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Comparative Pharmacologic Studies on Synthetic and Recombinant Inhibitors of Thrombin

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COMPARATIVE PHARMACOLOGIC STUDIES ON SYNTHETIC AND RECOMBINANT INHIBITORS OF THROMBIN

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DEDICATION

To my parents

TABLE OF CONTENTS

VOLUME I

Chapter

VOLUME II

LIST OF FIGURES

LIST OF TABLES

LIST OF STANDARD ABBREVIATIONS

μg	microgram
μm	micrometer
μM	micromoles/liter
μmol	micromoles
Å	angstrom
Arg	arginine
Asp	aspartate
Boc	t-butyloxycarbonyl
Ca^{++}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophopsphate
cGMP	cyclic guanosine monophosphate
cm	centimeter
dL	deciliter
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
fmol	femtomoles
g	gram

g	acceleration of gravity (9.8 m/s^2)
Glu	glutamine
Gly	glycine
GPC	gas permeation chromatography
H_2O	water
His	histidine
HPLC	high pressure liquid chromatography
I.V.	intravenous
Ile	isoleucine
k_{cat}	catalytic rate constant
k_d	dissociation rate constant
kg	kilogram
K_i	inhibition constant
Km	Michaelis constant
L	liter
Lys	lysine
M	moles/liter
M.W.	molecular weight
mg	milligram
ml	milliliter
ml	milliliter
mm	millimeter

mM	millimoles/liter
mmol	millimoles
mol	moles
mV	millivolt
mW	milliwatt
N	nitrogen
Na_2SO_4	sodium sulfate
$-NHSO_3$	aminosulfate
nm	nanometer
nM	nanomoles/liter
nmol	nanomoles
NMR	nuclear magnetic resonance
NO	nitric oxide
-OH	hydroxyl
$-OSO_3$	sulfate
pg	picogram
Phe	phenylalanine
pM	picomoles/liter
pmol	picomoles
pNA	para nitroaniline
Pro	proline
RBC	red blood cell

RI	refractive index
RNA	ribonucleic acid
S.C.	subcutaneous
S.E.M. (SEM)	standard error of mean
SD	standard deviation
Ser	serine
Tris	tris(hydroxymethyl)aminomethane
t½	biologcal half life
U	unit
USP	United States Pharmacopeia
UV	ultraviolet
Val	valine
WBC	white blood cell
®	registered trademark
™	trademark
°C	degree Celsius

LIST OF NON-STANDARD ABBREVIATIONS

α angle	the angle defined by the center line transversing the length of the TEG graph and the tangent from the initial point of divergence and the first inflection point of the tracing
ΔA_{405}	change in absorbance at 405 nm
5-HT	5-hydroxytryptamine
5U TT	5 unit thrombin time
Ac	acetyl
ACT	activated clotting time
AMI	acute myocardial infarction
ANOVA	analysis of variance
Anti-IIa	antithrombin
Anti-Xa	anti-factor Xa
APC	activated protein C
APTT	activated partial thromboplastin time
AT-III	antithrombin III
ATU	antithrombin unit
BBP	blood bank plasma
BD_2	dose producing bleeding of $2 \cdot 10^9$ RBC/L
b.i.d.	twice daily

C_3a	activated fragment of complement $_3a$
C_5a	activated fragment of complement $_5a$
$Ca^{++}TT$	calcium thrombin time
CPDA-1	mixture of 26.3 g/L trisodium citrate, 3.27 g/L citric acid, 31.9 g/L dextrose, 2.22 g/L monobasic sodium phosphate, and 0.275 g/L adenine
CT_{100}	concentration resulting in prolongation of clotting time to 100 sec
CU	casein unit
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
E.C.	enzyme commission
EACA	ϵ-aminocaproic acid (Amicar®)
ED_{50}	effective dose producing 50% of maximal effect
EGF	epidermal growth factor
ELISA	enzyme-linked immunoabsorbance assay
EPI	extrinsic pathway inhibitor (same as LACI and TFPI)
F_{1+2}	prothrombin fragments 1+2 assay
FEIBA®	factor eight inhibitor bypass activator (prothrombin complex containing factors II, VII, VIIa, IX and X)
Gla	γ-carboxyglutamic acid residue domain
GPIb	glycoprotein Ib
GPIIb/IIIa	glycoprotein IIb/IIIa
HC-II	heparin cofactor II
HIT	heparin induced thrombocytopenia

HMWK	high molecular weight kininogen
HV	hirudin variant
I.D. (ID)	internal diameter
IC_{50}	inhibitory concentration, producing 50% inhibition of maximal effect
IIa	thrombin
k value	the difference between the r and rk values of a TEG recording
IU	International Unit
KIU	kallikrein inactivator unit
KONYNE®	prothrombin complex consisting of factors II, VII, IX and X
LACI	lipoprotein associated coagulation inhibitor (same as EPI and TFPI)
LDL	low density lipoprotein
LMW	low molecular weight
LMWH	low molecular weight heparin
Me	methyl
min	minutes
msec	millisecond
N-terminal	amino-terminal
NHP	normal human plasma
NIH	National Institutes of Health
nkat	nanocatalytic unit
NRP	normal rabbit plasma
O.D.	optical density

PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor 1
PAI-2	plasminogen activator inhibitor 2
PAI-3	plasminogen activator inhibitor 3
PCI	protein C inhibitor
PDGF	platelet derived growth factor
PF3	platelet factor 3
PF4	platelet factor 4
PI	phosphatidyl inositol
PMH	porcine mucosal heparin
PPACK	D-Phe-Pro-ArgCH_2Cl
PPP	platelet poor plasma
proUK	pro-urokinase
PRP	platelet rich plasma
PT	prothrombin time
r value	the length from the beginning of the TEG recording until the point where the graph reaches a 2 mm divergence
r-hirudin	recombinant hirudin
rHV	recombinant hirudin variant
RIA	radioimmunoassay
rk value	the length from the beginning of the thrombelastogram until the point where the graph reaches a 2 cm divergence
rTF	recombinant tissue factor

sc-uPA	single chain urokinase type plasminogen activator
sec	second
serpin	serine protease inhibitor
tc-uPA	two chain urokinase type plasminogen activator
TEG	thrombelastogram
TF	tissue factor
TFPI	tissue factor pathway inhibitor (same as EPI and LACI)
t.i.d.	three times daily
TNF	tumor necrosis factor
tPA	tissue type plasminogen activator
TT	thrombin time
TXB_2	thromboxane B_2
uPA	urokinase type plasminogen activator
vWF	von Willebrand Factor

CHAPTER I

PURPOSE

Thrombin is known to play a key role in the development of various thrombotic conditions, such as stroke, myocardial infarction, pulmonary embolism and hypercoagulable states. Therefore it is the primary biochemical and pharmacologic target for developing newer antithrombotic drugs. One of the hypotheses underlying this approach is that sole inhibition of thrombin at blood and vascular sites is sufficient for the control of thrombogenesis and cellular activation processes. The purpose of this dissertation is to examine the hypothesis that inhibition of thrombin alone is sufficient for the control of thrombogenesis. This central question is addressed by determining the relative inhibitory effects of recombinant hirudin, a monospecific inhibitor of thrombin, along with the synthetic serine protease inhibitors (serpins) D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, argatroban and unfractionated heparin in the control of thrombogenesis. While the thrombin inhibitors included in this investigation represent varying degrees of specificities and potencies towards thrombin, heparin represents a poly-functional drug with mupltiple sites of action involving both the plasmatic proteases and cellular targets. Biochemical and pharmacologic models based on defined *in vivo* and *in vitro* experimental systems are used to investigate the comparative effects of each of these agents. This dissertation also provides supportive data on the relative role of non-specific serine protease inhibition in the mediation of the antithrombotic actions of

the newly developed thrombin specific peptide inhibitors.

Several antithrombin agents with different mechanisms of action are studied in terms of their specificity for inhibiting thrombin and related serine proteases such as factor Xa, activated protein C, plasminogen activator, plasmin and kallikrein. It is widely accepted that hirudin exhibits its antithrombotic action by solely inactivating thrombin. On the other hand, heparin which also is an effective antithrombotic agent exhibits a more complex mechanism of action through endogenous serpins such as antithrombin or heparin cofactor II (HC-II), to inactivate serine proteases. The synthetic peptides and peptidomimetic inhibitors used in this study are reported to be potent inhibitors of thrombin but are not monospecific for thrombin. Tripeptide inhibitors site-directed towards the catalytic region of thrombin vary in their specificity and potency for thrombin inhibition, which is largely dependent on their structure. The thrombin inhibitors chosen for this study provide structurally defined tools with different serpin profile which may be useful in identifying the activation processes which lead to a thrombogenic event. The relative efficacy of each one of these inhibitors in valid animal models of venous as well as arterial thrombosis provides a clear indication as to whether or not sole inhibition of thrombin is the most effective method to prevent thrombogenesis.

Many of these agents are in various phases of clinical development. However, a comparative account of their biochemical and pharmacological actions has not been available until now. Several studies indicate that these agents are not only different from heparin but marked differences among these inhibitors in terms of their mechanisms of

action and pharmacologic properties are predictable. Since each of the thrombin inhibitors exhibit a specific structural and biochemical profile, this investigation represents valuable information on the structure activity relationship for these agents. Furthermore, a direct comparison with heparin also provides additional insight into the role of endogenous macromolecular ligands and receptors in the mediation of the pharmacologic actions of the newly developed synthetic and recombinant agents.

Except for heparin, all of the agents included in this study are homogeneous, structurally characterized peptides or peptidomimetics, whose pharmacologic actions can be expressed in terms of molarity in relation to their interactions with endogenous enzymes, ligands and other sites. Thus, the anticoagulant and anti-protease actions of these agents can be quantified and compared in defined terms. A direct comparison of these data with heparin also provides new information on the mechanisms of mediation of the actions of these antithrombin agents.

The comparative anticoagulant profile of these agents does not correlate with their *in vivo* antithrombotic actions. Furthermore, the global clotting tests do not provide information on the precise site of action of anticoagulants but rather provide an indication of a general area of the protease cascade where inhibitory activities may take place.

One of the novel features of this investigation is the development and utilization of defined *in vitro* protease generation systems, which allow for the assessment of the relative importance of inhibition of specific factors in the coagulation cascade in defined and plasma based matrices. Furthermore, the studies on the inhibition of thrombin as well as its generation from specific precursors along with the studies on the broader

inhibitory spectrum of these agents on other purified proteases such as plasmin, activated protein C and kallikrein, adds to the understanding of the mechanisms of mediation of both the anticoagulant and antithrombotic actions of these agents.

Another important purpose of this research was to demonstrate the impact of the non-specific inhibition of regulatory serine proteases. While the amidolytic assays for the direct inhibition of many of the serine proteases and the generation of plasmin assays have been employed in isolated studies, a comparative investigation of these in a uniform manner has not been reported before. Furthermore, in this investigation all comparisons are made in a manner where the inhibitor levels as well as the enzymes (preformed or generated) can be directly compared at molar levels. Such a comparison can be used to project the relative impact of these thrombin inhibitors in the modulation of coagulation, fibrinolysis and kallikrein system.

Protease generation assays using *in vitro* plasmatic systems also provide a useful approach to characterize the multiple sites at which these thrombin inhibitors act. In the fibrinogen deficient human plasmatic systems, where thrombin is generated by activation of either the intrinsic or the extrinsic pathway, the difference in the pharmacologic actions of these antithrombin agents can be determined. These methods provide insights to the overall mode of actions of the thrombin inhibitors in a diluted plasma matrix where feedback modulatory actions of thrombin can be simultaneously studied.

Defined non-plasmatic protease generation systems have also been used to trigger the generation of thrombin is provided by extrinsic activation of prothrombin complexes (FEIBA® and KONYNE®), to determine the role of other plasmatic proteins in the

mediation of the anticoagulant effects of the thrombin inhibitors. Agents such as heparin, which require endogenous cofactors for the mediation of their inhibitory activities do not prevent the generation of thrombin or inhibit thrombin in these systems, whereas agents not specific for thrombin and possessing other serine protease inhibitory actions, are more effective in these assays.

To measure the relative *in vivo* antithrombotic actions of heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H, pharmacologic models of acute thrombosis are utilized. In the rabbit jugular vein stasis thrombosis model, the effectiveness of these agents in preventing clot formation is assessed in terms of potency, efficacy and duration of antithrombotic actions. However, the physiology of venous thrombosis differs from that of arterial thrombosis, thus, inhibition of thrombin and its generation and/or inhibition of other serine proteases may be of different importance in each model. Therefore, the antithrombotic actions of these agents are also studied in a rat laser-induced arterial thrombosis model. The results from these two models are useful in understanding not only the relative importance of direct thrombin inhibition as compared to the inhibition of thrombin generation and/or other serine proteases, but also the role of the proteases inhibited in the venous system as compared to the arterial system.

Since trace amounts of thrombin are required for the hemostatic function of platelets, thrombin inhibitors may also compromise this hemostatic process. At relatively higher dosage, indiscriminate inhibition of all effects of thrombin may lead to bleeding. Thus, the studies on the rabbit ear blood loss model can provide useful data on the

relative bleeding effects of these agents. Based on these observations, some projections on the role of platelets in the mediation of the thrombotic events and their modulation by thrombin inhibitors can also be made.

The animal model of venous thrombosis also allows for *ex vivo* analyses of blood samples so that a comparison of the pharmacodynamic actions (anticoagulant and antiprotease) with the antithrombotic effects of each antithrombin agent can be made. This is important in the understanding of the additional mechanisms of action for each agent. Additional correlations between the *ex vivo* antithrombin or related anticoagulant actions with the observed hemorrhagic actions in a rabbit model of ear blood loss provides data on the endogenous interactions of these agents resulting in bleeding effects. Thus, a safety/efficacy index can be developed for each of the agents studied.

