAD(HAPI M358R) or pBAD(HAPI RCL5) as a template and a forward primer coding for a hexahistidine tag, HV3 tridecapeptide sequence, glycine spacer and the first few amino acids of API as well as a reverse API primer (Table 1). These amplified $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5 sequences contained Nco1 and EcoRI restriction

sites. The PCR products were visualized on a 1% agarose gel to check for successful amplification before being cleaned with the Qiagen PCR purification kit. The PCR products as well as a mini-prepped pBAD/*Myc*-HisB vector were digested with EcoRI and Nco1. These products were then gel-extracted using the QIAquick Gel Extraction QIAGEN kit. Either the $HV3_{54-66}$API M358R or $HV3_{54-66}$API RCL5 gel extraction was then ligated into the pBAD vector with T4 DNA ligase. This ligation was transformed into Top10 competent cells by heat shocking the cells at 37°C. Potential clones were screened by restriction digest. If the restriction endonuclease digestion pattern observed correlated to that expected for the desired insert, the sample was sent to MOBIX for sequencing (Table 1) and confirmation of the desired $HV3_{54-66}$API M358R or $HV3_{54-66}$API RCL5 insert.

2.2.3 Expression of API M358R Fusion Proteins and Variants

Bacteria (50 mL) containing the desired construct were incubated at 37°C for 16h in Luria Broth containing 100 mg/mL ampicillin. Following this incubation a 1/50 subculture into 1-2 L of the appropriate media was performed. This was grown at 37°C until an OD of 0.5 was reached. At this point arabinose was added at a final concentration of 0.002% to induce expression and this was incubated at 37°C for another 4 hours before the cells were harvested by centrifugation for 15 min at 6000 x g using the GSA rotator in the Sorvall RC 5B Plus centrifuge.

2.2.4 Purification of Recombinant API Variants and Fusion Proteins

All constructs used were purified using a nickel column as described in Cunningham et al., 2002 (*110*) except the elution fractions were dialyzed overnight in 20mM sodium phosphate pH 6.8 prior to ion exchange chromatography using DEAE Sepharose. A NaCl gradient ranging from 0 to 300 nM in 20 mM sodium phosphate pH 6.8 was utilized for DEAE Sepharose purification. Fractions containing the protein of interest as determined by SDS-PAGE were pooled and concentrated using a Amicon Ultra-15 Centrifugal Filter Unit and stored at -80°C.

2.2.5 Quantification of Purified Protein by Bradford Assay or Extinction Coefficient

During a Bradford assay, a protein standard was created using eight dilutions of 2 mg/mL BSA ranging from 0.1 mg/mL – 1.0 mg/mL. Two or three diluted samples were created for the purified protein. To a 96-well plate 5 μL of each sample were added in duplicate including two blanks composed only of buffer. To each 5 μL sample 200 μL of Coomassie Plus Protein Assay Reagent was added. The plate was read using the KC4 software. Through measuring the OD of the wells and using the BSA dilutions as a standard the approximate concentration of the purified protein was extrapolated. Minor contaminants were observed in the HAPI and API M358R and RCL5 purifications. In these cases were impurities were observed, concentrations were adjusted according to the percent purity determined by densitometry using the Quantity One software (Bio Rad). Since no contaminants were detected in $HV3_{54\text{-}66}$API M358R and $HV3_{54\text{-}66}$API RCL5 preparations, the concentrations of these proteins were determined using the extinction

coefficient method. The purified recombinant protein was diluted and its absorbance at A_{280} was determined. The extinction coefficient and concentration of the protein were calculated as described by Pace, C. N. et al, 1995 (*111*).

2.2.6 Immunoblots to Determine Presence of His-tag or API

On an SDS-PAGE gel 100 ng of API fusion protein was visualized and a prestained ladder was used. The SDS-PAGE gel was transferred to a nitrocellulose membrane using an iBlot (Invitrogen). The nitrocellulose membrane was covered in 5% milk in TBST and incubated for 1 hour with shaking. To check for the presence of a His-tag, mouse anti-his antibody was diluted, 1 in 2000, in 10 mL of 5% milk/TBST and added to the membrane. This membrane was rocked for 1 hour at room temperature and then the membrane was washed 3 times with TBST for 5 minutes each. Next, the anti-mouse AP conjugated antibody was diluted 1 in 5000 and this solution was added to the membrane. This reaction was rocked for 1 hour at room temperature and then washed 3 times with TBST prior to development with NBT/BCIP (one tablet/10 mL of water). After 5 minutes the reaction was stopped with water. A similar process was used to detect α_1-PI. Instead, a 1/5000 dilution of sheep anti-human API HRP conjugated antibody was used. The membrane was developed with DAB solution (9.8 mL PBS, 200 μL of 1% cobalt chloride and 5 mg DAB). Just before use 10 μL of 30% hydrogen peroxide was added to the DAB solution.

2.2.7 Gel Based Serpin Enzyme Complex Analysis

The ability of $HV3_{54-66}$API M358R, HAPI M358R and API M358R to form SDS-stable complexes with α-thrombin was measured by incubating 1 μM serpin with 0.15 μM α-thrombin at room temperature at various time points over 5 minutes. Reactions were stopped with the addition of 4x SDS-PAGE dye. Reaction products were electrophoresed on 10% SDS-PAGE gels.

2.2.8 Kinetic Analysis of API Variants and Fusion Proteins

The second order rate constants for α-thrombin, γ-thrombin or APC inhibition by API M2358R fusion proteins and variants were determined as previously described (*110, 112*). In short, recombinant serpins (100-650 nM) were incubated with α-thrombin (10 nM), γ-thrombin (10 nM) or APC (25 nM) in PPNE buffer (20 mM Na_2HPO_4, pH 7.4, 100 nM NaCl, 0.1 mM EDTA, 0.1% poly(ethylene glycol) 8000) for various times. Reactions were quenched with chromogenic substrate (150 μM for S2238 and 400 μM for S2366) to determine residual protease activity. The change in absorbance over 5 minutes at 405 nm was measured with an EL808 plate reader (BioTek Instruments, Winooski, VT) and subsequently second order rate constants were determined. This was performed by graphing the time in seconds (x-axis) by $\ln(P_o/Pt)$ (y-axis). Linear regression was performed; data were only included in cases where the regression coefficient, R^2, ≥ 0.9. The slope was used in the equation K_2=slope/[serpin]x(1+[chromogenic sub]/Km)x60, where the Km was determined previously to be 2.9 μM by Jason Sutherland in his PhD thesis (2007). It should be noted a variation on the protocol was used for the APC

inhibition by the RCL5 serpin variants. In these cases 10 nM APC and 1000 fold excess of serpin (10 μM) was used.

Stoichiometries of α-thrombin inhibition by the recombinant serpins were determined by incubating inhibitor (0-800 nM) with 200 nM thrombin for two hours at room temperature. This reaction was quenched with 150 μM chromogenic substrate S2238. Residual thrombin activity was measured by reading the absorbance at 403 nm over 5 minutes. The number of serpin molecules required to inhibit one molecule of thrombin was calculated by plotting the percent activity of thrombin by the ratio of serpin to thrombin.

3. RESULTS

3.1 HV3 Peptide and HCII 1-75 Analysis

The peptides $HV3_{54-66}$, H_6HV3_{54-66}, $H_6HV3_{54-66}G_6$ and HCII 1-75 were characterized for their ability to bind to thrombin through TCTs and competitive binding assays (note that all the HV3 peptides also have MGS on the N-terminus). The concentrations of the HV3 peptides required to double the clotting time (DCT) in the TCT assay were calculated (Table 2). A graph was plotted showing the time required for the plasma to clot (y-axis) as a function of the concentration of the peptide (x-axis). Second order polynomial regression analysis was used to calculate the DCT (Figure 4). The addition of the H_6 tag to the N-terminus of $HV3_{54-66}$ was associated with a decrease in the DCT (0.93 μM to 0.45 μM). However, addition of the G_6 spacer to the C-terminus of $HV3_{54-66}$ resulted in an increase in the DCT (0.93 μM to 1.6 μM) (Table 2 & Figure 4). Furthermore, the peptide HCII 1-75 had the highest DCT at 12 μM. Similar results were ascertained when the peptides were compared as competitors of the binding of thrombin to immobilized HCII 1-75. A graph was plotted with the concentration of the peptide on the x-axis and the $A490/A_o490$ on the y-axis, where A_o is the absorbance when peptide concentration is zero. First order exponential decay analysis was used to determine the IC50 of each peptide (Figure 5; Note that the x-axis scale in the graph for HCII 1-75 differs from the graph for the HV3 peptides). The IC50 value for H_6HV3_{54-66} was similar to that for $HV3_{54-66}$ (0.8 μM compared to 0.9 μM). Conversely, the addition of the G_6 resulted in the elevation of the IC50 to 2.78 μM (Table 2 & Figure 5). Lastly, the peptide HCII 1-75 produced an IC50 value much greater than the HV3 peptides at 9.7 μM. The :

Table 2: Determination of relative thrombin binding by HV3 peptides and HCII 1-75. DCTs were calculated from TCTs depicted in Figure 4 and IC50 and K_i values were determined from competitive binding assay in Figure 5. Values are the mean of 3-4 determinations ± SD. Statistics were not performed because at least an n=5 is required for appropriate tests of normality (i.e. whether or not the data distribute following a proper Gaussian distribution).