Another important feature of this investigation is the utilization of recombinant tissue factor (rTF) in the *in vitro* biochemical assays and the *in vivo* rabbit venous thrombosis studies. The thrombogenic environment developed by using this trigger is rather defined and the role of heparin and antithrombin agents can be readily assessed in the rTF mediated thrombogenesis. The use of rTF in the stasis thrombosis model is more defined than the existing models using mixtures of enzymes or human serum. The tissue factor used in this investigation is produced by recombinant technology and is molecularly characterized and packaged in phospholipids. In the *in vivo* thrombogenic studies, it acts as a strong extrinsic pathway activator that leads to thrombogenic events. Thus, the data generated on rTF in both the biochemical and pharmacologic studies represent more defined information on the control of thrombogenesis with the new

antithrombin agents.

The experimental approaches included in this dissertation are designed to differentiate the pharmacological profile of antithrombin agents as a class from those of heparin. Furthermore, these studies also provide crucial data on the significant differences which exist amongst these agents. In addition, this dissertation also addresses the question of standardization, relative role of thrombin inhibitors in the mediation of antithrombotic actions and some of the non-thrombin mediated modulatory actions of these agents, such as their effects on the fibrinolytic network and activated protein C modulated regulatory processes.

The experimental methods included in this dissertation represent optimal and pharmacologically defined systems to obtain valid data in a systematic fashion on the biologic actions of these antithrombin agents of different origins. It is expected that the reported data in this dissertation will serve as a systematic approach for the valid pharmacologic investigation of newer antithrombin agents.

CHAPTER II

REVIEW OF LITERATURE

A. Overview of Hemostasis

Unicellular organisms derive their nutrients by simple physiochemical process and/or by phagocytotic process. However, more complex multicellular organisms require more complex mechanisms of essential nutrient delivery to their cells. The vasculature in mammals serves as a conduit via which nutrients reach individual cells. Evolution has shaped the vascular physiology with mechanisms to take corrective measures to cope with the disruptive events. Blood is a non-Newtonian fluid which flows through the vessels to provide oxygen and nutrients and regulate the vascular integrity. While the process of hemostasis (arrest of bleeding) will prevent blood loss, the fibrinolytic process facilitates the dissolution of clot as the healing process progresses, allowing the restoration of blood flow. Despite all of the mechanisms which serve to regulate hemostasis, uncontrolled activation of this process leads to a range of disorders, including both the thrombotic and hemorrhagic disorders.

It is now becoming clear that the role of the endothelial cell monolayer is rather important in the regulation of hemostasis and extends beyond providing a non-thrombogenic surface for the continuous flow of blood. The products of the vascular cells are essential in regulating various physiologic processes. Receptors expressed on

the surface of these endothelial cells regulate many of their hemostatic functions. Cell surface interactions on the endothelium and on other blood-borne cells control a multitude of natural processes, among these is the localized production and control of thrombin, the key enzyme in clot formation. The importance of these components is evident when there is a defect in one of these which can lead to a wide range of clinical disorders ranging from thrombosis and atherosclerosis to neurological disease, inflammatory and immune disorders, microangiopathy and vasculitis.

Focusing on the processes that regulate the flow of blood, while the recognition of the role of fibers has been noted since the time of Plato, the specifics of blood clotting started to become unravelled in the beginning of the 19th century (Bloom et al. 1994). By 1964, enough was known about specific coagulation proteins to develop the "waterfall" or "cascade" coagulation model (Macfarlane 1964), in which activation of one zymogen leads sequentially to the activation of another, until thrombin is generated to mediate the conversion of liquid fibrinogen to solid fibrin clots. Since then, an exponential increase in the knowledge of about these proteins has led to several modifications of the original simplified scheme to develop increasingly more complex networks. It has also become clear that these processes are not independent of other cellular components and that there is a constant interaction with many different cell types mediating equally diverse physiologic responses (Loscalzo and Schafer 1994).

The pathophysiology of thrombotic events is multicomponent and involves blood, vascular system and target sites. Vascular injury results in the localized alterations of the vessels and subsequent platelet activation. Activated cells mediate several direct or

signal transduction mediated processes resulting in the activation of platelets. Cellular activation also results in the release of various mediators which amplify vascular spasm and the coagulation process. Thus, anaphylatoxins (C_3a and C_5a), superoxide, leukotrienes (LTC4), thromboxane B_2 (TxB_2), serotonin, platelet factor 3 (PF3), platelet factor 4 (PF4), platelet activating factor (PAF), endothelin-1 and numerous cytokines play a role in the overall pathophysiology of the thrombotic process. Drugs that target various sites of the activation process can be developed to control thrombotic events. Because of the coupled pathophysiology, a single drug may not be able to target these sites to produce therapeutic actions. Furthermore, many of these mediators produce localized actions at cellular and subcellular levels. The feedback amplification process also plays an important role in the pathology of these disorders. This understanding has led to the concept of polytherapy in the management of thrombotic disorders.

Venous insufficiency, blood plasma related disorders, fibrinolytic deficit and an imbalance of regulatory proteins result in the activation of the hemostatic process. Post-surgical trauma, inflammation and sepsis also lead to the activation of the hemostatic process leading to venous thrombosis. The primary process in venous thrombosis is the generation of thrombin. Thus, drugs targeting coagulation protease activation are useful in the treatment of venous disorders (Fareed 1992). However, platelet and cellular activation contribute significantly to arterial thrombotic events, and therefore, drugs targeting those sites are important in the management of arterial thrombosis and microangiopathic disorders.

The newer developments in antithrombotic drugs are significant. Many advanced

techniques to develop antithrombotic drugs are used at the present time. Advances in biotechnology and separation techniques have also contributed to the development of newer antithrombotic drugs (Fareed et al. 1988-1992). These drugs may prove to have a better efficacy in the control of thrombogenesis and its treatment. Drugs and devices which have been or are being developed based on newer concepts and range from heparin related drugs and antiplatelet agents to endothelial lining and viscosity modulators.

Antithrombotic drugs represent a wide spectrum of natural, synthetic, semi-synthetic and biotechnology produced agents with marked differences in chemical composition, physicochemical properties, biochemical actions and pharmacologic effects. The use of physical means to treat thrombotic disorders, and advanced means of drug delivery, add to the expanding nature of this area.

The endogenous actions of the antithrombotic drugs are remarkably complex. It is no longer valid to assume that an antithrombotic drug must produce an anticoagulant action in blood as the conventional heparin and oral anticoagulants. Many of these drugs, such as platelet inhibitors, do not produce any alteration of blood clotting parameters, yet they are effective therapeutic agents because of their interactions with the various elements of the vasculature and other blood components. Another perspective is that several of these agents require endogenous transformation to become active products. Therefore, it becomes important to rely on the pharmacodynamic actions of these agents rather than on their in vitro characteristics to assess potency or efficacy of the product. Hematologic modulation plays a key role in the mediation of the antithrombotic actions of these drugs involving red cells, white cells, platelets and blood

proteins. This is particularly true for the case of trauma induced thrombotic disorders where multiple processes are involved in thrombogenesis.

B. Role of Serine Proteases in the Regulation of Coagulation, Fibrinolytic, Kallikrein and Complement Pathways

Blood is a complex liquid tissue that serves as an interface between its own cells and those of the organ systems which it perfuses to mediate many vital physiologic processes, one of which is prevention of excessive bleeding following trauma, a process which is usually referred to as hemostasis. A crucial component of hemostasis is the coagulation system. Upon activation, this system results in clotting of liquid blood. The processes involved in the activation of the coagulation system are enzymatic in nature and are mostly mediated through a series of coagulation factors (proteins) and cofactors whose activities are enhanced by Ca^{++} and phospholipids. The activation of the coagulation system leads to formation of a key active coagulation factor, thrombin. Thrombin is essential in converting liquid fibrinogen to fibrin clots which are deposited in platelet plugs to arrest bleeding. In addition, thrombin plays a key role in many other physiologic and pathologic processes.

Thrombin is generated in the blood through two separate enzymatic pathways (Ratnoff et al. 1964, Macfarlane et al. 1964). In the intrinsic pathway, components intrinsic to whole blood (circulating in blood) are involved through contact activation, in the reactions leading to thrombin generation. In the extrinsic pathway, components extrinsic to whole blood (not normally in contact with whole blood) are generated or activated to initiate a cascade of events leading to thrombin formation. These pathways

coalesce at the level of activation of a common enzyme, factor Xa, which activates prothrombin leading to thrombin formation. An integrated version of the coagulation, fibrinolytic and kallikrein systems is illustrated in Fig 1.

All of the enzymes involved in blood coagulation normally circulate in inactive proenzyme forms as zymogens. When a zymogen is activated to an enzyme, this enzyme further activates another zymogen to its active enzymatic state, thus propagating the coagulation cascade. The activated form of the zymogen is denoted with a lower case "a" following the name of the zymogen.

The extrinsic pathway is initiated physiologically when membrane surface bound tissue factor (TF), on tissues outside the circulatory system, comes in contact and forms a complex with blood born factor VIIa through trauma (Bloom et al. 1994). In the presence of Ca^{++} this complex is able to directly activate factor X to factor Xa, also shown in Fig.1. Beside the activation of coagulation, this complex is also involved in cellular activation at various sites.

The kallikrein-kinin system is a system of protease interactions in the blood, whose major physiological function is to produce bradykinin and other low molecular weight kinins. The effects of these peptides on the circulatory system include increased vascular permeability, vasodilation of smaller blood vessels, pain, smooth muscle contraction and synthesis and enhancement of prostaglandins (Bloom et al. 1994). This system also interacts with both the coagulation system and the fibrinolytic system in a complex manner. The kallikrein-kinin system can be stimulated by a variety of factors such as tissue damage, allergic reactions, viral infections and inflammatory processes that

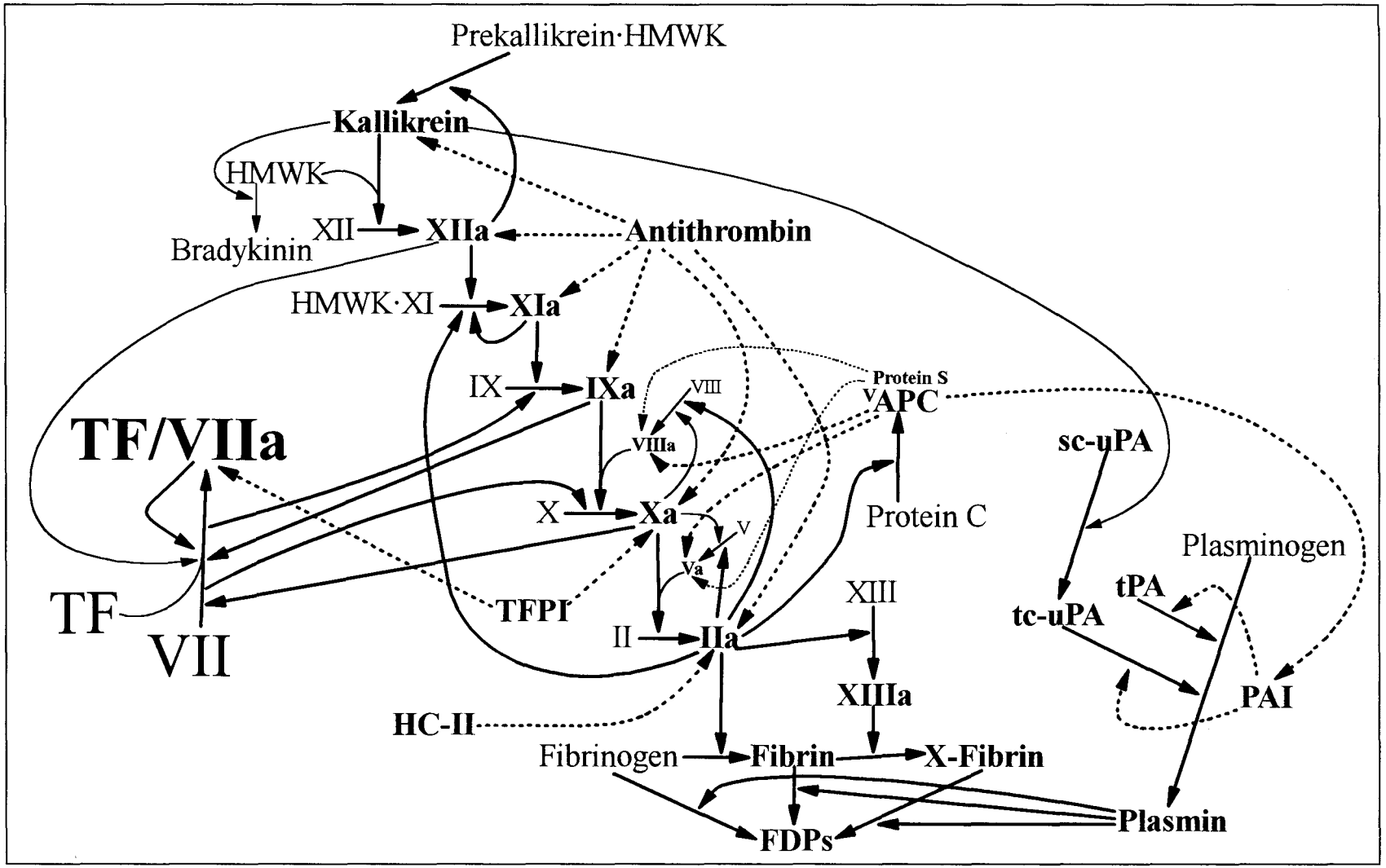

Fig. 1. The coagulation and fibrinolytic system. The interactions and feedback amplifications within the coagulation system are depicted, as are its interactions with the fibrinolytic system. - - - indicates inhibition by the major endogenous serpins.

lead to plasmatic generation of bradykinin (via proteolytic action of plasma kallikrein on high molecular weight kininogen or HMWK) and direct activation of the complement system (kallikrein can directly activate C5) (Bloom et al. 1994). Of relevant interest is the generation of plasma kallikrein, which is generated through the proteolytic action of factor XIIa on prekallikrein, as depicted in Fig.1. Factor XIIa is generated from factor XII upon interaction with negatively charged surfaces or collagen as with tissue damage and is accelerated by HMWK. The generated kallikrein, with HMWK as a catalyst, can then further catalyze the activation of factor XII to factor XIIa. Thus, the kallikrein-kinin system is linked to the intrinsic activation pathway via kallikrein and factor XII (Loscalzo et al. 1994).

The intrinsic pathway is initiated physiologically by disruption of the vessel wall inner lining and exposure of connective tissue elements which provide the negatively charged surface necessary for the activation of factor XII by kallikrein and HMWK (Loscalzo et al. 1994), as seen in Fig.1. Factor XIIa in turn activates factor XI to factor XIa in the presence of Ca^{++} and HMWK. Kallikrein, factors XII and XI and HMWK comprise the group of contact factors. Factor XIa converts factor IX to factor IXa in the presence of Ca^{++}. Factor IXa binds to a membrane surface bound cofactor, factor VIIIa, to form the tenase complex, which activates factor X to factor Xa.

The extrinsic and intrinsic pathways are not completely independent up to the point of formation of factor Xa and interact at different levels during feedback processes. For example, factor XIIa from the intrinsic pathway is also capable of activating factor VII to VIIa in the extrinsic pathway and factor VIIa is capable of converting factor IX to IXa in the intrinsic pathway. Furthermore, factor IXa from the intrinsic pathway is

capable of activating factor V in the extrinsic pathway. Thus, enzymatic activations are complex and feedback controls are abundant.

The activation of the two pathways leads to formation of factor Xa which is a component of the common pathway. Factor Xa binds to membrane bound factor Va in the presence of Ca^{++} to form the prothrombinase complex. This complex converts prothrombin to thrombin which converts fibrinogen to fibrin clot. In addition to prothrombin activation, factor Xa also mediates the activation of factor VII in the extrinsic pathway, cofactor VIII in the intrinsic pathway and cofactor V in common pathway. The function of thrombin in the coagulation cascade is not restricted to conversion of fibrinogen to fibrin, but also includes further activation of cofactors V and VIII (as does factor Xa), as well as activation of factors XI and XIII. The common pathway ends in stabilization of the formed fibrin by factor XIIIa mediated cross-linking of the fibrin strands.

The coagulation system is controlled at various levels by four major physiological inhibitors, also shown in Fig. 1: antithrombin, heparin cofactor II (HC-II), tissue factor pathway inhibitor (TFPI) and activated protein C (APC). Antithrombin mediates its anticoagulant effects by inhibiting thrombin and factor Xa and to a lesser extent factors XIIa, XIa, IXa and kallikrein. HC-II directly inhibits only thrombin, while TFPI forms an inactive complex with TF and factor Xa. The inhibitory mechanism of APC is more complex in that while this enzyme is activated by thrombin and it degrades cofactors VIIIa and Va (which are necessary for factor X and prothrombin activation), it requires two cofactors for its anticoagulant activities: protein S and factor V (which it degrades in the activated form). Thus, activation of coagulation and formation of thrombin also

triggers its inactivation.

The fibrinolytic system, balances the coagulation system by ultimately resulting in fibrin clot dissolution. The two systems regulate and balance each other intricately and offset towards one or the other system leads to thrombosis or bleeding respectively.

The enzymes involved in fibrinolysis, similar to the ones involved in coagulation, also circulate in their inactive forms under normal physiological conditions. In an oversimplified scheme, shown in Fig.1, fibrinolysis is initiated with the formation of fibrin clots where plasminogen and thrombin become entrapped. Plasminogen binds to fibrin weakly but activators such as tissue plasminogen activator and urokinase, which also diffuse into the clot, accelerate the binding of plasminogen to fibrin and mediate its activation to plasmin (Bloom et al. 1994). Plasmin then can degrade fibrin to fibrin degradation products. The interaction of the kallikrein -kinin system with the fibrinolytic system is at the level of urokinase, where kallikrein can directly activate single chain pro-urokinase to the two chain urokinase (Bloom et al. 1994), also depicted in Fig. 1.

1. Biochemical Characteristics of Serine Proteases

All of the blood coagulation factors and enzymes involved in fibrinolysis belong to the family of serine proteases with the following exceptions: tissue factor, factors V and VIII and HMWK which function as cofactors, factor XIII which is a transamidase and fibrinogen which is a non-enzymatic glycoprotein. The blood coagulation serine proteases share some common features in terms of their structure and mode of action. All are synthesized in the liver, except for Von Willebrand factor (vWF), initially with a signal peptide which allows for their translocation to the endoplasmic reticulum, where

this peptide is cleaved (Loscalzo et al. 1994). Some of the characteristics of serine proteases involved in coagulation are summarized in Table 1.

The catalytic domain of all blood clotting enzymes is highly homologous to that of trypsin (Furie et al. 1982). This domain is composed of a specific site for recognition of macromolecular substrates referred to as the specificity pocket and an active site referred to as the catalytic triad, which serves for the conversion of inactive zymogens to active enzymes via cleavage of specific bonds. The blood clotting enzymes are trypsin-like enzymes, meaning that they recognize and cleave peptide bonds involving arginine. They are also serine proteases, meaning that their active site in the catalytic domain is composed of a specific catalytic triad: serine, aspartate and histidine (Stroud et al. 1974).

Other common features shared among blood coagulation enzymes are epidermal growth factor (EGF) domains which consist of 3 disulfide bonds in a characteristic covalent structure (Gregory 1977). Factors VII, IX, X, XII, protein C and protein S, as well as tPA and pro-urokinase (proUK) from the fibrinolytic system, all have at least 1 EGF domain. These structures may mediate the binding of factor IX to endothelial cells (Ryan et al. 1989). Another structure shared among many blood proteins is the Kringle domain, also composed of 3 disulfide bonds in a characteristic covalent structure but involving double the amount of amino acids involved in EGF domains and with a different three dimensional structure. This type of domain is thought to be important in protein complex formation (Parket al. 1986). Among the blood proteins possessing such a structure are prothrombin (Magnusson et al. 1973), factor XII (Cool et al. 1987), plasminogen (Malinowski 1984), pro-urokinase (Holmes et al. 1985) and tissue- type plasminogen activator (Ny et al. 1984).

Table 1 -- Characteristics of Procoagulant and Pro-fibrinolytic Proteins

	Enzyme Commission #	MW	Proenzyme Plasma Level	Natural Substrates	Enzyme Type
Kallikrein	3.4.21.34	85,000	0.6 μM	Factor XII, sc-uPA, C5	Serine protease
HMWK	-	120,000	0.7 μM	-	Cofactor for prekallikrein, kallikrein and factor XI
Factor XIIa	3.4.21.38	80,000	0.4 μM	Prekallikrein, factors VII and XI, C1	Serine protease
Factor XIa	3.4.21.27	160,000	0.025 μM	Factors XII XI and IX	Serine protease
Factor IXa	3.4.21.22	45,000	0.09 μM	Factors X, VII and VIIIa	Serine protease
Factor VIIIa	-	166,000	0.3 pM	-	Cofactor for factor IXa
Factor VIIa	3.4.21.21	50,000	0.01 μM	Factors X, IX and VII	Serine protease
Tissue Factor	-	37,000	0 μM	-	Cofactor for TF
Factor Xa	3.4.21.6	48,000	0.17 μM	Prothrombin, factors XII, X, VIII, V and VIIIa, protein C	Serine protease
Factor Va	-	180,000	0.03 μM	-	Cofactor for factor Xa
Thrombin	3.4.21.5	36,000	2 μM	Fibrinogen, factors V, VII, VIII, XI and XIII, protein C	Serine protease
Fibrinogen	-	340,000	6-12 μM	-	Non-enzymatic glycoprotein
Factor XIIIa	2.3.2.13	150,000	0.03 μM	Fibrin, $\alpha_2$plasmin inhibitor, fibronectin, von Willebrand factor, vitronectin, actin, myosin, lipoprotein(a)	Transglutaminase
Plasmin	3.4.21.7	92,000	2 μM	Fibrin, plasminogen	Serine protease
tPA	3.4.21.68	68,000	70 pM	Plasminogen	Serine protease
uPA	3.4.21.73	33,000	150 pM	Plasminogen	Serine protease

The proenzyme forms of these proteases have the same MW as the active enzymes, with the following exceptions: factor XI (MW of 55,000), factor VIII and factor V (MW of 330,000), factor X (MW of 59,000), prothrombin (MW of 72,000), factor XIII (MW of 320,000) and sc-uPA (MW of 54,000). Loscalzo and Schafer 1994, Bloom et al. 1994, High and Roberts 1995.

Prothrombin, factors VII, IX, X, and the inhibitory proteins, namely protein C and protein S require vitamin K for their synthesis (Stenflo et al. 1974, Nelsestuen et al. 1974). These are the vitamin K-dependent proteins and the requirement for vitamin K stems from a characteristic string of 10-12 glutamic acid residues of these proteins in the first 45 amino acids of their amino termini. Vitamin K is required for the carboxylation of these residues to form dicarboxylic acid side chains (γ-carboxyglutamic acid residues or Gla domains) which are necessary for binding Ca^{++} and assembly on membrane surfaces in the presence of these ions. The carboxylation of these residues is catalyzed by a membrane bound hepatic microsomal γ-carboxylase in the presence of reduced vitamin K, as a post-translational step in the synthesis of these proteins.

2. Role of Serine Proteases and Cofactors in the Hemostatic and Hemorrhagic Processes

Certain defects in the coagulation and fibrinolytic systems as well as other causes can lead to development of thrombosis. The pathophysiology of thrombosis in the arterial system is quite different from that in the venous system and it is important that they are recognized and understood for effective differential clinical management.

Arterial thrombosis is frequently associated with atherosclerosis (Davies et al. 1985). Thrombosis is initiated by disruption of atherosclerotic plaques which exposes the subendothelium to the blood (Falk et al. 1983, Fuster et al. 1988, Davies et al. 1984, 1985). This leads to activation of platelets and their aggregation, followed by stabilization of this platelet aggregate by fibrin generated from the activation of the coagulation cascade (Davies et al. 1986). Therefore, the plug formed at the site of atherosclerotic lesioning is a platelet-rich clot. This clot may remain attached to the site

of injury and may grow to completely obstruct the blood vessel. Thrombogenic factors involved in this kind of thrombosis are damage to the vessel wall exposure of TF and connective tissue elements with subsequent activation of coagulation. Treatment of arterial thrombotic disorders include antiplatelet agents (to prevent further activation of platelets which are the building block of arterial thrombi), anticoagulants (to inhibit the coagulation cascade, highly controversial but with positive results -Resnekov et al. 1989, Théroux et al. 1988) and thrombolytic therapy (to accelerate fibrinolysis, Cairns et al. 1989). When these non-invasive treatments fail to re-open an occluded vessel, invasive procedures are employed such as endarterectomy, embolectomy, arterial bypass surgery and angioplasty. More recently, to stabilize the vessels non-thrombogenic supporting devices such as stents are also used.

There is always a low basal level (subclinical) of coagulation activation produced even in normal subjects (Conway et al. 1987) but the activated coagulation factors are normally cleared by flowing blood, or controlled by plasma inhibitors and inhibitors on the surface of endothelial cells (Esmon et al. 1987). When blood coagulation exceeds the anticoagulant system and the fibrinolytic system, thrombosis occurs, most frequently in the large venous sinuses of the calf and in the deep vein valve cusps of the calf. The thrombi formed in such locations are composed primarily of fibrin and red cells (no platelets) (Frieman 1987). The thrombogenic factors involved are activation of blood coagulation, venous stasis and vessel wall damage. Activation of blood coagulation may occur as a result of disseminated malignant disease (Gordon et al. 1975) or even infusion of activated factor concentrates (Kingdon et al. 1979). Venous stasis (impediment in

venous return) can be the result of immobility, increased venous pressure, venous dilation, venous obstruction or increased blood viscosity (Leonard 1987). Vessel wall damage may occur after surgical procedures or after trauma and results in the expression of tissue factor activity which leads to activation of coagulation and thrombus formation.

Pharmacological treatment of venous thrombosis consists of thrombolytic and anticoagulant therapy. The most common preventable cause of death in a hospital setting is venous thromboembolism (Salzman et al, 1987), where a venous thrombus breaks off to produce satellites which lodge in pulmonary vessels causing a variety of pathologic effects which may lead to death if untreated. Prophylaxis of venous thromboembolism consists of anticoagulants in conjunction with intermittent leg compression.