Peptide	**DCT (μM)**	**IC50 (μM)**	**K_i (μM)**
$HV3_{54-66}$	0.93 ± 0.05	0.9 ± 0.2	0.9 ± 0.2
H_6HV3_{54-66}	0.45 ± 0.01	0.8 ± 0.2	0.7 ± 0.2
$H_6HV3_{54-66}G_6$	1.6 ± 0.1	2.8 ± 1.0	2.7 ± 1.0
HCII 1-75	12 ± 4	9.7 ± 3.0	9.5 ± 3.0

Figure 4: Comparison of the ability of HV3 peptides and HCII 1-75 to inhibit thrombin. TCT performed where increasing concentrations of inhibitor peptide were added to human plasma and time to clot was measured upon thrombin addition. Second order polynomial regression analysis was conducted. The mean of three determinations ± standard deviation (SD) is shown.

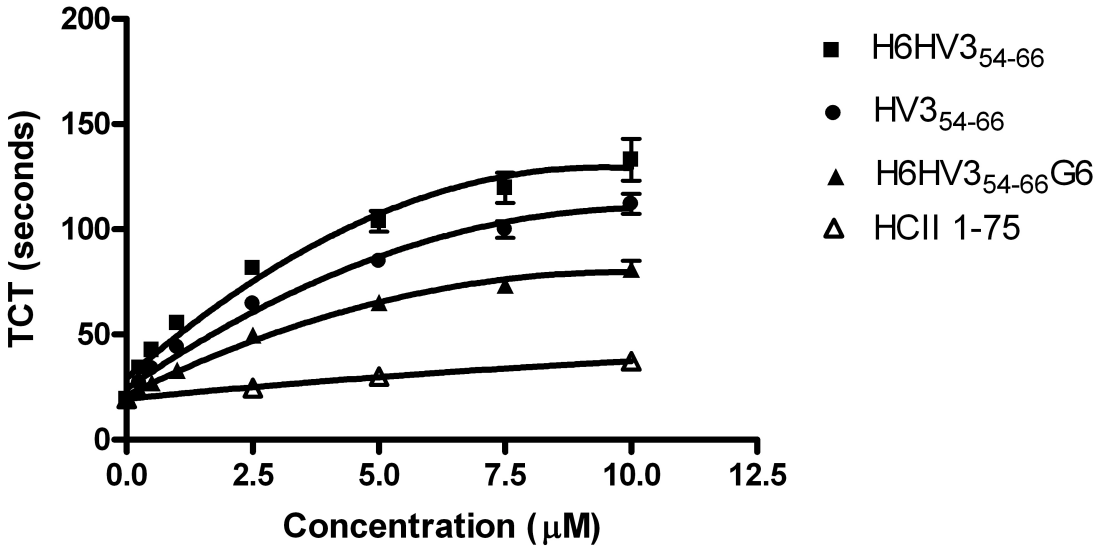
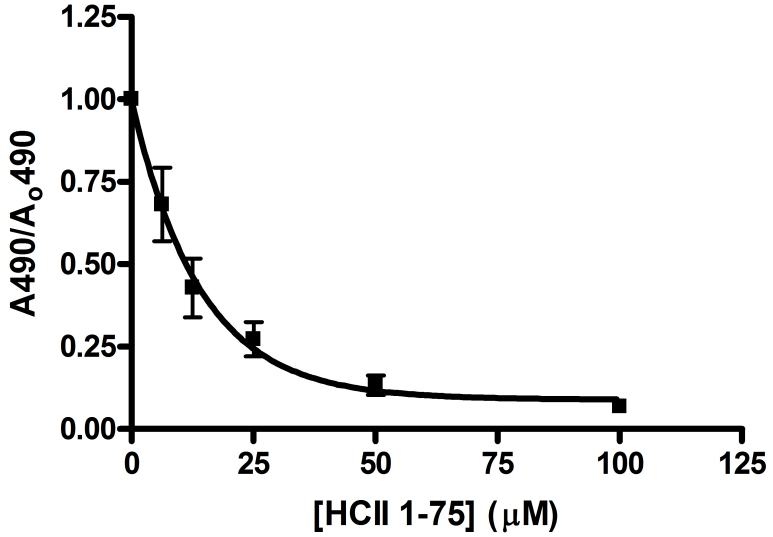
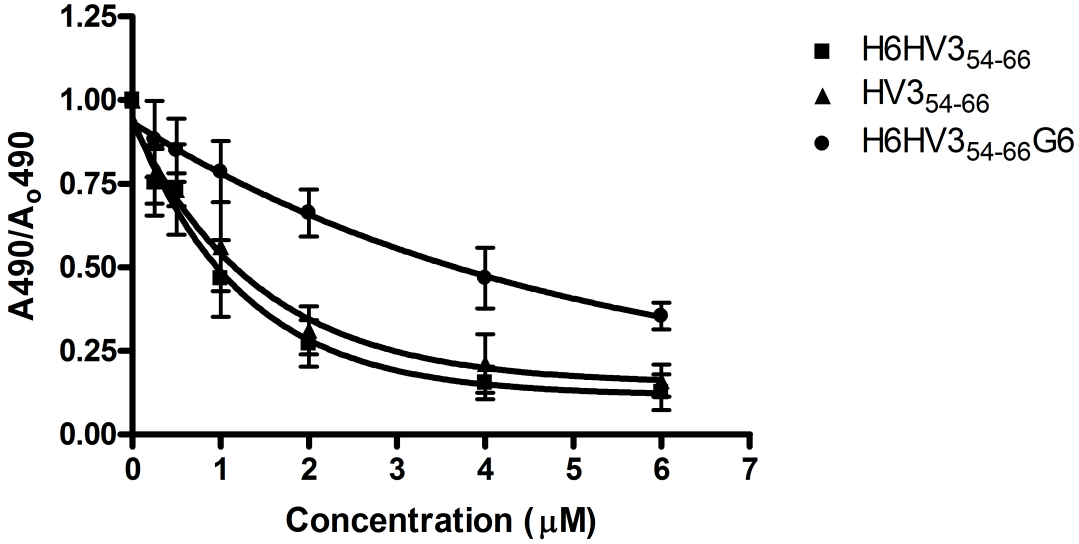

200
150
100
50
0
TCT (seconds)
0.0
2.5
5.0
7.5
10.0
12.5
Concentration (μM)
H6HV3 54-66
HV3 54-66
H6HV3 54-66 G6
HCII 1-75

Figure 5: Competitive binding assay comparing the ability of HV3 peptides and HCII 1-75 to bind thrombin. Thrombin and inhibitor peptide were incubated in HCII 1-75 coated wells. Thrombin bound to HCII 1-75 was detected with sheep anti-human thrombin peroxidase conjugated antibody and the absorbance was read at 490 nm. First order exponential decay regression analysis is shown. The mean of 4 determinations ± SD is shown.


1.25
1.00
0.75
0.50
0.25
0.00
A490/A$_o$490
0
25
50
75
100
125
[HCII 1-75] (μM)


1.25
1.00
0.75
0.50
0.25
0.00
A490/A$_o$490
0
1
2
3
4
5
6
7
Concentration (μM)
H6HV3$_{54-66}$
HV3$_{54-66}$
H6HV3$_{54-66}$G6

inhibition constant (K_i) was also calculated using the IC50 values with the equation K_i = IC50/(1+ (S/K_D)) where S was the concentration of thrombin used in the competition experiments (5 nM) and K_D, 340 nM, as calculated from Amanda Boyle's SPR (surface plasma resonance) experiments in her Master's thesis (2010). These K_i values closely reflect the IC50 determinations at 0.9 μM for $HV3_{54-66,}$ 0.7 μM for H_6HV3_{54-66} , 2.7 μM for $H_6HV3_{54-66}G_6$ and 9.5 μM for HCII 1-75.

3.2 Expression of $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5

3.2.1 Cloning of $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5

HV3API M358R and HV3API RCL5 were successfully cloned into the pBADMyc-HisB vector. These fusions were created by using HAPI M358R and HAPI RCL5, previously cloned into the pBADMyc-HisB vector, as a template in the PCR reaction along with a primer containing the $MGSH_6HV3_{54-66}G_6$ sequence at the 5' end (as described in section 2.2.1). This product was then ligated into the pBADMyc-HisB using restriction sites Nco1 and EcoRI before transformation into Top10 *E. coli* for expression. The appropriate construction of both HV3API M358R and HV3API RCL5 expression vectors was verified by DNA sequencing (data not shown). All α_1-PI M358R variants and fusion proteins used in this study are depicted in Figure 6.

3.2.2 Purification of $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5

Expression of recombinant hexahistidine tagged $HV3_{54-66}$API RCL5 was induced with 0.002 % arabinose. The first two lanes right of the marker in Figure 7 show bacterial lysates containing pBADmychisB plasmid with the HV3API RCL5 insert that was either

Figure 6: Schematic of proteins used in this study. All proteins are a variation of α_1-PI M358R. They either have an alteration in the RCL, denoted RCL5, where key residues were exchanged for those from AT, an extension on the N-terminus, or a combination of both. The N-terminal extension is either the first 75 residues on the N-terminus of HCII (HAPI proteins) or the C-terminal tridecapeptide of hirudin (HV3API proteins). All proteins are his-tagged and those with an N-terminal addition have a glycine-6 spacer between the two constituents.