A hypercoagulable state can arise not only from the above clinical disorders, but may also be a result of an inherited thrombotic disorder. These inherited disorders may be deficiencies in protein C or protein S, antithrombin, plasminogen and fibrinogen. Taking a closer look at the coagulation enzymes, it is evident that activation of certain enzymes has a stronger impact than that of others. This point is illustrated best when comparing the coagulation disorders arising from deficiencies or disorders in various blood coagulation factors.

a. High Molecular Weight Kininogen (HMWK)

In the coagulation cascade, HMWK functions as a cofactor for prekallikrein, kallikrein and factor XI. Prekallikrein and factor XI circulate in the blood as complexes with HMWK (which mediates surface binding) while the presence of HMWK catalyzes the activation of factor XII by kallikrein. Apart from its role in the coagulation cascade,

HMWK is also the precursor to the vasoactive peptide bradykinin, which results from the proteolytic action of kallikrein on HMWK. Deficiency in HMWK is a rare autosomal recessive deficiency and the individuals characterized with this disorder do not suffer from bleeding complications, despite markedly prolonged coagulation tests (Cheung et al, 1993, Hayashi et al, 1990). No association between HMWK deficiency and hypertension has been made.

b. Prekallikrein

Prekallikrein circulates in the blood as a complex with HMWK and is activated to kallikrein by the action of factor XIIa (Bloom et al. 1994). Kallikrein then, in the presence of HMWK further activates factor XII. In addition, kallikrein also proteolytically releases bradykinin from HMWK, directly activates C5 in the complement system and activates pro-urokinase to urokinase in the fibrinolytic system. Thus, deficiency in this factor may be expected to be precipitate disorders associated with all of these systems. However, individuals diagnosed with this deficiency appear to have no bleeding diatheses, even though the APTT is prolonged, and although intrinsic fibrinolytic activity is reduced, the association with thromboembolic complications is questionable (Wuillemin et al. 1993, Hess et al. 1991, Castaman et al. 1990, Bouma et al. 1986, Sollo and Sallem, 1985). This deficiency is transmitted in an autosomal recessive manner.

c. Factor XII

Factor XII is activated by kallikrein (Meier et al. 1977, Griffin et al. 1978,

Fujikawa et al. 1980) and this conversion of factor XII to factor XIIa is accelerated by HMWK and negatively charged surfaces or collagen (Ratnoff et al. 1966, Wilner et al. 1968). Factor XIIa functions to convert factor XI to factor XIa and prekallikrein to kallikrein. In addition, factor XIIa can directly stimulate the complement system, by activating C1. As with deficiencies in HMWK or prekallikrein, deficiency in this factor (Ratnoff et al. 1955) may be discovered in preoperative evaluations and is not associated with a bleeding history nor with excess bleeding during surgical procedures. Factor XI's coagulant function is not impaired even if its physiological activator (factor XIIa) is absent. Patients lacking factor XII also have low plasminogen activity which, however, is not associated with defective fibrinolysis.

d. Factor XI

Factor XI circulates in blood as a complex with HMWK, as does prekallikrein. Factor XIa (resulting from the action of factor XIIa on factor XI, described above) functions to catalyze the activation of factor IX to factor IXa (Bloom et al. 1994). Deficiency in factor XI (Rosenthal et al. 1953) is inherited and is due to abnormalities in the gene of this factor which may lead to heterozygote, compound heterozygote or homozygote patients. Bleeding complications arise only after trauma and the severity of these episodes varies proportionally with the deficiency in the factor. However, low levels of factor XI are not always associated with bleeding and this may be due to platelet membrane factor XI-like activity or the interaction with factor VIIa in the extrinsic pathway which can also activate factor IX.

e. Factor IX

Factor IX is activated by either factor XIa (Fujikawa et al. 1974, DiScipio et al. 1978, Davie et al. 1987) or factor VIIa/tissue factor complex (Österud et al. 1977), always in the presence of Ca^{++} while it is in solution (in contrast to the other vitamin K dependent clotting factors which have to be bound to be activated). Both factors IX and IXa bind on platelet surfaces or the surface of endothelial cells. This binding to cell surfaces is enhanced by factors V, VIII and X and renders factor IXa about 3 times more active than in solution. The activation of factor IX is slow compared to the rest of the coagulation steps, which signifies its importance in the control of the coagulation cascade. At the site of injury, factor IX is slowly activated which leads to inefficient activation of factor X on cell surfaces. This activated factor Xa then leads to activation of factor VII/tissue factor complex which then can further activate both factors IX and X leading to "burst" of coagulant activity (Warn-Cramer et al. 1986).

Deficiency in factor IX is sex-linked recessive and may arise from defects in its gene on chromosome X , such as point mutations, gene deletions, missense mutations and gene insertions, and are the cause of the bleeding disorder termed Hemophilia B and Christmas disease. Hemophilia B (Mandel et al. 1988) can also result from production of normal levels of factor IX but defective post-translational factor IX protein processing that may affect the γ-carboxylation, EGF domain and function, zymogen activation, substrate recognition and enzyme activity (Green et al. 1989). Hemophilia B patients suffer from excessive bleeding in the form of easy bruising and frequent hematomas that often spread after a few days and may become life-threatening. Current treatment

approaches include factor IX concentrates, fresh-frozen plasma or supernatant from cryoprecipitates.

f. Factor VIII/von Willebrand Factor Complex

Although factor VIII is a cofactor and not a serine protease, it deserves attention since deficiencies in this protein lead to Hemophilia A. The circulating form of this cofactor is inactive and it is activated to factor VIIIa by thrombin or factor Xa (Pittman et al. 1988). Factor VIII is an unstable protein when free in solution and it circulates as a stable complex with von Willebrand factor (vWF), a completely different protein under separate genetic control (Kaufman et al. 1988, Weiss et al. 1977, Brinkhous et al. 1985, Koedam et al. 1989). The two proteins have different physiologic functions as well: vWF is essential for platelet adhesion to subendothelium, whereas factor VIIIa serves as a cofactor for factor IXa to activate factor X. Whereas phospholipid surfaces serve to anchor and concentrate factors IXa and X (enzyme and substrate respectively) factor VIIIa binds to both previous factors, increasing the forward rate of catalysis of factor X to Xa by factor IXa. Factor VIIIa is thus able to accelerate the activation of factor X by factor IXa by 10,000 in the presence of Ca^{++} and phospholipids.

Hemophilia A is sex linked and is due to defects of the factor VIII gene located on the X chromosome, such as deletions, insertions, base mutations, transitions and point mutations (White et al. 1989, Antonarakis et al. 1988, Vehar et al. 1989, Gitschier et al. 1989). The bleeding abnormalities associated with factor VIII deficiency include joint and muscle hemorrhages that lead to disabling long-term events, easy bruising and prolonged post-operational bleeding. The platelet function is normal and therefore there

is no excessive bleeding from minor cuts. Current clinical treatment strategies include immobilization, compression, ϵ-aminocaproic acid (EACA) which is an anti-fibrinolytic agent and in more severe cases factor VIII or factor IX concentrates.

g. Tissue Factor

Tissue factor (TF) is a 30,000 integral membrane glycoprotein that serves as a cofactor and is required for the activation of factor VII (Broze et al. 1985, Guha et al. 1986, Bach et al. 1986, Nemerson 1988). In addition to catalyzing the activation of factor VII, TF also enhances the catalytic activity of factor VIIa (Andree and Nemerson, 1995), since factor VIIa alone has negligible coagulant activity. The crystal structure of the TF/factor VIIa has been recently reported (Banner et al. 1996). While TF is expressed on the surface of a variety of cell types, non of these cells are normally in contact with blood. Basal coagulation levels are due to circulating factor VIIa, which corresponds to about 1% of the total factor VII antigen. However, since blood is not physiologically exposed to TF and free factor VIIa has minimal activity, only low level of coagulation is normally in effect and is readily counteracted by endogenous inhibitors and the basal fibrinolytic system.

Other functions of TF are currently unknown, however various cells are believed to have TF receptors. Many of the post-surgical and post-interventional thrombotic events are due to the release of TF. Increased levels of TF are associated with several pathologic conditions such as cancer, sepsis and inflammation (Bloom et al. 1994). Cellular necrosis also results in an increase of TF as the cells in the traumatized area lyse and release endogenous cell surface-bound TF. TF has been mapped by using

immunohistochemical methods in various tissues, such as the brain, kidney, spleen, liver, skin, gut, vessels, adrenal glands and peripheral nerves (Feleck et al. 1990). TF is produced constitutively by cells that are normally separated from blood by the vascular endothelium. It is present in the subendothelial sites and is only exposed after endothelial damage. TF is also produced in response to a host of stimuli, including endotoxin, complement C5a, immune complexes, interleukin-l and tumor necrosis factor (Fleck et al. 1990) in a variety of cells. In addition, TF is also generated under some pathological conditions by monocytes, some tumor cells, foam cells of atherosclerotic lesions, smooth muscle cells and their mesenchymal derivatives (Koyama et al. 1994, Takahashi et al. 1994, Adamson et al. 1994, Carty et al. 1990, Marmur et al. 1993, Muller et al. 1993, Callander et al. 1992, Lockwood et al. 1991). No deficiencies in this factor have been reported.

h. Factor VII

After factor VII has complexed with TF, it is most rapidly activated by factor Xa, although factors VIIa (auto-activation), IXa, XIIa and thrombin also activate this factor. Factor VIIa/TF complex then activates factor X directly or via activation of factor IX to IXa (Radcliffe et al. 1973, Jesty et al. 1974). A small amount of generated factor Xa serves as feedback loop to activate factor VII to VIIa, resulting in amplification of extrinsically generated thrombin. Deficiency in factor VII is a rare autosomal recessive disorder and results from point mutations or partial gene deletions and can lead to worse bleeding than deficiency in factor IX (Ragni et al. 1981, Briet et al. 1987, Bernardi et al. 1994, Chaing et al. 1994). The clinical symptoms are variable and poorly

understood, ranging from none (Ohiwa et al. 1994) to cerebral hemorrhage (Papa et al. 1994) to thromboembolism Martini et 1992, Mitropoulos et al. 1992). Factor VII deficiency is the only deficiency that results in prolongation of the PT with a normal APTT. Therapy of factor VII deficient patients consists of factor IX concentrate or plasma supplementation.

i. Factor X

Factor X is a Ca^{++} dependent protein that interacts with acidic phospholipids. Factor X and factor Xa bind to endothelial cells and are endocytosed. While factor Xa is degraded by lysosomes, factor X reappears on the cell surface. The activation of factor X to factor Xa is the point where the intrinsic and extrinsic pathways of coagulation join, since it is activated by either factor VIIa/TF complex or by factor IXa/factor VIIIa complex. Factor V is a cofactor necessary for the catalytic activity of factor Xa and is activated to factor Va by thrombin. Factor Xa can then form a complex with factor Va to form the prothrombinase complex, and this complex catalyzes the conversion of prothrombin to active thrombin, which then in turn converts fibrinogen to fibrin.

The concentration of factor V in plasma is lower than that of factor X (Nesheim et al. 1981, Miletich et al. 1981) and without TF available (as under normal conditions) only the VIIIa/IXa complex can catalyze the activation of factor X (Drake et al. 1989). Thus, as in the PT assay, the supplementation of large exogenous TF, after complexation with the basal factor VIIa, causes a direct activation of factor X that is then able to saturate all of the available factor V. The generated factor Xa can then further catalyze

the activation of factor VII, thus resulting in an amplified loop of the extrinsic pathway of coagulation, leading to generation of massive amounts of thrombin with the end result of formation of thrombi.

Factor X deficiency (Hougie et al. 1956) is an autosomal recessive trait resulting from 4 point mutations to its gene (Watzke et al. 1990, Reddy et al. 1989). Treatment regimens include fresh-frozen plasma or factor IX concentrate supplementation.

j. Factor XIII

Factor XIII is a transglutaminase that, once activated by thrombin to factor XIIIa, it cross links fibrin strands, thus making them more stable and less susceptible to proteolytic digestion by plasmin. Factor XIII deficiency is a rare autosomal recessive disorder that results in bleeding diathesis and delayed hemorrhages in soft tissues after a primary plug is successfully formed. About fourteen different mutations have been reported and most of them are due to point mutations (Mikkola et al. 1996).

k. Plasmin

Just as thrombin is the key enzyme in the coagulation cascade, plasmin is the key enzyme in the fibrinolytic system. Plasmin results from the two-step activation of plasminogen, upon the action of plasmin and plasminogen activators (tissue type plasminogen activator and urokinase type plasminogen activator). The activators cleave the Arg^{561}-Val^{562} bond which on the plasminogen molecule converts it Glu-plasmin (a less active form of plasmin) and on the Lys-plasminogen molecule converts it to fully active plasmin. Plasmin cleaves the Lys^{77}-Lys^{78} bond, which on the plasminogen molecule

converts it to Lys-plasminogen and on Glu-plasmin converts it to fully active plasmin. Plasmin then acts on both fibrin and fibrinogen to produce degradation products, which is the mechanism by which plasmin mediates clot dissolution (Bloom et al. 1994). Genetic variations in the plasminogen molecule are present in the normal population and they are not associated with pathologic states. However, mutation resulting in dysfunctional plasminogen, associated with thromboembolic complications, have been isolated in Japanese populations and in one European Jewish patient (Robbins 1992).

1. Tissue Type Plasminogen Activator (tPA)

tPA is a serine protease of the fibrinolytic system (Pennica et al. 1983). tPA is a relatively insignificant enzyme when not bound to fibrin, but after binding to fibrin it is able to activate plasminogen to plasmin (Hoylaerts et al. 1982). Plasmin (also a serine protease) then can degrade fibrin, thus exposing new tPA binding sites on the underlying fibrin (Higgins et al. 1987). In addition to binding to fibrin, tPA also binds to receptors on endothelial cells as does plasminogen which may provide focal points for plasmin generation. An association between the contact system and the stimulation of the fibrinolytic system is made at this point: bradykinin, resulting from the action of kallikrein on HMWK, directly stimulates the release of tPA from endothelial cells (Tranquille et al. 1989), so that when intrinsic activation of the coagulation cascade occurs, the fibrinolytic system is also activated. Furthermore, thrombin is the most potent inducer of secretion of tissue-type plasminogen activator (tPA) from endothelial cells (Mosher 1990).

Although no reports on mutant tPA production have been made, reduced tPA

production or decreased release of tPA may contribute to thrombosis in about 10-20% of patients with deep vein thrombosis who have an inadequate fibrinolytic response (Juhan-Vague et al. 1987). However, there is little evidence to support this hypothesis and the current thinking is that reduction of available tPA is mostly due to increased complexation with plasminogen activator inhibitors, secondary to increase in plasminogen activator inhibitors.

m. Urokinase Type Plasminogen Activator (uPA)

Single-chain urokinase type plasminogen activator (sc-uPA) acts on plasminogen to convert it to plasmin Lijnen et al, 1987). Plasmin then converts sc-uPA to a two chain derivative (tc-uPA, Lijnen et al. 1987). This tc-uPA further cleaves plasminogen to plasmin. The difference between tc-uPA and sc-uPA is that the former accelerates the conversion of plasminogen to plasmin without being specific towards fibrin while sc-uPA is specific towards fibrin (Lijnen et al. 1986). An additional association between the contact system and the fibrinolytic system is made at this level: kallikrein can directly mediate the conversion of sc-uPA to tc-UPA, so that intrinsic activation of coagulation also precipitates fibrinolytic activation (Bloom et al. 1994).

3. Regulation of Serine Proteases by Serpins

Blood coagulation is controlled by many physiologic inhibitors, called serpins (serine protease inhibitors). Among these are protein C and protein S, antithrombin (antithrombin-III), Heparin Cofactor II, C_1inactivator (C_1esterase inhibitor), $\alpha_2$plasmin inhibitor ($\alpha_2$antiplasmin), $\alpha_1$proteinase inhibitor ($\alpha_1$antitrypsin) and $\alpha_2$macroglobulin.

Some of the characteristics of these inhibitors are listed in Table 2. Acquired and congenital deficiency of these factors predisposes to thrombotic or bleeding diseases.

a. Antithrombin

Thrombin is inactivated by a class of serpins called antithrombins. The most significant of these inhibitors is antithrombin (also referred to as antithrombin III) which inactivates heparin bound thrombin faster than free thrombin (Pomerantz and Owen 1978, Griffith 1982, Nesheim 1983). The activity of antithrombin is catalyzed by the presence of heparin which acts as a cofactor by binding to antithrombin to form a non-covalent ternary complex. Additional actions of antithrombin include inhibition of factors IXa, Xa, XIa, XIIa, kallikrein and plasmin.

Deficiency in antithrombin, congenital heterozygous or acquired, leads to thrombotic episodes when the hemostatic system is stressed. Hereditary deficiencies of antithrombin include quantitative and qualitative abnormalities and their classification is controversial at present, although many of them have been molecularly analyzed and over 50 point mutations have been reported (Lane et al. 1993). Treatment of patients with thromboembolic disorders due to deficiency in antithrombin consists of replacing the levels with antithrombin concentrates and heparin.

b. Heparin Cofactor II

Thrombin is also inactivated by heparin cofactor II (HC-II). HC-II is present in plasma at concentrations approximately half of antithrombin. HC-II has a similar mechanism of action to that of antithrombin in that its action is catalyzed in the presence

Table 2 -- Characteristics of Inhibitors of Procoagulant and Pro-fibrinolytic Proteins

	MW	Plasma Level	Natural Targets
Antithrombin	58,000	2.6 μM	Thrombin, factors IXa, Xa, XIa and XIIa, kallikrein, plasmin
HC-II	66,000	0.09 μM	Thrombin
TFPI	33,000	3.5 nM	Factor VIIa/TF and factor Xa
APC	62,000	0.08 μM	Factor Va and VIIIa, PAI-1 (enzyme commission # 3.4.21.69)
Factor V	330,000	0.03 μM	cofactor for APC
Protein S	75,000	0.33 μM	cofactor for APC, factor Va, factor Xa
$\alpha_2$plasmin inhibitor	65,000	0.6-1.2 μM	Plasmin, thrombin, kallikrein, tPA, uPA, trypsin, chymotrypsin
PAI-1	52,000	0.4 μM	APC, tPA, uPA
PAI-2	47,000	$<$0.001 μM	uPA, tPA, plasmin
PCI	57,000	0.06-0.12 μM	Thrombin, factors Xa and XIa, kallikrein, trypsin, tPA, uPA, APC
PN-1	50,000	0	Thrombin, factor Xa, trypsin, kallikrein, tPA, uPA, plasmin
$\alpha_2$macroglobulin	845,000	22-50 μM	Thrombin, factor Xa, kallikrein, plasmin, tPA, trypsin, elastase, collagenase, cathepsin C, APC
$\alpha_1$proteinase inhibitor	104,000	13-31 μM	Thrombin, kallikrein, factors Xa, XIa, trypsin, plasmin, elastase, cathepsin G, APC
C_1inactivator	725,000	0.25 μM	Factor XIa, complement system

Loscalzo and Schafer 1994, Bloom et al. 1994, High and Roberts 1995.2

of heparin (Blinder et al. 1988). The physiologic role of antithrombin and HC-II differ in that HC-II is a specific inhibitor of thrombin without reacting with other serine proteases, while in contrast to antithrombin, it can slowly inactivate chymotrypsin-like proteases (Parker et al. 1985, Church et al. 1985). Only two deficiencies of this cofactor have been molecularly analyzed, HCII Oslo (Blinder et al. 1989) which is asymptomatic and HCII Awaji (Kondo et al. 1996) which is associated with coronary artery disease (Matsuo et al. 1992).

c. Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) , also known as extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI), is present in plasma in different molecular forms which are primarily determined by its association with circulating lipoproteins. TFPI is a Kunitz-type protease inhibitor (Wun et al. 1988, Girard et al. 1989). One domain of this protease binds to the factor VIIa/TF complex while another binds to factor Xa, thus blocking both the extrinsic pathway leading to thrombin formation and the amplification of the same pathway by factor Xa (Broze et al. 1988, Hjort et al. 1957). Therefore, while antithrombin controls the final stage of coagulation, TFPI regulates the activation of coagulation (Abildgaard 1995). TFPI is present in the plasma at ng/ml concentrations (Warr et al, 1989). The vascular endothelium secretes TFPI in culture (Broze et al. 1987, Warr et al. 1989) but the stimuli that lead to release or increase in plasma TFPI are not well defined and neither is their mechanism of action. It is known that intravenous and subcutaneous heparin as well as low molecular weight heparins, acidic glycosaminoglycans and their synthetic analogues,

cause the release of TFPI bound to the luminal surface of the vascular endothelium (Sandset et al. 1988, Fareed et al. 1994, Hoppensteadt et al. 1995, Samama et al. 1994, Jeske et al. 1995). Recently, there has been a proposal of a heparin-recognition site on the TFPI molecule, but further studies are needed to support this model (Harenberg et al. 1995).

d. Protein C

Protein C and protein S are vitamin K dependent proteins produced in the liver. Protein C is slowly activated by free thrombin to activated protein C (APC), but when thrombin is bound to its endothelial receptor, thrombomodulin, the activation of protein C is catalyzed (Esmon et al. 1989). The function of APC is to inactivate factors Va and VIIIa (the two cofactors of the coagulation system) via proteolysis. Factor Va bound to factor Xa is protected from protein Ca inactivation, as is factor VIIIa bound to factor IXa (Walker et al. 1987, Bertina et al. 1984, Rick et al. 1988). However, when factor Va is bound to the endothelial surface it catalyzes the activation of protein C (Maruyama et al. 1984).

Another function of APC is to complex with plasminogen activator inhibitor 3 (PAI-3), also referred to as protein C inhibitor (PCI), which renders both proteins inactive (De Fouw et al. 1987). While PAI-3 is the major APC inhibitor, plasminogen activator inhibitor 1 (PAI-1) also inhibits APC. Other inactivators of APC include $\alpha_2$macroglobulin and $\alpha_1$proteinase inhibitor ($\alpha_1$antitrypsin).

Acquired and congenital deficiencies of APC have been reported to cause thrombotic syndromes such as DVT, microangiopathy and necrosis due to a deficit of

protein C or its production (Griffin et al. 1981, Broekmans et al. 1983). At least 55 different mutations of protein C have been discovered and they are transmitted as autosomal dominant traits (Gandrille et al. 1995). These mutations result in variable degrees of plasma protein C levels and the individuals with these mutations may be asymptomatic or they may develop thrombotic episodes such as purpura fulminans, skin necrosis and disseminated intravascular coagulation (Gandrille et al. 1995).

e. Protein S

APC is potentiated by protein S, which is a cofactor synthesized and secreted in the liver and endothelial cells. In addition, protein S is capable of independently inactivating factor Va (Heeb et al. 1993) and factor VIIIa (Heeb et al. 1994). Its activity is regulated by the C4b-binding protein, which forms an inactive complex with proteins S. Familial protein S deficiency is associated with recurrent venous thrombosis (Comp and Esmon, 1984, Schwartz et al. 1984) and with arterial thrombosis (Girolami et al. 1989, Allaart et al. 1990).

f. Factor V

Another essential cofactor for the activity of APC is factor V in its native proenzyme form. Thus, while the active form of factor V serves as a cofactor for factor Xa to mediate procoagulant activities, factor V is a cofactor for APC that mediates the inactivation of factor Va (and VIIIa). More recently, a new syndrome known as APC resistance has been identified and is claimed to be the most common cause of inherited venous thrombosis (Svensson and Dahlback 1994). Most of the individuals exhibiting

APC resistance possess a point mutation of factor V represented by a substitution of Arg^{506} with Gln (Factor V Leiden). In contrast to the normal factor V molecule, the mutant form does not retain its regulatory function as a cofactor in the mediation of APC's effects in the inactivation of factor Va and factor VIIIa. In addition, the mutant form of factor V is activatable by thrombin to factor Va and it retains its procoagulant activity as a cofactor for factor Xa. Furthermore, the mutant form of factor Va is non-susceptible to the digestive action of APC. Another recently characterized factor V mutation resulting in factor V deficiency has been characterized, in which a missense mutation of Ala^{221}-to-Val occurs (Factor VNew Brunswick, Murray et al. 1995).

g. α_2Plasmin Inhibitor

Free plasmin is rapidly inactivated by $\alpha_2$plasmin inhibitor (or $\alpha_2$antiplasmin), another serpin (Collen et al. 1986, Wiman et al. 1978). When plasmin is bound to fibrin, the binding sites for $\alpha_2$plasmin inhibitor on plasmin are occupied so that inactivation is slow (Wiman et al. 1978). $\alpha_2$plasmin inhibitor also inhibits tPA. Aprotinin is a synthetic inactivator of plasmin, approved in 1995 by the U.S. FDA for clinical usage during cardiac bypass surgery.

Additional plasmin inhibitors include plasminogen activator inhibitor 1, protease nexin 1, $\alpha_2$macroglobulin, $\alpha_1$proteinase inhibitor and C_1inactivator.

h. Plasminogen Activator Inhibitors

Plasminogen activator inhibitor 1 (PAI-1) is the primary inhibitor of tPA and uPA in plasma (Lawrence et al. 1989, Pannekoek et al. 1986, Kruithof et al. 1984). In

addition, PAI-1 inhibits plasmin, thrombin and APC. The major source of PAI-1 is vascular smooth muscle cells and it is also released from activated platelet α granules. In endotoxemia and other pathological disorders endothelial cells are the major site of PAI-1 synthesis.

Plasminogen activator inhibitor 2 (PAI-2), or placental PAI, is produced by the placenta and is significantly increased during pregnancy. PAI-2 inhibits tc-uPA and tPA, but the physiological function of this inhibitor is unknown.

Plasminogen activator inhibitor 3 (PAI-3) or protein C inhibitor (PCI), is synthesized in the liver and inactivates several serine proteases including APC, kallikrein, thrombin, factors Xa and XIa, uPA and tPA. PCI is the major inhibitor of APC.

C. Thrombin: Structure, Function, Physiologic, Pathologic and Pharmacologic Mechanisms

1. Biology of Thrombin Formation and Structure of the Thrombin Molecule

Negatively charged phospholipids such as on the surface of activated platelets promote factor activation leading to thrombin formation. More specifically, thrombin is formed by the proteolytic cleavage of prothrombin by the prothrombinase complex, which is the complex of factor Xa, Va, Ca^{++} and phospholipids, that cleaves prothrombin twice (Suttie et al. 1977). It is the amino terminal prothrombin fragment that results in active α thrombin (Magnusson et al. 1975) and in contrast to most activated serine proteases, thrombin loses most of its activation peptide which forms the small A chain of the molecule (Dodt et al. 1990). A diagrammatic representation of the tertiary conformation of the thrombin molecule (Fenton et al. 1988) is depicted in Fig. 2.