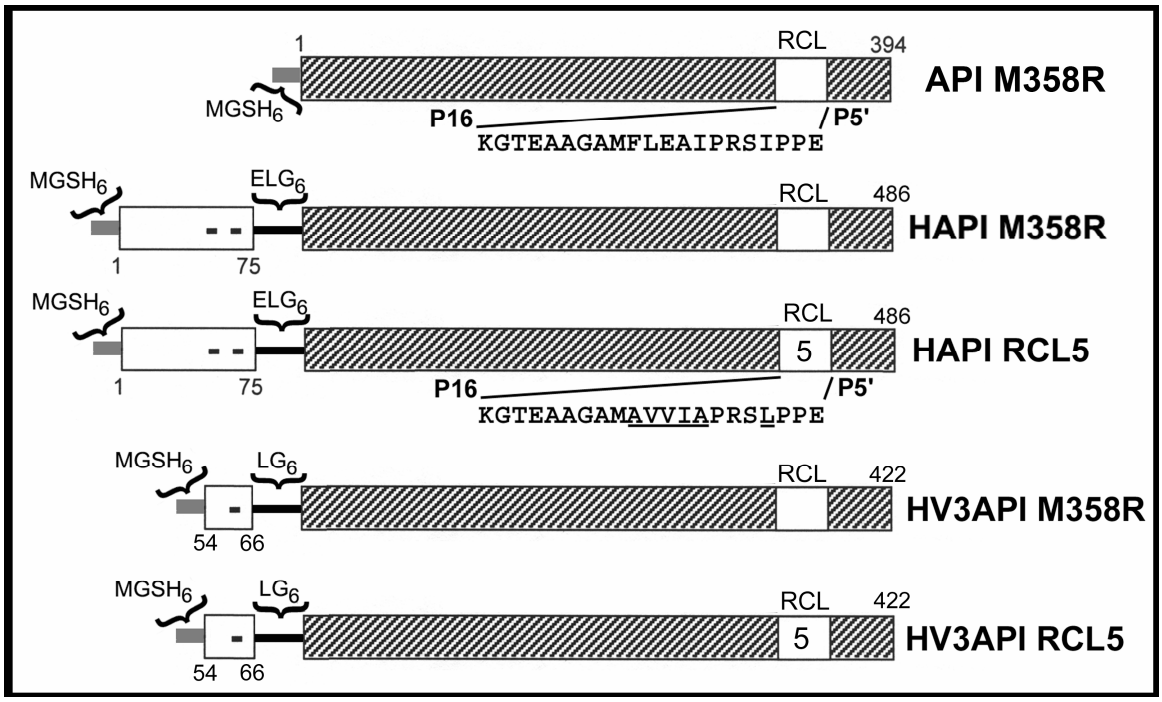
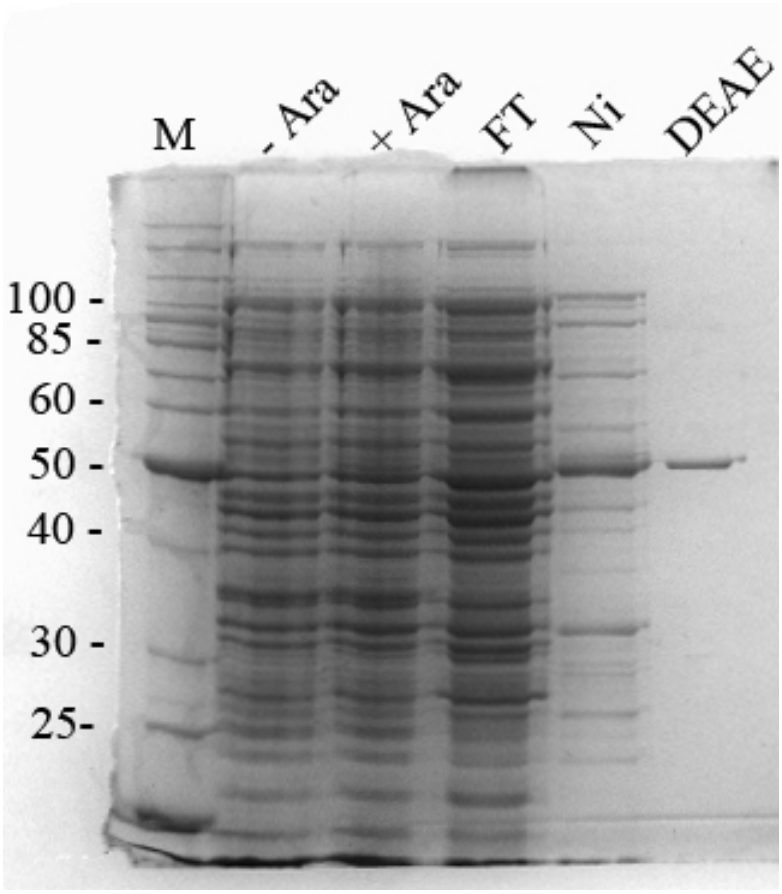

1
RCL
394
MGSH6
API M358R
P16
/P5'
KGTEAAGAMFLEAIPRSIPPE
MGSH6
ELG6
RCL
486
HAPI M358R
1
75
MGSH6
ELG6
RCL
486
5
HAPI RCL5
1
75
P16
/P5'
KGTEAAGAMAVVIAPRSLPPE
MGSH6
LG6
RCL
422
HV3API M358R
54
66
MGSH6
LG6
RCL
422
5
HV3API RCL5
54
66

Figure 7: Gel analysis depicting the purification profile of HV3API RCL5. A 10% SDS-PAGE gel stained with Coomassie Blue is shown. The –Ara and + Ara lanes show total bacterial lysates from bacteria expressing HV3API RCL5 that were grown in the absence or presence of 0.002% arabinose. Samples from steps in the purification process of HV3API RCL5 are shown (FT, flow through; Ni, sample of imidazole elution from Ni-NTA affinity column; DEAE, final product after DEAE-Sepharose ion exchange column). Approximately 500 ng of purified HV3API RCL5 (DEAE) were electrophoresed. M, molecular marker and labeled as appropriate in kDa.


M
- Ara
+ Ara
FT
Ni
DEAE
100 -
85 -
60 -
50 -
40 -
30 -
25-

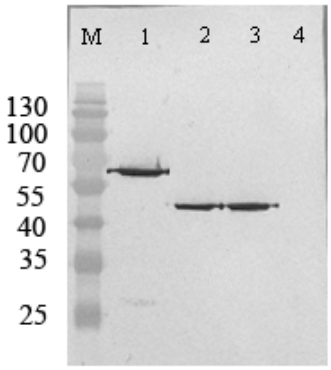
grown in the absence or presence of arabinose (-Ara/+Ara). Upon expression HV3API RCL5 was first purified using a nickel affinity column. The lane, FT, in Figure 7 shows the flow-through from the nickel column containing all the constituents that did not bind to the Ni-NTA resin. The lane denoted Ni shows an imidazole-eluted fraction containing a band of the appropriate size at 50 kDa, as well as various contaminants, as observed by SDS-PAGE. The elution fractions from this purification step were pooled and underwent dialysis with the appropriate buffer. Following this step, ion exchange chromatography with a DEAE- Sepharose column was performed for further purification. In Figure 7 the "DEAE" lane represents the final purified HV3API RCL5 product after concentration of all the DEAE-Sepharose elution fractions. It was determined that the concentration of purified $HV3_{54-66}$API RCL5 was approximately 2.2 mg/mL by using the extinction coefficient method. The total volume of protein acquired was about 600 µL and therefore the total protein yield was approximately 0.66 mg/L bacterial culture extracted. A second batch of $HV3_{54-66}$API RCL5 was purified resulting in a yield of 0.366 mg/L bacterial culture extracted. Two batches of $HV3_{54-66}$API RCL5 were purified in a similar manner resulting approximately 0.8 mg/L of bacterial culture extracted from both purifications. All batches of purified HV3API M358R and HV3API RCL5 contained no contaminating bands detectable by Coomassie Blue staining of electrophoresed samples. The concentrations of other recombinant serpins were determined by Bradford assay.

3.2.3 Immunoblot Analysis

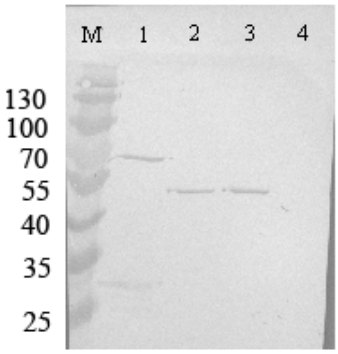
Anti-His and anti-API immunoblots were performed on the purified proteins. On both the anti-His (Figure 8A) and anti-API (Figure 8B) immunoblots a distinct band

Figure 8: Immunoblot analysis of HV3API proteins. 100 ng of each sample was electrophoresed on a SDS-PAGE gel that was then transferred to a nitrocellulose membrane. M, molecular marker and labeled as appropriate in kDa. Lane 1, HAPI M358R; lane 2, HV3API M358R; lane 3, HV3API RCL5; lane 4, GST. Bands are present around 65 kDa for HAPI M358R and 50 kDa for HV3API M358R and HV3API RCL5. In panel A Mouse anti-his antibody was used followed by anti-mouse AP conjugated antibody. In panel B, Sheep anti-human α_1-PI HRP conjugated antibody was used.