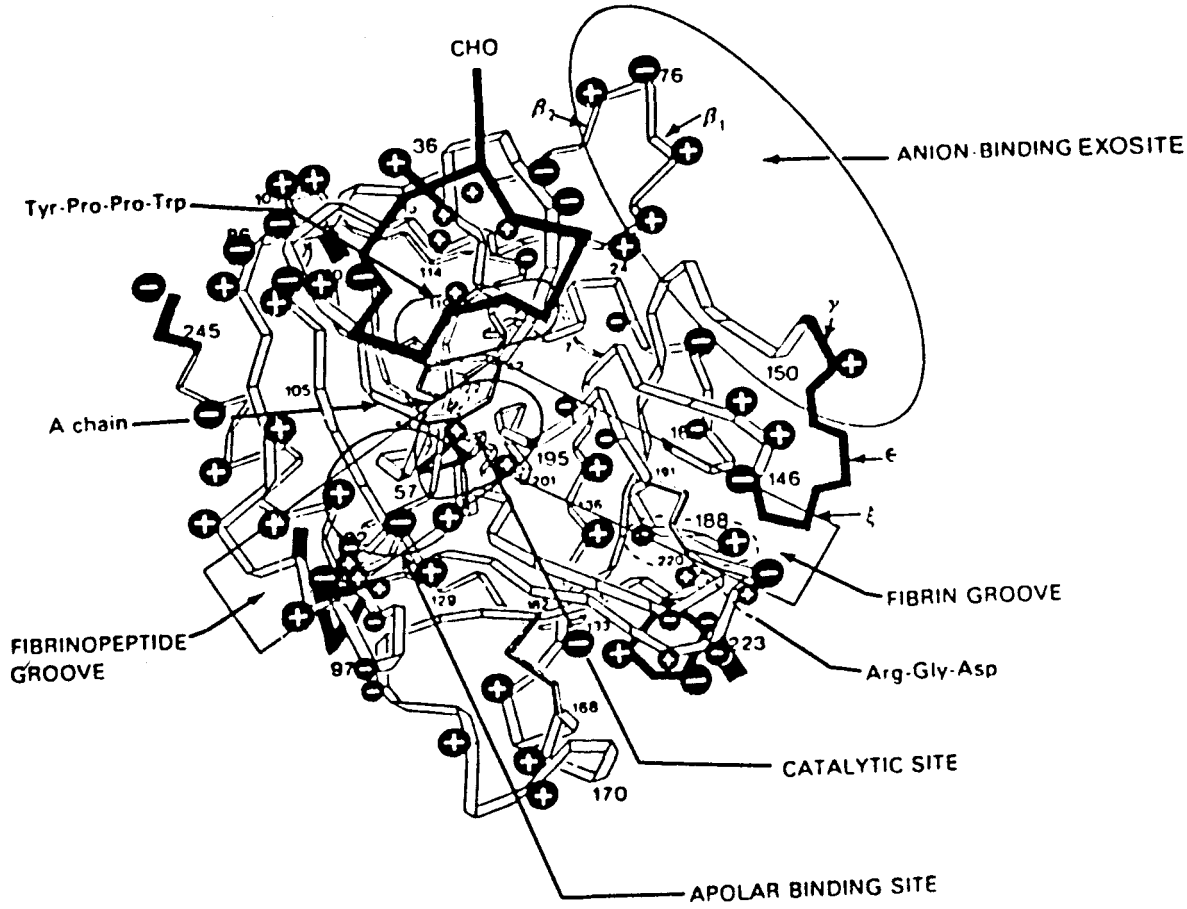

Fig. 2. Three dimensional model of human α-thrombin (Fenton 1988). Among other important structural features, the proteolytic cleavage sites ß and γ (autoproteolytic digestion or trypsin), ϵ (elastase), ξ (chymotrypsin or cathepsin G) are depicted near the anion-binding site.

α thrombin is different from the other serine proteases in its mode of proteolytic action: this protein possesses three important exosites adjacent to its catalytic site which serve to align protein substrates and inhibitors optimally. The human thrombin molecule is composed of two chains, A (36 residues) and B (259 residues), linked together by a single disulfide bridge. The function of the A chain remains unknown although it may be implicated in conformational stability of the protein. From crystallographic (Bode et al. 1989) and computer-generated models (Fenton et al. 1986, Bing et al. 1981, Furie et al. 1982) of the thrombin molecule, the structure and nature of the molecule has been characterized. The B chain of thrombin assumes a round conformation with a substrate groove resembling a deep narrow canyon. The catalytic site of thrombin, composed of the catalytic triad His-57, Asp-102, Ser-195, lies in the center of the global mass, in the groove. When fibrinogen fits into this groove and it is cleaved at the catalytic site of thrombin, the right half of the groove is occupied by fibrin (fibrin groove, carboxy terminus of fibrinogen) while the left half is occupied by the fibrinopeptide (fibrinopeptide groove, amino terminus of fibrinogen). The interior of the groove is composed of apolar residues and lined on the exterior with charged groups (Fenton 1986). The region above the catalytic site is also very hydrophobic. The site of fibrinopeptide groove next to the catalytic site is notably hydrophobic and forms the apolar binding site of thrombin (Berliner et al. 1977, Sonder et al. 1984). On the opposite site of the catalytic site across from the apolar binding site is the anion binding exosite I, which is composed of a long peptide segment rich in arginines and lysines (Fenton et al. 1986). This cluster of positively charged residues is involved in fibrinogen

recognition and hirudin complexation (Fenton et al. 1988). α thrombin possesses another anion binding exosite II which seems to play a synergistic role with the anion binding exosite I in the binding of heparin (Church et al. 1989). This second anion binding exosite is located above the catalytic groove. Thrombin also has a single carbohydrate attachment for carboxylation but no known function has been yet attributed to this moiety (Horne et al. 1984). Another domain of ambiguous importance is the Arg-Gly-Asp sequence which may be involved in thrombin receptor recognition under certain circumstances.

Differences between the bovine and the human thrombin molecule are distinct in structure and lead to functional differences. The structural differences were delineated with electron spin resonance and fluorescence studies (Nienaber and Berliner, 1991). These studies revealed that the active site region of the bovine molecule is more apolar but more accessible. Furthermore, a hydrophobic insertion loop penetrates and thus obstructs the human thrombin active site. This is not observed with the bovine molecule and this difference accounts for the inability of aprotinin to inhibit the human form but not the bovine variant. Deletion of this insertion loop in the human thrombin resulted in the mutant des-PPW thrombin, which remains a specific serine protease but is sensitive to aprotinin, indicating that other structural factors are also involved in the narrow specificity of thrombin (Le Bonniec et al. 1993). The apolar binding site on the human and bovine α thrombin molecules also differs, as does the γ cleavage site that converts human α thrombin to γ thrombin (Nienaber and Berliner, 1991). In the bovine α thrombin, the γ cleavage site does not exist and therefore γ bovine thrombin cannot be formed (Fenton and Bing, 1986).

2. Molecular Variants of Thrombin

Human α thrombin degrades either auto-proteolytically (slowly) or via the action of trypsin (rapidly) to ß and then to γ thrombin with loss of its clotting activity (Fenton et al. 1977, a and b). The cleavage sites for formation of both thrombins lie in the anion binding exosite I (fibrinogen recognition site, Fenton 1986, Fenton 1988). Since trypsin does not normally circulate in the bloodstream and since autoproteolytic cleavage is slow, these forms of thrombin (ß and γ) are not found *in vivo* (Fenton and Bing, 1986). Since bovine thrombin lacks the γ cleavage site, only ß bovine thrombin can be formed (Fenton and Bing, 1986). Leukocyte elastase and cathepsin G cleave human α thrombin at sites before the γ site to yield ϵ and ζ thrombin respectively (Brower et al. 1987, Brezniak et al. 1990). The elastase-mediated conversion is enhanced by heparin and occurs at physiologically relevant concentrations, so that ϵ form may occur *in vivo* to mediate non-clotting thrombin activities (Fenton and Bing, 1986). ζ thrombin retains about 85% of the α thrombin clotting activity (Brezniak et al. 1990). Except for ζ thrombin, the previous forms that result from the α form after proteolytic cleavage, have no clotting activity but retain high amidolytic and esterolytic activities (Fenton and Bing, 1986).

3. Thrombin Standardization

Highly purified preparations of human α thrombin are prepared by the same procedure (Fenton et al. 1977) and supplied from the same source around the world for standardization purposes. However, the first standardization of human thrombin was attempted in 1942 (Seegers and Smith, 1942), with the definition of the Iowa Unit as the amount of thrombin that clots 1 ml of standardized fibrinogen solution (bovine plasma

based) in 15 sec at 28°C. The Division of Biologics Control, National Institutes of Health, U.S. Public Health Service issued specifications for commercial thrombin preparations in 1946, attempting to define the NIH unit equal to the Iowa unit. The two units are not equal and 1 NIH unit equals about 1.25 Iowa units. Both of these units are based on the clotting activity of thrombin. The NIH standard thrombin is also referred to as the US standard. In 1975, the Expert Committee on Biological Standardization of the World Health Organisation established an International Standard for thrombin, which is used to define the thrombin International Unit (IU, Gaffney and Edgell, 1995). Another International Standard for thrombin was established in 1991. Although the IU was originally defined to approximate the NIH unit, 1 NIH unit equals 1.15 IU (Gaffney and Edgell, 1995). Currently, both units are used in the literature and the reagent and pharmaceutical grade products are standardized in terms of NIH units.

4. Physiologic Regulation of Thrombin Formation

Thrombin has a unique mechanism of regulation of its generation from prothrombin. As mentioned above, thrombin is generated from prothrombin by the double proteolytic activity of factor Xa in the prothrombinase complex. The activated thrombin can then activate factors V and VIII to amplify its generation to a burst of coagulant activity (Mosesson et al. 1990 a, b). These steps are the targets of the natural antithrombins (antithrombin and HC-II). Thrombin down-regulates its own activity by binding to thrombomodulin, which results in loss of thrombin's ability to convert fibrinogen to fibrin. Furthermore, thrombomodulin bound thrombin activates protein C, which then can degrade factors V and VIII with protein S as a cofactor, thus blocking

the generation of thrombin (Esmon et al. 1987).

5. Cellular Interactions of Thrombin

In addition to proteolytic activities, thrombin exhibits several non-enzymatic or hormonal activities. Thrombin activates platelets and other factors to promote hemostasis. In vitro, thrombin addition to platelets causes phosphatidyl inositol (PI) hydrolysis, eicosanoid formation, protein phosphorylation, increase in cytosolic free Ca^{++}, change in their shape, granule secretion and fibrinogen receptor expression. Thrombin also suppresses cAMP synthesis and has other effects as well, such as on endothelial cells (stimulation of PGI_2 formation, proliferation, angiogenesis, neovascularization), fibroblasts (production of cAMP, proliferation, chemotaxis), and vascular smooth muscle cells, all of which may be implicated in platelet activation, for example PGI_2 inhibits platelet activation by stimulating cAMP synthesis. Other hormonal activities of thrombin include monocyte (Bar-Shavit et al. 1983) and neutrophil chemotaxis and aggregation (Bizios et al. 1984), mitogenesis in certain macrophage-like cells (Bar-Shavit et al. 1983), albumin transport across endothelial cell monolayers (Malik et al. 1986) and inhibition of neurite outgrowth (Gurwitz et al. 1988, Hawkins et al. 1986).

6. Thrombin Receptors

Thrombin activates a variety of receptors. It interacts with a specific high affinity receptor on platelets to cleave the amino terminus thus exposing a tethered ligand which activates the receptor (Vu et al. 1991). This receptor is linked to an inhibitory G protein

and activation of it results to inhibition of adenylate cyclase and decrease in cAMP (Seiler et al. 1992). The same receptor also regulates the Na^+/H^+ exchange in platelets during stimulation with thrombin. This receptor is thought to be linked through another G protein to phospholipase A_2 which may be the main source of arachidonate production (Siess et al 1984, Silk et al. 1989, Kajiyama et al. 1989). Thrombin also interacts with a lower affinity receptor on the platelets, but this one is thought to be linked through a G protein to phospholipase C and protein kinase C (McGowan et al. 1986). Activation of this receptor could be the source of the thrombin-induced elevations in cytoplasmic Ca^{++} levels.

Cultured fibroblasts also have a high affinity thrombin receptor, activation of which leads to increase in cAMP (Carney et al. 1978, 1984, 1992), which is identical to the high affinity receptor found on platelets. The same thrombin receptor is also expressed by endothelial cells, smooth muscle cells and lymphocytes (Brass 1995). Thrombomodulin is the thrombin specific receptor found on endothelial cells (Awbrey et al. 1979). This receptor recognizes the fibrinogen recognition site on thrombin which is independent of its catalytic site (Hofsteenge et al. 1986). There appear to be over 1 million low affinity receptors with a k_d of about 30 nM, on the endothelial cells (Isaacs et al. 1981, Bauer et al. 1983).

D. Pharmacologic Control of Thrombin's Action.

Understanding the mechanisms through which thrombin mediates its various activities, as well as knowing the structural requirements for these actions, provides the basis of the strategies for the modulation of thrombin's action. Thrombin mediated

effects can thus be controlled at four different levels: (1) blockade of the thrombin receptors, (2) physiological antagonism of the effects mediated by thrombin, (3) inhibition of the generation of thrombin and (4) direct inhibition of formed thrombin.

1. Inhibition of Thrombin Receptors

Thrombin has multiple receptors in a variety of tissues. Some of these receptors have been characterized and studied extensively, but most remain insufficiently characterized. The obvious thrombin receptor targets is the newly cloned thrombin receptor found on platelets and endothelial cells (Vu et al, 1991). Effective antagonists of the cloned thrombin receptor are currently under investigation. Aplysillin A (1,4-D-Phe-1,3-butadiene disulfate ester) is a compound isolated from a deep water sponge, with a reported IC_{50} for binding to the thrombin receptor of 20 μM (Gulavita et al. 1995). Another thrombin receptor inhibitor, C186-65 (3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-amide), has been recently reported to be effective in inhibiting vascular smooth muscle relaxation and platelet aggregation, phospolipase A2 and the Na^+/H^+ exchange, all mediated through the thrombin receptor (Tesfamariam 1994, Seilet et al. 1995). A phage selection technique has also resulted in the isolation of a thrombin receptor inhibitor that was effective in antagonizing platelet aggregation, serotonin release and tyrosine phosphorylation (Doorbar and Winter 1994). The clinical potential of these thrombin receptor inhibitors is unknown.

2. Inhibition of Cellular Processes Mediated by Thrombin

Targeting of the cellular processes mediated by thrombin provide an alternative

mode of pharmacological control of the actions of thrombin. This approach is complicated by the fact that thrombin mediates a spectrum of cellular processes ranging from platelet activation to stimulation of proliferation of various cell types. An agent that has such effects and is being used clinically is defibrotide. The exact modes of action and the full pharmacologic potential of this agent is currently being explored (Ulutin et al. 1988). This agent is different from the usual antithrombotic and anticoagulants in that it is a functional antagonist of thrombogenesis and not necessarily a direct antithrombin. This approach of pharmacological management of thrombin becomes even more complicated when considering the effects of thrombin on platelets and whether thrombin is the main activator of platelets or other activators play an equally important role. If thrombin is indeed the most important activator of platelets as suggested by Badimon et al. (1990), then single thrombin targeting would be an efficient method for controlling arterial thrombosis (in which platelet activation is the key event). However, this has not been proven and management of platelet activation with antiplatelet agents such as cyclooxygenase inhibitors are still widely used with success. On the other hand, Cadroy et al. (1989) have indicated with their work that thrombus formation is a complex situation where inhibition of platelets and coagulation pathways may be required for optimal management of thrombosis.

3. Inhibition of Thrombin Generation

Inhibition of the formation of thrombin may prove to be more effective in prevention and management of thrombotic disorders than direct inhibition of thrombin after it has formed. The issue of whether monospecific targeting of thrombin is the most

effective method for controlling thrombogenesis is a controversial one, and inhibition of other proteases contributing to the generation of thrombin is under study. By inhibiting the formation of thrombin, the hormone-like effects that it mediates may also be prevented and complications may be avoided. In inhibiting the formation of thrombin, the target sites should be the serine proteases that lead to this event. The candidates for this type of targeting would be factor VIIa and/or TF and factors IXa, VIIIa, Xa and Va.

Considerable interest has been shown in the role of TFPI and in animal models of DIC it has been found to be effective (Rapaport et al. 1985, 1989), but the clinical usefulness of this recombinantly produced agent is not established. Recently, a report of selective factor VIIa/TF inhibitors, derived from mutagenesis of the inhibitor of the Alzheimer's amyloid ß-protein precursor, demonstrated that one of these proteins is specific for factor VIIa/TF or factor VIIa, with no activity against thrombin, factors Xa and XIa and kallikrein, but with some activity against plasmin (Lazarus et al. 1995). These inhibitors exhibit promising anticoagulant effects, but their *in vivo* effects have not been published (Refino et al. 1995, Kirchhofer et al. 1995). The same group has used alanine-scan mutagenesis to develop a specific factor VIIa inhibitor that resembles TF. This inhibitor inhibits selectively the extrinsic pathway (Kelley et al. 1995) but *in vivo* studies are not complete. An anti-rabbit TF antibody has been recently developed (Himber et al. 1996) and was found to produce stringer *in vivo* antithrombotic effects than heparin in a rabbit model of arterial thrombosis.

The same group that isolated the factor VIIa/TF inhibitors based on the inhibitor of the Alzheimer's amyloid ß-protein precursor, also isolated during these studies an

inhibitor of kallikrein, which also has weaker activities against factor XIa (Lazarus et al. 1995). This compound is also comparable to the factor VIIa/TF inhibitors in terms of anticoagulant effects (Refino et al. 1995, Kirchhofer et al. 1995).

Although APC has been synthesized by recombinant technology, only the purified product from human plasma has been used clinically in individual case studies of patients with inherited protein C deficiency (Alhenc-Gelas et al. 1995, De Stefano et al. 1993) or patients with purpura fulminans in meningococcemia (Rivad et al. 1995). An alternative approach resulting in activation of endogenous protein C has been reported (Gibbs et al. 1995): a thrombin mutant (E229K) has been developed which has lost its fibrinogen-cleaving ability but has retained its thrombomodulin-dependent activating capability for protein C. This compound produces dose-dependent and reversible anticoagulant effects in Cynomolgus monkeys. Based on the same principle of developing a thrombin mutant which lacks its procoagulant activity while retaining its protein C activating capabilities, a series of thrombin mutant molecules have been constructed where the insertion loop that obstructs the catalytic site and the Na^+ binding site on thrombin have been mutated (Guinto et al. 1995). The *in vivo* effects of these mutants remain to be examined.

Focusing on the role of APC in the coagulation system, another approach reported recently was the design of a protein C mutant which can be activated by factor Xa, instead of thrombin (Friedrich et al. 1996). This factor Xa activatable protein C mutant was found to produce *in vitro* anticoagulant effects but the potential of this agent is unclear at this time.

Beside the direct inhibition of thrombin, an additional approach to develop new antithrombotic agents is the inhibition of factor Xa. It is known for some time that low molecular weight high antithrombin affinity heparin fractions, upon complexing with antithrombin, produce selective inhibition of factor Xa (Barrowcliffe et al. 1986). Based on this, a synthetic heparin pentasaccharide has been developed. This pentasaccharide represents an indirect factor Xa inhibitor requiring the presence of plasmatic antithrombin for its biological effects. Many different structural analogues of this pentasaccharide with varying anti-Xa potency have been developed. This pentasaccharide is currently in phase II clinical trials for the post hip replacement prophylaxis (personal communication). It is also interesting to note that pentasaccharide also inhibits thrombi generation and almost has no effects on formed thrombin (Lormeau and Herault 1993).

The direct factor Xa inhibitors include both the recombinant and synthetic agents. Recombinant tick anticoagulant peptide (TAP) represents an anticoagulant principle which was first isolated from the soft tick *Ornithodoros moubata*. This 60 amino acid containing peptide exhibits reversible tight binding to factor Xa (Vlasuk et al. 1991). It has also been shown to exhibit varying degrees of antithrombotic actions in different animal models. Despite its safety and low immunogenicity, this agent is not developed clinically until now.

Another recombinant anti-Xa agent is antistatin, which represents a 15,000 peptide isolated from the mexican leech *Hirudo officinalis* (Dunwiddie et al. 1989). This peptide is found to be active in various animal models of thrombosis (Schaffer et al 1991). However, in contrast to TAP, it is strongly immunogenic and thus, its development is

carried out only in preclinical stages.

Novel factor Xa inhibitors from the saliva of the medicinal leech *Hirudo medicinalis* have also been reported (Rigbi et al. 1995). These inhibitors share 50% homology and have similar patterns of inhibition, which include in addition to factor Xa, chymotrypsin and trypsin. However, apart from these enzymes, these inhibitors are selective for factor Xa.

The synthetic anti-Xa agents include both the peptidic and peptidomimetic agents. A series of orally active highly specific factor Xa inhibitors have been reported (Ostrem et al. 1995). These include the series of compounds referred to as SEL followed by a number which inhibit the free and prothrombinase factor Xa. These compounds are of a low molecular weight (550-750). One of these agents has been found to be a selective factor Xa inhibitor with lesser activities towards thrombin, plasmin and APC. While the antithrombotic effects and the pharmacokinetics of this agent are species-dependent, this agent exhibits oral and S.C. bioavailability.

Another peptidomimetic factor Xa inhibitor, DX 9065A, is a bis-amidino derivative with relatively specific anti-Xa activities (Hara et al. 1994, Yamazaki et al. 1994). After I.V. and oral administration, this compound prolonged the global clotting assays and did not exhibit any antithrombin activity. In animal models of thrombosis and DIC, this agent produced dose-dependent antithrombotic effects.

In contrast to thrombin inhibitors, the factor Xa inhibitors are claimed to exhibit much lesser bleeding effects. Furthermore, these agents are also reported to be superior inhibitors of thrombin generation (Prasa et al. 1996).

A novel broad-spectrum serine protease inhibitor has been isolated from *Eschericia coli*, ecotin (Lauwereys et al. 1993). Notably, the inhibitory constant of this agent for factor Xa is in the pM range. While ecotin inhibits chymotrypsin, elastase, kallikrein, factor XIIa and XIa, it has no activities against APC, tPA and uPA. This compound has been shown to be an effective dose-dependent antithrombotic agent in a rat model of chemically induced arterial thrombosis.

Aprotinin is a broad-spectrum protease inhibitor that has been approved by the U.S. FDA for clinical use as an anti-fibrinolytic drug during cardiac surgery. The primary structure of aprotinin is depicted in Fig. 3, lower panel. Based on the structure of aprotinin, a series of mutants have been synthesized that have inhibitory activities against coagulation enzymes in the nM range (Stassen et al. 1995). One of these mutants was coupled to PEG to optimize biological half life, without affecting its inhibitory activities. These aprotinin mutants were shown to inhibit factors XIa, Xa and TF/VIIa, as well as plasmin, trypsin and kallikrein. However, they possessed no detectable activity against thrombin, uPA and APC.

4. Inhibition of Formed Thrombin

The most direct method of controlling the actions mediated by thrombin is by inhibiting this enzyme directly. The thrombin inhibitors described earlier above are all designed or selected for this direct approach in controlling thrombin. Understanding the structure-activity relationship of thrombin is very important in this approach since inhibitors of different epitopes of the thrombin molecule result in differential management of thrombin's spectrum of activity (eg. although the anion-binding exosite of thrombin

```
        1                                   10
HV-1  Val-Val-Tyr-Thr-Asp-Cys-Thr-Glu-Ser-Gly-Gln-Asn-Leu-Cys-Leu-Cys-Glu-Gly-
HV-2  Ile-Thr
HV-3  Ile-Thr

            20                                  30
HV-1  Ser-Asn-Val-Cys-Gly-Gln-Gly-Asn-Lys-Cys-Ile-Leu-Gly-Ser-Asp-Gly-Glu-Lys-
HV-2                      Lys                                 Asn     Lys-Gly
HV-3                      Lys                                 Gln     Lys-Asp

                  40                                  50
HV-1  Asn-Gln-Cys-Val-Thr-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-Asp-Gly-
HV-2                                        Asn     Glu             Asn
HV-3                                                                Gln
                        60          *
HV-1  Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-  -Tyr-Leu-Gln
HV-2
HV-3                Pro             Asp-Ala     Asp-Glu
```

```
            1                                   10
Aprotinin:  Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala

                      20                                          30
            Arg-Ile-Ile-Arg-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr

                                    40
            Gly-Gly-Cys-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys

            50
            Met-Arg-Thr-Cys-Gly-Gly-Ala
```

Fig. 3. Primary structures of natural hirudin variants 1, 2 and 3 (HV-1, Dodt et al. 1984, HV-2, Harvey et al. 1986 and HV-3, Dodt et al. 1986) and bovine aprotinin (Stassen et al. 1995).

* denotes sulfation, which is absent in the recombinant forms of these molecules.

may be blocked, its catalytic activity towards smaller substrates may not be prevented).

E. Thrombin Inhibitors

The development of substrate-related synthetic inhibitors of thrombin started with the discovery of the specificity of thrombin for hydrolysis of arginyl bonds. There is a broad spectrum of interests from which the thrombin inhibitors related work stems, including the identification of reactive groups in active site, demonstration of similarities and differences between binding areas of related enzymes, selective inhibition of one protease in the presence of another and *in vivo* anticoagulation. Physical methods such as NMR and x-ray crystallography for topography of the thrombin active site have also contributes substantially to these developments. Direct inhibitors of thrombin can be classified according to source and structure into endogenous, analogues of natural substrates, recombinant and synthetic inhibitors. Some of these are directed against the catalytic site of thrombin while others bind to the exosites of thrombin. In addition, some are reversible inhibitors while others are irreversible.

1. Inhibitors of Plasma Origin

The natural inhibitors of thrombin belong to a class of serpins called antithrombins. The most important ones are antithrombin and HC-II (which recognizes the anion binding site as well as the catalytic site of thrombin, Hortin et al. 1989). The mechanisms of actions of these is discussed previously. In addition to the above thrombin inhibitors, thrombin forms complexes and is inhibited by two other proteins secreted form platelets upon activation: platelet protease nexin and thrombospondin

(Detwiler et al. 1992). Platelet protease nexin is secreted from activated platelets and forms a 77,000 MW complex with thrombin through thrombin's anion binding exosite to inactivate it (Detwiler et al. 1992). Thrombospondin is a 420,000 MW glycoprotein secreted by activated platelets and binds to many cells and proteins (Lawler 1986) and is incorporated into polymerizing fibrin (Bale et al. 1985) and extracellular matrix (Jaffe et al. 1983). The nexin-thrombin complex is further complexed with thrombospondin via a disulfide bond (Detwiler et al. 1992).

2. Synthetic Inhibitors

Prior to the development of peptide-based inhibitors of thrombin, several non-peptidic inhibitors have been described (Geratz and Tidwell, 1977). These non-peptidic inhibitors of thrombin can be classified according to the extent of the domain they occupy (Geratz and Tidwell, 1977). There are three important binding sites in the catalytic domains of the arginine specific proteases (Schechter and Berger, 1967):

1) The specificity pocket to which the protonated arginine side chain of a substrate is attracted, located at the entrance of the enzyme's catalytic mechanism (the pocket consisting of His-57, Asp-102, Ser-195).

2) The area to left of the specificity pocket which accommodates the amino terminal leaving group of substrate (towards the apolar binding exosite or fibrinopeptide groove). This is the S1 site of the enzyme and corresponds to the P1 site of the substrate. The sites following the S1 and P1 sites, away from the catalytic site, are designated S2, S3... and P2, P3 on the enzyme and the substrate respectively.

3) The area to the right of the specificity pocket which accepts the carboxy terminus of

the substrate (towards the anion binding exosite or fibrin groove). This is the S1' site of the enzyme and corresponds to the P1' site of the substrate. The sites following the S1' and P1' sites, away from the catalytic site, are designated S2', S3'... and P2', P3' on the enzyme and substrate respectively.