A



B



migrating around 50 kDa was noted in lanes containing $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5. A distinct band was also noted in the lanes containing HAPI M358R (positive control) just under 70 kDa for both immunoblots and no band was observed in the lanes containing GST (negative control).

3.3 Characterization of the Interaction Between API M358R Variants and Thrombin

API M358R, HAPI M358R and $HV3_{54-66}$API M358R formed SDS-stable serpin-enzyme complex with thrombin as observed in Figure 9 (panels A-C). The formation of a novel protein species with retarded mobility (SEC) was noted in the presence of thrombin after only 10 seconds of incubation in all three instances. The density of this elevated band increased over 60 seconds and then remained constant over 300 seconds as observed in the $HV3_{54-}$ and API M358R SDS-PAGE gels. However, the density of the SEC band remained constant over the course of the 300 seconds in the HAPI M358R SDS-PAGE gel (panel B). The formation of a third band that migrated slightly more rapidly (CS, cleaved serpin) than the band representing the serpin (S) was also noted in these gels starting at 10 seconds. This band remained relatively constant over 300 seconds and was most prominent in the HV3API M358R gel and absent from the HAPI M358R gel. There is also a very faint band, marked E (enzyme), just below 40 kDa present in all three gels. The density of this band gradually diminishes and is absent by 30 seconds in the API M358R gel, by 20 seconds in the HV3API M358R gel and by 10 seconds in the HAPI M358R gel.

Figure 9: Formation of SDS -stable serpin-enzyme complex between API M358R variants and thrombin. Serpins (1 μM) were incubated with 0.15 μM thrombin for the indicated times from 0 to 300 seconds (5 minutes) before the reaction was terminated with 4x SDS dye. These samples were then electrophoresed on a 10% SDS-PAGE gel and stained with Coomassie Blue. SEC, serpin-enzyme complex; S, unreacted serpin; CS, cleaved serpin. M, molecular weight marker are shown and labeled as appropriate in kDa. Panel A, α_1PI M358R incubated with thrombin; panel B, HAPI M358R incubated with thrombin, Panel C, HV3API M358R incubated with thrombin.

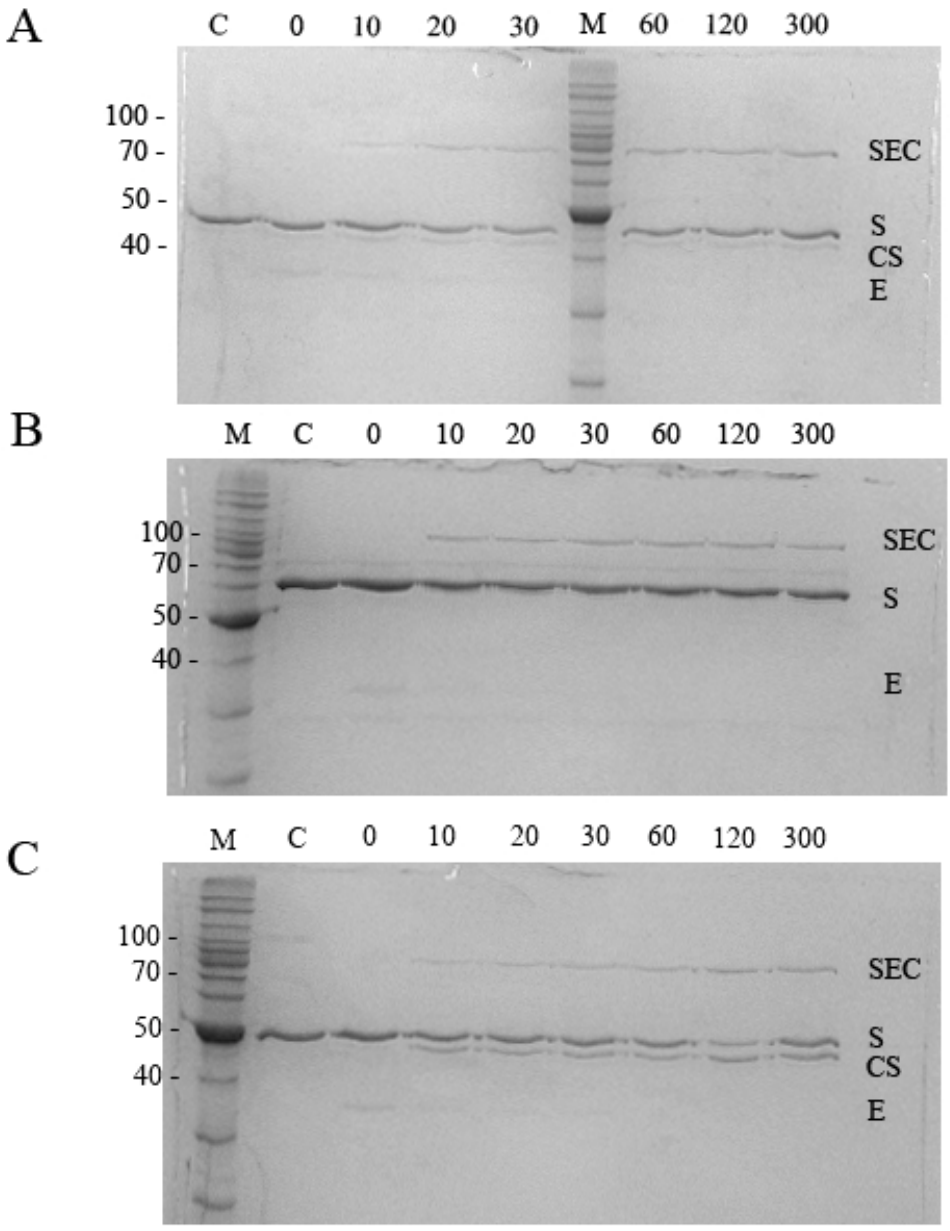

A
C 0 10 20 30 M 60 120 300
100 -
70 -
50 -
40 -
SEC
S
CS
E
B
M C 0 10 20 30 60 120 300
100 -
70 -
50 -
40 -
SEC
S
E
C
M C 0 10 20 30 60 120 300
100 -
70 -
50 -
40 -
SEC
S
CS
E

Figure 10: Kinetic analysis of the inhibition of α and γ thrombin and APC by API fusion proteins and variants. Panel A shows an example of the raw data for the kinetics of γ-thrombin inhibition by HV3API M358R that was used to generate the graph in panel B. The example graph in panel B plots time (x-axis) versus $\ln(P_o/Pt)$ (y-axis) and was analyzed by linear regression; the slope generated from this analysis was used in the equation, $k_2 = (m/[serpin, M]) *60$ to determine the second order rate constant (k_2). P_o, initial thrombin activity; Pt, thrombin activity at time t; R^2, regression coefficient.

A

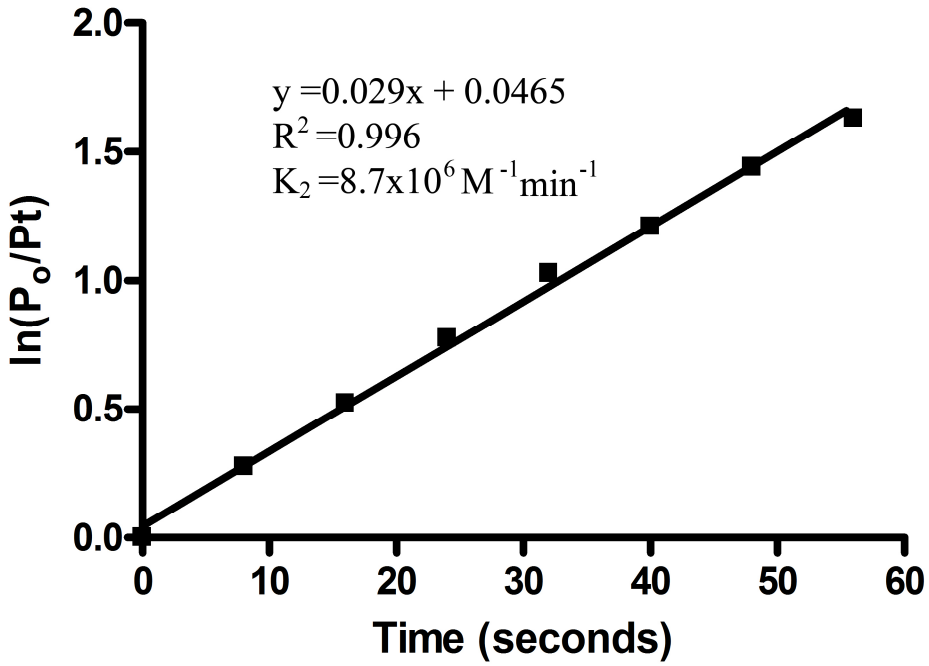
Time (sec)	Mean V	ln(Po/Pt)
56	5.56	1.63
48	6.71	1.44
40	8.4	1.21
32	10.1	1.08
24	13.0	0.777
16	16.8	0.522
8	21.5	0.275
0	28.2	0

[HV3API M358R] = 200nM

[-IIa] = 10 nM

Po = Pt at time 0 seconds

B



3.4 Kinetics of API Fusion Proteins and Variants

3.4.1 Determination of Second Order Rate Constants

Second order rate constants (K_2) were determined for the inhibition of α and γ thrombin and APC by the API variants and fusion proteins to ascertain their relativeinhibitory capabilities. Graphs were generated relating time on the x-axis and $\ln(P_o/Pt)$ on the y-axis, where P_o is the initial thrombin or APC activity and Pt is the activity at t seconds as exemplified in Figure 10B. The values for the y-axis were determined using the mean velocities (mean Vs) generated (Figure 10A) from reading the absorbance at 405 nm over 5 minutes upon addition of chromogenic substrate (see section 2.2.7). The slope from the graph determined using linear regression was used in the following equation: K_2= slope/[serpin]x60.

3.4.2 Kinetic Analysis of Thrombin Inhibition by API Variants and Fusion Proteins

Upon kinetic characterization of the recombinant serpins it was noted that the $HV3_{54-66}$ peptide fusion to API M358R was associated with an elevation of the second order rate constant of α-thrombin inhibition by 3.3 fold compared to API M358R. In addition, HV3API RCL5 exhibited a 1.9 fold increase in the rate of α-thrombin inhibition compared to α_1PI RCL5 (Table 3). Furthermore, HAPI M358R and HAPI RCL5 showed a greater elevation of second order rate constants for α-thrombin inhibition compared to the HV3API proteins (8.5 fold compared to API M358R and 3.5 fold for API RCL5).

Table 3: Kinetic analysis of protease inhibition by API fusion proteins and variants. The mean of at least 5 determinations ± SD is shown. [a]Values from Sutherland et al., 2007, [b] Values represent mean of 3 determinations ± SD. * Values statistically significant from α_1-PI counterpart as determined by t-test.

serpin	α-thrombin K_2 ($x10^6$ $M^{-1}min^{-1}$)	γ_T-thrombin K_2 ($x10^6 M^{-1}min^{-1}$)	APC K_2 ($x10^6$ $M^{-1}min^{-1}$)	α-thrombin/ APC ratio
HV3API M358R	72 ± 15*	11 ± 3	1.9 ± 0.2	39
HAPI M358R	186 ± 32*	34 ± 14	1.7 ± 0.2	109
API M358R	22 ± 4	5.9 ± 0.1	1.7 ± 0.1	13
HV3API RCL5	66 ± 8*	21 ± 3	0.016 ± 0.003[b]	4,125
HAPI RCL5	122 ± 37*	27 ± 2	0.013 ± 0.002[a]	9,385
API RCL5	35 ± 8	15 ± 0.4	0.0038 ± 0.0013[a]	9,211

When analyzing the results by a two-tailed t-test a significant difference was noted between HV3API M358R and API M358R (P=0.0022) and between HV3PI RCL5 and API RCL5 (P=0.0001). There was also a significant difference noted between the HAPI proteins and their API counterparts. HAPI M358R maintained the greatest average second order rate constant for α-thrombin inhibition with its being 2.6-fold greater than the K_2 for HV3API M358R (Table 3).

As observed previously, the augmented α-thrombin inhibition rates determined by second order rate constants were reduced when γ_T-thrombin, containing an incomplete exosite 1, was used instead. The fold increase dropped from 8.6 to 5.8 for HAPI M358R and from 3.5 to 1.7 for HAPI RCL5 (Table 3). Similarly, the rate was reduced for HV3API M358R (3.4 fold to 1.9 fold). However, the difference in rates of thrombin inhibition for HV3API RCL5 was quite similar (Table 3) between α-thrombin and γ_T-thrombin (1.9 fold compared to 1.4 fold). In each case, the reduction in rate constant associated with substituting γ_T-thrombin for α-thrombin for a given HCII 1-75- or HV3-fused serpin compared to its unfused counterpart was found to be statistically significant by using a two-tailed t-test. An unpaired t-test was used when comparing α and γ_T-thrombin kinetics for HAPI M358R and the non-parametric Mann-Whitney test was used when interpreting data for HAPI RCL5, HV3API RCL5 and HV3API M358R, because of differences in the distribution within the data sets.

3.4.3 Kinetic Analysis of APC Inhibition by α_1-PI Variants and Fusion Proteins

The rates of APC inhibition for HAPI M358R, HV3API M358R and API M358R were similar as judged by second order rate constants. The α-thrombin to APC ratio was

greatest for HAPI M358R at 109 followed by HV3API M358R at 39 and lowest for API M358R at 13 (Table 3). The rate of APC inhibition for HV3API RCL5 was determined to be $0.016x10^6\ M^{-1}\ min^{-1}$. This is similar to the rate for HAPI RCL5 ($0.013x10^6\ M^{-1}\ min^{-1}$) and slightly higher than the rate for API RCL5 ($0.0038x10^6\ M^{-1}\ min^{-1}$) as determined in Sutherland et al., 2007 (*91*). The α-thrombin to APC ratio for HV3API RCL5 is 4,125; this may be much greater than all the M358R variants but is still considerably lower compared to HAPI RCL5 and API RCL5 at 9,385 and 9,211 respectively (Table 3).

3.4.4 Determination and Analysis of the Stoichiometry of Inhibition of α_1-PI Variants and Fusion Proteins

The stoichiometry of inhibition was calculated for HV3API M358R, HV3API RCL5, HAPI M358R and API M358R. Graphs were plotted with the ratio of serpin to thrombin on the x-axis and the percent of thrombin activity on the y-axis as exemplified in Figure 11B. The SI was determined by analyzing the graph with liner regression and by using the equation of the line to determine the value of x (ratio of serpin to thrombin) when y (thrombin activity) is equal to zero. The mean velocity (mean V) at the various serpin to thrombin ratios was determined as exemplified in Figure 11A upon addition of chromogenic substrate and reading the absorbance at 405 nm over 5 minutes (see section 2.2.7). These values were used to determine the percent activity of thrombin by dividing the mean V by the mean V when thrombin activity is 100 % and multiplying this value by 100 ($(SI = meanV/meanV_o)*100$). The mean SI of HAPI M358R was determined to be 2.5. This value was slightly lower for API M358R at 2.1 and slightly higher for both HV3API M358R and HV3API RCL5 at 3.1 (Table 4). However, there was no significant

Figure 11: Stoichiometry of inhibition of recombinant serpins against thrombin. 200 nM thrombin was incubated with 0-800 nM inhibitory serpin for two hours at room temperature before quenching with 150 μM chromogenic substrate S2238 to calculate residual thrombin activity. Panel A shows an example of the raw data that was used to generate the plot in panel B. The example graph in panel B plots the ratio of HV3API RCL5 to thrombin (x-axis) by the percent activity of thrombin (y-axis). This graph was analyzed with linear regression to determine the serpin to thrombin ratio (SI) when the percent thrombin activity has reached zero. R^2, regression coefficient.

A

[HV3API rcl5] : [IIa]	Mean V	% IIa Activity
0	128.4	100
0.5	111.5	86.9
1	92.8	72.3
1.5	70.0	54.6
2	43.6	33.9
2.5	25.5	19.9
3	1.86	1.44
4	0	0

% activity = (mean V/mean Vo)*100

B

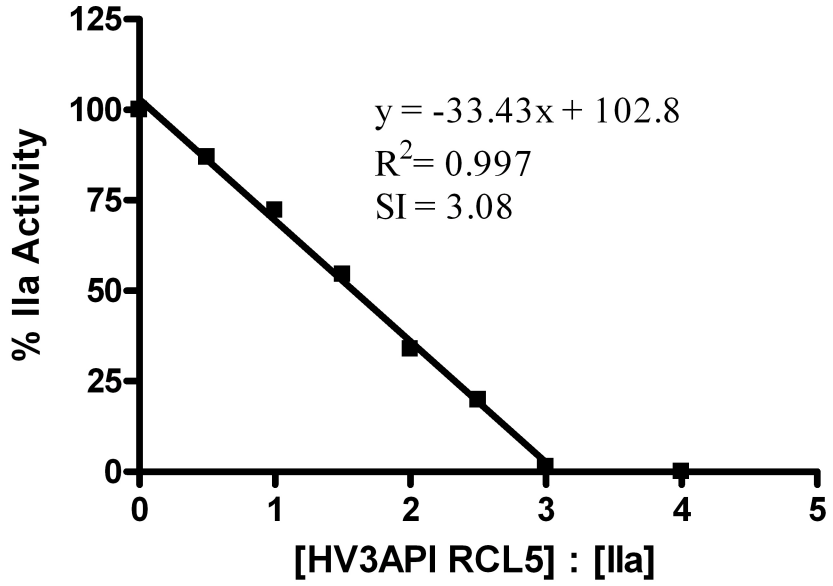


Table 4: Stoichiometry of inhibition of recombinant serpins. Determined by linear regression analysis of graphs such as the example shown in Figure 11. The mean of 6-8 determinations ± SD is shown.*Values statistically significant from API M358R as determined by ANOVA.

Serpin	**SI**
HV3API M358R	3.1 ± 0.3*
HV3API RCL5	3.1 ± 0.2*
HAPI M358R	2.5 ± 0.2
API M358R	2.1 ± 0.1

difference in the SI values between HAPI M358R and API M358R as determined by Dunn's multiple comparisons test, but there is a significant difference between API M358R and the HV3API proteins.

3.5 Analysis of Competitive Binding Assay Comparing the Ability of Full-length Serpins and HCII 1-75 to Bind PPACK-thrombin

The ability of HV3API RCL5, HAPI RCL5, $H_6HV3_{54-66}G_6$ and HCII 1-75 to bind thrombin exosite 1 was compared in a binding assay involving PPACK-thrombin. The serpin concentration (x-axis) was plotted by the absorbance ratio at 490 nm ($A490/A_o490$) where A_o is the absorbance when the serpin concentration was zero. First order exponential decay analysis was used to determine the IC50 (Figure 12). The K_i was determined using the IC50 and inputting it into the equation, $K_i = IC50/(1+ (S/K_D))$ as described in section 2.2.8.2. The K_i values were very similar to the IC50. These values were lowest for HV3API RCL5 at 1.10 μM (IC50, 1.11 μM), followed by $H_6HV3_{54-66}G_6$ at 3.2 μM (IC50, 3.3μM) and HCII 1-75 at 9.1 μM (IC50, 9.2 μM). The K_i and IC50 obtained for HAPI RCL5 were more than double those from HCII 1-75 with a K_i of 21.2 μM and an IC50 of 21.5 μM (Table 5); however, the HAPI RCL5 values are likely less reliable than those of the other proteins, given that they were determined only a single time due to the very high concentrations of this protein required to detect any inhibition of binding.

Figure 12: Competitive binding assay comparing the ability of full-length serpins and HCII 1-75 to bind PPACK –thrombin. First order exponential decay analysis was used to determine the IC50. Similarly to Figure 5, PPACK-thrombin and inhibitor peptide were incubated in HCII 1-75 coated wells. PPACK-thrombin bound to HCII 1-75 was detected with sheep anti-human thrombin peroxidase conjugated antibody and the absorbance was read at 490 nm. The mean of 3 determinations ± SD is shown in all cases, except for that represented by the dotted line, where n=1.

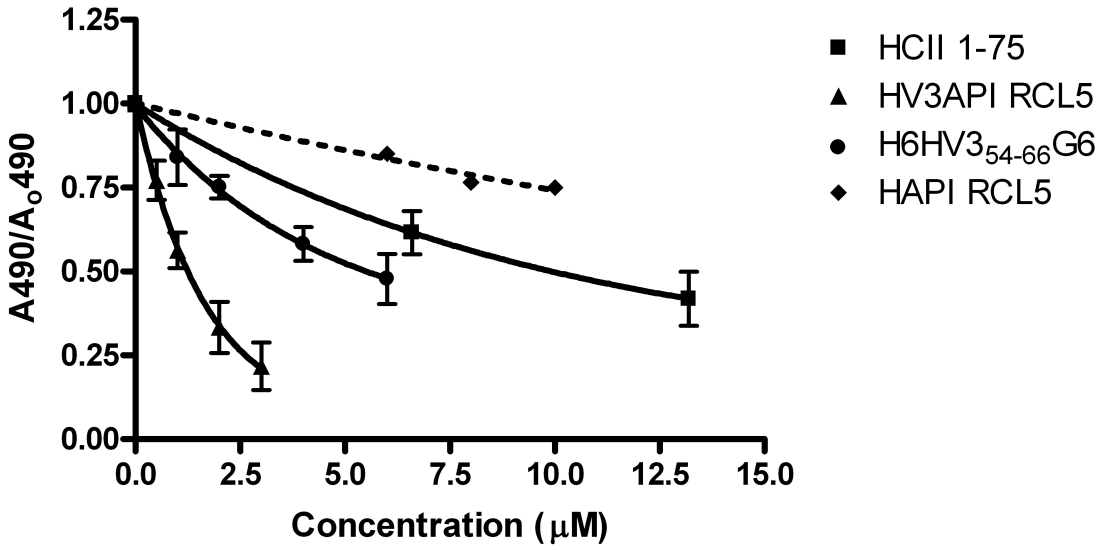

1.25
1.00
0.75
0.50
0.25
0.00
A490/A$_o$490
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Concentration (μM)
HCII 1-75
HV3API RCL5
H6HV3$_{54-66}$G6
HAPI RCL5

Table 5: Comparison of the ability of serpins and peptides to bind exosite 1 of thrombin. A competitive binding assay was used to determine IC50 and K_i values. The mean of 3 determinations ± SD is shown. [a]Value represents one determination.

Serpin/Peptide	**IC50 (μM)**	**K_i (μM)**
HV3API RCL5	1.11 ± 0.06	1.10 ± 0.05
$H_6HV3_{54-66}G_6$	3.3 ± 1.4	3.2 ± 1.4
HAPI RCL5	21.5[a]	21.2[a]
HCII 1-75	9.2 ± 2.0	9.1 ± 2.0

4. DISCUSSION

The aim of this project was to enhance API M358R's activity and/or specificity towards thrombin. Previously the N-terminal tail of HCII, that binds exosite 1 of thrombin, was fused to the N-terminus of API M358R, creating HAPI M358R (*89*). The addition of the N-terminal tail resulted in an enhancement of API M358R's thrombin-inhibitory properties. This augmentation was attributed to the serpin's new ability to bind exosite 1, because its enhanced ability to inhibit thrombin was not as substantial when γ_T-thrombin, a thrombin derivative with a disrupted exosite 1, was substituted for α-thrombin (*89*). Since hirudin is known to be the most potent natural thrombin inhibitor and also binds exosite 1 via its C-terminal residues, it was hypothesized that the addition of the C-terminal exosite 1 binding residues of hirudin to API M358R would augment its thrombin-inhibitory properties to a greater extent than that of HAPI M358R. It was also proposed to try this fusion with API RCL5, since the RCL mutation results in a significant reduction of API's anti-APC activity, thereby increasing its specificity towards thrombin. The relative abilities of the HV3 peptides and HCII 1-75 to bind to exosite 1 were first considered, to determine if there was preliminary experimental support for this hypothesis.

4.1 Relative abilities of HV3 peptides and HCII 1-75 to Inhibit and Bind Thrombin

The ability of the $HV3_{54-66}$ peptides and HCII 1-75 to inhibit thrombin-mediated clotting, as well as their ability to inhibit thrombin binding to immobilized HCII 1-75 were compared using TCTs and competitive binding assays. These two assays produced

similar results. With respect to clotting, all three HV3-related peptides doubled the TCT at concentrations 8- to 26-fold lower than HCII 1-75 (Table 2). Similarly, all three HV3-related peptides were more effective at binding thrombin exosite-1 than HCII 1-75, as indicated by H_6HV3_{54-66} was the most effective at binding exosite 1 since it had the lowest IC50 and K_i values 4- to 13-fold lower than that of HCII 1-75 (0.8 μM, 0.7 μM), followed very closely by $HV3_{54-66}$ with an IC50 and K_i of 0.9 μM. $H_6HV3_{54-66}G_6$ had the highest IC50 and K_i values (2.8 μM and 2.7 μM) of the HV3 proteins and therefore was least effective at binding exosite 1 (Table 2). The mathematically derived values match the visual inspection of the graphs shown in Figures 4 and 5. It can be observed that H_6HV3_{54-66} > $HV3_{54-66}$ > $H_6HV3_{54-66}G_6$> HCII 1-75 in terms of binding to exosite 1, assuming that inhibition of clotting arises from competition of binding between fibrinogen and thrombin exosite-1. The addition of a component (H_6) to the N-terminus did not have a significant effect on the binding ability, while the addition of a component to the C-terminus (G_6) resulted in a minor reduction in the ability of $HV3_{54-66}$ to bind exosite 1. These results demonstrated that the previously reported tight interaction between the unsulfated dodecapeptide of hirudin variant 1 and thrombin exosite 1 (*113*) was also observed with the corresponding peptide of hirudin variant 3. While the addition of the C-terminal glycine spacer diminished the binding affinity to certain extent, all the hirudin peptides bound thrombin more effectively than the N-terminal HCII extension. Thus our initial experiments with HV3-related peptides supported the hypothesis that these peptides were more effective exosite-1 ligands than HCII 1-75. Though $H_6HV3_{54-66}G_6$ had the lowest affinity for exosite 1 of the HV3 peptides it still has a highly

augmented ability to bind thrombin compared to HCII 1-75. This peptide had a considerably higher DCT and K_i at 12.0 μM and 9.5 μM, respectively. As expected, all the hirudin peptides bound thrombin more effectively than the N-terminal HCII extension.

4.2 Designing HV3API Fusion Proteins

The data obtained from the TCTs and competitive binding assays affected the design of this project. It was decided that $MSGH_6$ be added to the N-terminus of the peptides for purification purposes and that a G_6 linker be used to separate the $HV3_{54-66}$ from either API M358R or API RCL5 (Figure 6). The MSG tripeptide was necessary to provide an initiator Met residue for polypeptide synthesis *in vivo* in bacteria, and the SG dipeptide was necessitated by DNA manipulations. The $MSGH_6$ nonapeptide was also used in previously described HAPI M358R and HAPI RCL5 fusion proteins, as was the G_6 spacer, which was introduced between HCII 1-75 and API M358R in an effort to limit potential steric hindrance between the exosite-1-binding motif and the serpin. Though the addition of the G_6 reduced the HV3 peptide's exosite 1 binding capacity, when it formed the peptide's C-terminus, this design was what had been used in previous APIfusion proteins (Figure 6) and it was decided to retain it to make for a fair comparison between HV3API and HAPI fusion proteins. Furthermore, $H_6HV3_{54-66}G_6$ still bound and inhibited thrombin more proficiently than HCII 1-75. Thus, it was hypothesized that the addition of $H_6HV3_{54-66}G_6$ to API M358R (and API RCL5) would result in an enhancement in the specificity as a thrombin inhibitor to a greater extent than observed for HAPI M358R

(and HAPI RCL5). To test this hypothesis, $HV3_{54-66}$API M358R and $HV3_{54-66}$API RCL5 were cloned into pBADmychisB vectors for expression.

4.3 Expression, Purification and Characterization of HV3API Fusion Proteins

The systems that were used to express and purify the HV3API fusion proteins were similar to those used previously for HAPI M358R (*89*). Both HV3API proteins contained a hexahistidine tag on the N-terminus for facilitation of purification by means of nickel affinity columns. DEAE- Sepharose was used as a resin for the subsequent purification step (Figure 7). Similarly to the HAPI proteins, the HV3API proteins are negatively charged; thus, anion exchange chromatography, in which negatively charged proteins bind to a positively charged surface, was appropriately chosen for successful purification. The isolated band observed in the DEAE fraction migrated near 50 kDa, which reflected the expected size of HV3API M358R at 47.3 kDa and HV3API RCL5 at 47.1 kDa. These candidate proteins also reacted in both anti-hexahistidine and anti- API immunoblots (Figure 8A and B), thereby suggesting the presence of a histidine tag as well as API. These results taken together indicate that the HV3API proteins were successfully expressed and purified. However, note that though the arabinose induced expression of the HV3API proteins worked very efficiently the prokaryotic system did not sulfate the tyrosine at residue 64 of the HV3 extension; this lack of sulfation differs from naturally occurring hirudins but is consistent with the non-sulfated nature of the HV3 peptides utilized in the experiments shown in Figures 4 and 5.

4.4 Characterization of the Formation of Serpin-Enzyme Complexes for API M358R Fusion Proteins

Serpin-enzyme complexes have been shown previously to be irreversible and SDS-stable (*114, 115*). As expected, API M358R, HAPI M358R and HV3API M358R were shown to form irreversible, SDS-stable serpin-enzyme complexes (SECs) with thrombin as noted in Figure 9A-C by the persistence of this complex over 5 minutes. When thrombin was incubated with these recombinant serpins, the appearance of a band of decreased mobility of 85 kDa, for API M358R and HV3API M358R, and around 100 kDa for HAPI M358R was noted; the mobility of this band reflects a species containing both thrombin and the serpin. It is difficult to gauge the relative rate of SECs formation using this method. However, a few differences and similarities are noticed between the three gels. The SEC for both HV3API M358R and API M358R was first noted at 10 seconds and the density of this band increased until about 60 seconds indicating that they may have similar rates of thrombin inhibition. The SEC for HAPI M358R and thrombin was also first noted at 10 seconds; however, the density of this band had peaked at 10 seconds and remained constant over the rest of the 5 minute period. The presence of cleaved serpin (CL) noted with HV3API M358R and API M358R by the appearance of the band with slightly higher mobility, is a common observance in recombinant serpins (*89, 114*); it illustrates that serpin molecules can act as a substrate instead of an inhibitor and thus, the stoichiometry of inhibition is greater than 1. Overall, from these gels it appeared as though HAPI M358R had the greatest rate of thrombin inhibition. A loading test was performed prior to the SEC complex experiments to ascertain the required

conditions for the gels (data not shown), but nonetheless, the loading concentration appears to be slightly higher for HAPI M358R than the other two serpins and consequently these observations may not be precise; furthermore, it was difficult to observe differences in rates of inhibition with this method when these rates are similar. To accurately determine the relative thrombin inhibitory abilities of these serpins kinetic analysis was next performed.

4.5 Kinetic Analysis of API Fusion Proteins and Variants

4.5.1 Analysis of the Rate of α-thrombin Inhibition

The kinetic analysis of these API fusion proteins and variants produced unexpected results that did not support the hypothesis that HV3 tridecapeptide-containing fusion proteins would be more rapid inhibitors of thrombin than their HCII 1-75-containing counterparts. As anticipated, HV3API M358R was a superior thrombin inhibitor to API M358R as noted by the 3.3 fold increase in K_2 values (Table 3). However, contrary to the hypothesis that replacing HCII 1-75 with the HV3 tridecapeptide would yield an improved fusion protein, HAPI M358R was still the best thrombin inhibitor, with a 8.5 fold advantage in the rate of thrombin inhibition over α_1-PI M358R. The RCL5 variants produced similar results, but overall their fold increase over API RCL5was lower at 1.9 for HV3API RCL5 and 3.5 for HAPI M358R. The fold increases in K_2 values for HV3API variants compared to their API counterparts were shown to be statistically significant through the use of t-tests. This statistical approach was used to isolate the effects of HV3 addition alone. T-tests were also employed to show

a statistically significant increase in the K_2 values for the HAPI variants compared to their API counterparts, thus showing the effect of the HCII 1-75 addition alone.

4.5.2 Comparison of Stoichiometry of Inhibition

A second important indicator of the potency of an inhibitor is the number of inhibitor molecules that are required to inactivate the target molecule. This stoichiometry of inhibition (SI) is ideally one for natural serpins inhibiting their most appropriate physiological cognate proteases, but it is commonly higher with recombinant proteins. For instance, Han, J. et al. 1997 (*116*) reported an SI for wild type recombinant HCII complexed with DS as 3.8. Similarly Ciaccia, et al. 1997 (*115*) reported the SI for recombinant wild type HCII complexed with GAG to range from 1.8 to 3. In this study it was determined that API M358R had a relatively low SI value of 2.1 and the addition of the HCII acidic tail to α_1PI M358R resulted in a slightly elevated SI of 2.5, but this elevation was not statistically significant; these SI values reflect those reported in Sutherland et al., 2006 (*89*). In many cases where a SI of one was reported for API , this protein was isolated from inclusion bodies and then refolded (*117, 118*). Perhaps the folding of API in the cytoplasm of *E. coli*, the approach taken by our laboratory, is not as optimal as the refolding that occurs upon removal from inclusion bodies and the denaturants used in their solubilization; consequently an SI closer to 2 was observed in this study. The HCII 1-75 addition may have resulted in a slightly greater SI since the fusion protein is not naturally occurring; in contrast, HCII 1-75 co-evolved with the rest of HCII in its natural setting. The addition of the $HV3_{54-66}$ peptide to α_1PI, however,

resulted in a statistically significant increase in SI to 3.1 for both HV3API RCL5 and HV3API M358R (Table 4). The HV3 peptide fusion to the API variants may have negatively impacted the protein's folding ability. Not only is this fusion not naturally occurring, but the HV3 orientation is also altered since it is biologically a C-terminal peptide but was fused to the N-terminus of API. Nevertheless, the SIs for all these API variants examined in this study range from 2 to 3 and therefore do not largely differ from each other; moreover since the fusion of HV3 peptides did not greatly alter the stoichiometries, their effects can be interpreted independently of SI considerations.

4.5.3 APC Kinetic Analysis to Determine Thrombin Specificity

The capability of API M358R to inhibit APC likely detracts from its potential antithrombotic effects. Therefore, to optimize this inhibitor the ratio of rate of thrombin inhibition to APC inhibition arguably needed to be increased. Since the second order rate constants for APC inhibition by HV3API M358R, HAPI M358R and API M358R were all similar, ranging from 1.7 to 1.9×10^6 M^{-1} min^{-1} (Table 2), HAPI M358R still had the highest thrombin to APC ratio of 109 and was therefore the most specific thrombin inhibitor of the M358R variants followed by HV3API M358R and API M358R. However, HV3API RCL5 inhibited APC at a lower rate compared to all the M358R variants as noted by its K_2 value of 0.016×10^6 M^{-1} min^{-1} and thus, the thrombin to APC ratio was considerably higher at 4,125. Although HV3API RCL5 was more specific for thrombin than any of the M358R variants, it was still the least specific of the RCL5 variants when compared to kinetic data from Sutherland et al, 2007 (Table 3). Since the

HV3API proteins did not inhibit thrombin as well as their HAPI counterparts but inhibited APC at a similar rate, they were not as specific for thrombin as the HAPI proteins. Creating a high APC to thrombin ratio is likely critical for the optimization of the API fusion proteins as a specific thrombin inhibitor, and this parameter was not improved by substitution of HV3 for HCII 1-75 in serpin fusion proteins.

4.5.4 Role of Exosite 1 Binding in the Antithrombotic Ability of the API Fusion Proteins and Variants

Analysis of the γ_T-thrombin kinetics indicated that the enhanced thrombin inhibition by HV3 peptide fusion proteins compared to API M358R and API RCL5reflected their exosite 1 binding capability. Overall, the recombinant serpins tested had a reduced capacity to inhibit γ_T-thrombin (Table 3). This form of thrombin contains an incomplete exosite 1 due to proteolysis of α-thrombin by trypsin (*90, 91*). The elevation in the rate of thrombin inhibition by HV3API M358R compared to API M358R dropped when γ_T-thrombin was used instead of α-thrombin; a 3.3 fold difference was noted between HV3API M358R and API M358R for α-thrombin compared to a 1.9 fold difference for γ_T-thrombin. Accordingly, HV3API M358R's enhanced ability to inhibit thrombin correlated with its exosite 1 binding capability by means of the HV3 peptide extension. A significant fold reduction was also observed between α- thrombin and γ_T-thrombin for HAPI M358R (8.5 fold to 5.8 fold), HV3API RCL5 (1.8 fold to 1.4 fold) and HAPI RCL5 (3.5 fold to 1.7 fold). Thus, the enhanced inhibitory ability of these fusion proteins was also, at least in part, a result of the HCII 1-75 or HV3 peptide extension binding to exosite 1. However, if exosite 1 binding capacity were a direct

correlation to thrombin inhibition, then the HV3API proteins should be superior thrombin inhibitors compared to the HAPI variants, since $HV3_{54-66}$ binds thrombin with a higher affinity than HCII 1-75. Consequently, other factors need to be considered for a full explanation as to how these fusions alter the antithrombotic ability of API M358R and API RCL5. Nonetheless, the data extrapolated from the γ_T-thrombin kinetics illustrates the importance of exosite 1 binding in the potency of thrombin inhibition by these recombinant serpins and that it contributes in part to their mechanism of action.

4.6 Comparing the Ability of HV3API RCL5 and HAPI RCL5 to Bind Exosite 1

Analysis of the γ_T-thrombin kinetics reveals that HV3API RCL5 and HV3API M358R did bind to exosite 1 and this was at least partly responsible for the enhancement of the rate of thrombin inhibition compared to their API counterparts. However, since the HV3API proteins did not inhibit thrombin as well as the relative HAPI proteins, we formed the working hypothesis that adding $HV3_{54-66}$ to API M358R or APIRCL5 reduced the hirudin peptide's exosite 1 binding capacity. $HV3_{54-66}$, unlike HCII 1-75, found on the N-terminus of HCII, is found on the C-terminus of hirudin and consequently, perhaps placing it on the N-terminus affected its ability to bind thrombin. To test this hypothesis HV3API RCL5, HAPI RCL5, $H_6HV3_{54-66}G_6$ and HCII 1-75 were examined in a competitive binding assay in which these inhibitors compete with immobilized HCII 1-75 to bind PPACK-thrombin. With the binding site of thrombin blocked by PPACK, the RCL cannot interact at this site and only the exosite 1 affinity should have been in play. Unexpectedly HV3API RCL5 had the lowest K_i (1.1 μM) and therefore bound exosite 1 with the highest affinity while HAPI RCL5 had the highest K_i (21.2 μM) and thereby the

lowest affinity for PPACK-thrombin. $H_6HV3_{54-66}G_6$ and HCII 1-75 had K_i values that were in between (3.2 μM, 9.1 μM) and similar to those calculated previously using PPACK-free thrombin. Though the ability to bind exosite 1 does play a role in enhancing the rate of inhibition of thrombin, this affinity for exosite 1 does not directly correlate to the thrombin inhibitory properties of the serpin. These results suggest that the RCL plays a more critical role in the inhibitory mechanism of the chimeric serpins examined in this study than the chimeric serpin's ability to bind exosite 1. For instance, HCII 1-75 has two acidic repeats while $HV3_{54-66}$ only has one; this variation in their composition could result in a difference in exosite 1 binding and these diverse conformations may alter the accessibility of the RCL to the active site of thrombin. Furthermore, the HCII tail may have coevolved with its RCL and as a result is better suited than the HV3 peptide to coordinate with the rest of the serpin to arrange an optimal conformation. Similarly, the C-terminal peptide of HV3 binds very tightly to exosite 1 and though this works well in conjunction with the N-terminal portion of HV3, it may bind too tightly to orient attached API M358R in the optimal conformation to inhibit thrombin – especially if the HV3 moiety of the chimeric serpins binds exosite 1 as the first step of the inhibitory reaction, potentially locking the rest of the serpin into a less productive RCL – active site interaction than in either HAPI M358R or GAG-activated HCII.

4.7 Potential for Examining HV3API M358R and HV3API RCL5 *invivo*

The HV3API variants did not have as high inhibitory activity as the HAPI variants but were still able to inhibit thrombin effectively. This raises the question of their effectiveness in animal models and thus their potential therapeutic ability. Between the

two variants, HV3API RCL5 would likely have better potential as an antithrombotic drug than HV3API M358R since its thrombin: APC ratio is much higher at 4,125 compared to 39. Furthermore, recently HAPI RCL5 and API M358R were tested in mice as antithrombotic agents in Sheffield et al, 2012 (*119*). HAPI RCL5 was more effective than API M358R at reducing fibrin(ogen) deposition and clot size and in increasing the time to occlusion in both venous animal models. Since HV3API RCL5 produced similar kinetic results as HAPI RCL5, it would likely also be successful at limiting venous thrombus formation. However, existing agents such as recombinant APC and hirudin were just as efficient or more efficient at limiting thrombus formation as HAPI RCL5. Hence, in order to be worthy of investigation in animal models an API RCL5 derivative would need to have greater specificity towards thrombin than HAPI RCL5. Since HV3API RCL5 has a reduced rate of inhibition towards thrombin compared to HAPI RCL5, it is not likely that it would show enhanced efficacy in animal model testing.

5. CONCLUSIONS AND FUTURE DIRECTIONS

To enhance the antithrombotic properties of HAPI M358R and HAPI RCL5, the HCII 1-75 fusion was exchanged for the C-terminal hirudin peptide, $HV3_{54-66}$, that was confirmed to possess a greater exosite 1 affinity in initial experiments. However, through the use of kinetics to determine the second order rate constants, the hypothesis was disproven as it was determined that HAPI M358R and HAPI RCL5 still possessed the highest rate of thrombin inhibition. On the other hand, the ability of the HV3API proteins to bind thrombin was at least in part responsible for their augmented antithrombin activity compared to the API M358R variants as was expected. Also, as was hypothesized, the rate of APC inhibition by the HV3API proteins was no greater than their respective HAPI proteins. Furthermore, through performing competitive binding assays with PPACK-thrombin it was observed that HV3API RCL5 was able to bind thrombin with a much higher affinity than HAPI RCL5. Thus the results of this study reinforce the idea that the ability of these serpins to bind thrombin does not directly reflect their ability to inhibit thrombin; other factors such as the accessibility of the RCL to the active site likely play a more important role in thrombin inhibition. Importantly, this study provides a second, novel example confirming the concept that a thrombin-inhibitory serpin's activity can be augmented by fusion of an exosite-1-binding motif.

Future experiments attempting to enhance the HV3API variants' ability to inhibit thrombin should focus on providing the chimeric serpin with more flexibility after engagement of thrombin exosite-1 by the attached ligand moiety. For instance, a larger

spacer between $HV3_{54-66}$ and API M358R or API RCL5 could increase flexibility between the HV3 peptide and the API body. This improved mobility could allow the RCL to access the active site more effectively when the N-terminus of the fusion protein is engaged with thrombin exosite 1. Furthermore, this change could potentially alter how the HV3 peptide binds to exosite 1 and thereby positively influence the orientation of the API body towards the active site. Possible spacers could include at least 2 repeats of $(G_6)n$ or $(G_4S)n$, by analogy to spacers used in single chain antibodies or in optimized factor IX-albumin fusion proteins (*120*). Another idea that could be tested would be to fuse the $HV3_{54-66}$ peptide to the C-terminus of API RCL5 and/or API M358R. The HV3 peptide is naturally found on the C-terminus of hirudin and thus could potentially bind to exosite 1 more effectively in this orientation. Furthermore, a large spacer, in this context, would likely be ideal since positioning the HV3 peptide at the C-terminus would render it very close to the RCL. It is expected that a large spacer would allow for sufficient mobility of the RCL to access the active site of thrombin. Such modifications to my current experimental design could enhance the accessibility of the APIRCL to the thrombin active site and alter the binding of the HV3 peptide to exosite 1, thus augmenting the potential antithrombotic properties of this novel serpin.

Another approach to developing a novel anticoagulant is to look at a different serpin platform such as Protease Nexin-1 (PN1). When this serpin is complexed with heparin it is the fastest known endogenous inhibitor of thrombin, with a K_2 value of 1.0 $x10^9\ M^{-1}\ s^{-1}$ ($6.0\ x10^{10}\ M^{-1}\ min^{-1}$) (*121*). Supplementation with this serpin could be clinically applicable if it did not require heparin as a cofactor. Binding of heparin to helix

D of PN1 results in the optimal alignment of the P1-P1' residues with the active site of thrombin (*121*). Approaches that could be employed to make PN1 heparin independent include: mutation of the RCL to better align with thrombin; mutation by charge-neutralization of the heparin binding domain, attempting to induce the conformational changes wrought by heparin binding; and mutation to cause the two relevant strands of β-sheet A to align more closely, causing the RCL to be fully expelled and accessible to thrombin. Thus the modification of PN1 could have therapeutic potential, possibly via alterations of a less extensive nature than the mutations explored in this study.

6. References

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7. APPENDIX

Dear Leigh Ann,
Thank you for your request. We are happy to allow the use of our Cascade. We only ask that you acknowledge via written citation that it is courtesy of Enzyme Research Laboratories.

Best regards,
-Chris

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