The classification of the synthetic inhibitors can then fall into three categories (Geratz and Tidwell, 1977), according to the extent of the catalytic domain of thrombin that they occupy.

a. Compounds Restricted to the Specificity Pocket of the Catalytic Domain of Thrombin

Synthetic cationic compounds were used to decipher the spatial relationship of the specificity site of thrombin and establish an optimal structure for hydrophobic binding and ionic interactions with the negatively charged aspartate moiety at the bottom of the pocket (Geratz and Tidwell, 1977). Of the benzene analogues, the ones with an amidino side chain rather than an aminomethyl or a guanidino side chain, exhibited a stronger K_i (Geratz and Tidwell, 1977). This led to benzamidine incorporation as a building block in more complex thrombin inhibitors. Ring systems other than benzene, had an even tighter fit due to the improved hydrophobic interaction with the pocket (Geratz and Tidwell, 1977). Introduction of a pyruvic acid group in the para position to the amidino side chain of benzamidine led to an increase in inhibitory potency. The augmented inhibition extended to plasmin, trypsin and enterokinase (Geratz and Tidwell, 1977).

An alternative building block for these molecules was arginine and its modified forms. The D-configuration of arginine had little activity while chemical modifications on the guanidino part or the α-amino part of L-arginine decreased the antithrombin

activity (Tonomura et al. 1980).

b. Compounds Extending Unidirectionally Beyond the Specificity Pocket of the Catalytic Site of Thrombin

These compounds were molecularly designed by attaching a side chain to a starter compound (benzamidine mostly). The side chains were aliphatic, aromatic or aryl aliphatic and extended usually to the right od the specificity pocket. Only the agents resulting from the link-up of two benzamidine groupings were active (Geratz and Tidwell, 1977), more so than benzamidine alone, and they led to the study of factors affecting potency (length, nature and bulkiness of central chain, presence of various substituents, position of amidino groups). Diamidines proved effective and it was shown that the second amidino group is essential for full potency, especially for inhibition of kallikrein (Geratz and Tidwell, 1977).

Based on the work done with arginine as a building block, C-terminal modifications of L-arginine were synthesized and studied. Ester compounds were easily hydrolyzed by thrombin and other trypsin-like enzymes, so non-hydrolyzable tertiary amides that were found to combine only with thrombin were focused on (Okamoto et al. 1980, Kikumoto et al. 1980, Tonomura et al. 1980).

Modification on the N-terminal of L-arginine were also studied (Okamoto et al. 1980, Kikumoto et al. 1980). Dansyl group incorporation as well as other bulky aromatic substituents to the arginine increased the antithrombin activity of the arginine.

c. Compounds Extending Bidirectionally Beyond the Specificity Pocket of the Catalytic Domain of Thrombin

Since the most efficient natural substrates of thrombin bind to thrombin in the binding groove on both sides of specificity pocket, the idea of furnishing diamidines with a side chain that provides additional interaction with the thrombin groove led to the alteration of the location of the amidino groups, substitution of the halogen and replacement of one amidino moiety by a nitro group. In general, amidines exhibited inhibitory effects against any arginyl or lysyl specific protease, but had a preference for one protease over others. The degree of differential inhibition reflected the topographical variations in active sites of related enzymes.

An alternative building block used to develop small inhibitors for the catalytic site of thrombin is amidinopiperidine. Modifications of this molecule yielded competitive inhibitors of thrombin with Ki's of 20-50 nM (Hilpert et al. 1994). Further modifications of the central building block has led to the development of napsagatran (Ro 46-6240) as a reversible thrombin inhibitor with a Ki of 0.27nM. This compound has been found to be an effective antithrombotic agent in a variety of animal models (Gast et al. 1994, Carteaux et al. 1995).

The compounds synthesized based on the L-arginine as the building block and incorporating both C and N-terminal optimal modifications, were found to be highly toxic due to inhibition of butyl cholinesterase (Hijikata-Okunomiya and Okamoto, 1992). The introduction of a COOH group on the carboxy terminal resulted in decreased affinity for butyl cholinesterase (and therefore less toxicity) and after further modifications an isomer named argatroban (MD805 or MCI9038 or argipidine), depicted in Fig. 4, was generated

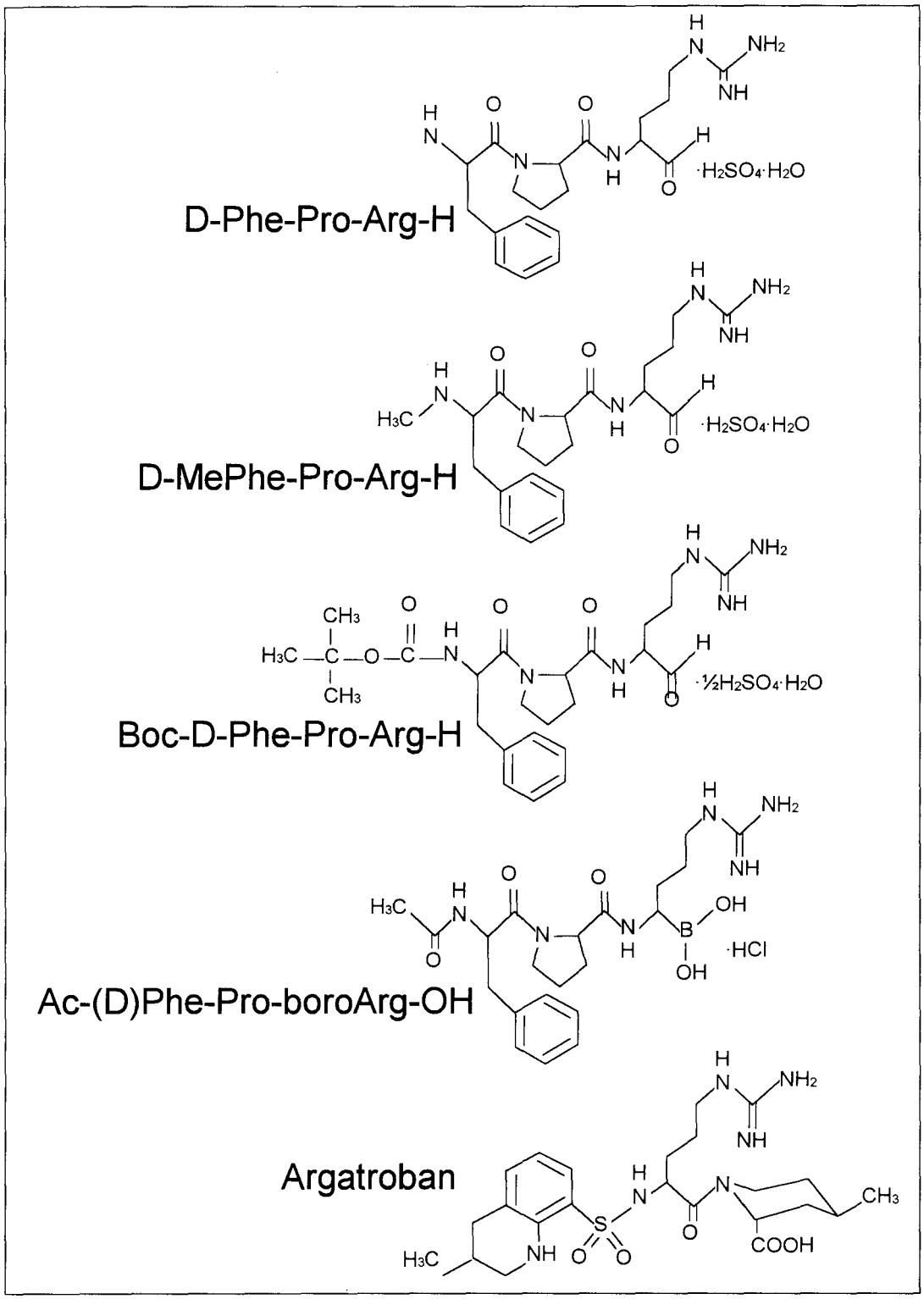

Fig. 4. Chemical structures of thrombin inhibitors: D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH and argatroban.

as a selective inhibitor of thrombin (K_i=0.039 μM, Hijikata-Okunomiya and Okamoto, 1992). This compound also has a sizeable affinity for trypsin (K_i=5.0 μM, Hijikata-Okunomiya and Okamoto, 1992). It has been characterized pharmacologically and has a plasma half life of 40 minutes (in humans, Tamao et al. 1986 a, b). Argatroban is also effective in preventing thrombus formation in various animal models at low concentrations (> 1 μM, Maruyama 1990, Hijikata-Okunomiya and Okamoto 1992, Hara et al. 1994, Kawai et al. 1995). This compound is being tested clinically for several indications (Yonekawa et al. 1986, Kumon et al. 1984, Oshiro et al. 1983, Matsui et al. 1992).

A new low molecular weight compound, that competitively inhibits thrombin with a Ki of 15 nM is inogatran (pINN, Teger-Nilsson et al. 1995). Preclinical and phase I studies have been completed with this compound in numerous investigations (Gustafsson et al. 1995, Eriksson et al. 1995). This agents was found to be selective for thrombin when compared to other serine proteases. Inogatran was also found to have more effective antithrombotic effects than hirudin and hirulog in an *in vivo* rat model of arterial thrombosis. No interaction with fibrinolytic processes were noted when inogatran was studied in rat and dog models of thrombolysis. The half life of inogatran is about one hour (Teger-Nilsson et al. 1995). This compound also has a substantial oral bioavailability that is species and gender specific (Gustafsson et al. 1995, Eriksson et al. 1995).

3. Peptide Inhibitors

With the elucidation of the primary structure of procoagulant proteins and the

identification of the proteolytic activation cleavage sites, specific chromogenic substrates for thrombin and factor Xa were developed by synthesis of peptides mimicking the amino acid sequence adjacent to the substrate's cleavage site and attaching a para nitro-aniline (pNA) group on the carboxy terminus of these peptides (Bang and Mattler, 1977). A comprehensive study reported on the effects of many peptide inhibitors belonging to various classes on a spectrum of serine proteases using clotting and novel amidolytic systems (Fareed et al. 1981).

a. Aldehyde Derivatives

In 1975 a series of tripeptide aldehydes containing arginine were developed as the first reversible peptide thrombin inhibitors. The prototype compound to be synthesized was D-Phe-Pro-Arg-H (GYKI 14166, Bajusz et al. 1975), depicted in Fig. 3, which although was a selective and potent inhibitor of thrombin, was very unstable in neutral aqueous solution where it cyclized and was inactivated (Bajusz et al. 1989). To prevent cyclization, a derivative was synthesized with a protective amino terminal t-butyloxycarbonyl (Boc) group: Boc-D-Phe-Pro-Arg-H (GYKI 14451, Bajusz et al. 1978), also depicted in Fig. 3. This compound was more stable than its parent compound but was not as specific for thrombin since it inhibited plasmin as well. In order to achieve compounds that are both stable and specific for thrombin, a series of N-alkyl derivatives were synthesized (a basic amino terminus promotes thrombin specificity) and from this series the methyl derivative D-MePhe-Pro-Arg-H (GYKI 14766, Bajusz et al. 1987), depicted in Fig. 3, was found to be as potent and selective reversible inhibitor of thrombin as the prototype aldehyde. The K_i for the aldehyde derivatives is around 0.1

μM. The aldehydes D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H have been studied pharmacologically. From toxicity studies in mice and rabbits, the LD_{50} for these compounds was about 40-45 mg/kg. Both agents were devoid of hemorrhagic effects as determined by a rabbit ear bleeding model. The unprotected aldehyde however did produced a blood pressure lowering effect (40% decrease) when injected I.V. which may have been due to contaminants. When these aldehydes were administered to rabbits and dogs orally, they produced persistent anticoagulant effects, although the bioavailability may have been low. Similar results indicated a high bioavailability when these compounds were administered subcutaneously. The biologic half life of both aldehydes after I.V. administration to primates was estimated to be around 90-180 minutes. The anticoagulant activities of the compounds were evident in the global clotting tests and were independent of antithrombin (Bajusz et al. 1990, Bagdy et al. 1992). Both agents inhibited thrombin-induced platelet aggregation in a concentration dependent manner, but the platelet count was not reduced after systemic administration. All three of the tripeptide aldehydes had antithrombotic activities as tested in various models of thrombosis (rats, rabbits, baboons). The free aldehyde as well as the methylated derivative were effective antithrombotics after I.V., S.C. and P.O. administration while the Boc derivative was active only after I.V. administration.

An analogue of the D-MePhe-Pro-Arg-H aldehyde, D-1-Piq-Pro-Arg-H (LY303496), is being developed as an orally effective thrombin inhibitor. This compound has been shown to be an effective antithrombotic agent in rat models of deep venous thrombosis (Wilson et al. 1995) and arterial thrombosis (Kurz et al. 1995).

b. Chloromethyl Ketone Derivatives

Peptides of arginine chloromethyl ketones correspond to the primary structure of physiological substrates of target proteases (Kettner and Shaw 1977). Chloromethyl ketones inactivate serine proteases by the formation of an intermediate reversible substrate-like complex with the protease followed by irreversible alkylation of the active site histidine. Peptides of arginine chloromethyl ketone are more effective than lysine analogues since thrombin hydrolyzes only specific arginine bonds in its substrates. Thus, arginine in the P1 position of the substrates is more effective. Factor XIII and prothrombin cleavage sites as well as other thrombin substrates have proline at the P2 site while the A and B chains of fibrinogen do not have this proline in the P2 site. After testing a series of these cleavage site analogues it has been found that the tripeptide chloromethyl ketone analogues of factor XIII cleavage site (Val-Pro-Arg-CH_2Cl) and the prothrombin cleavage site (Ile-Pro-Arg-CH_2Cl and Val-Ile-Pro-Arg-CH_2Cl) are the most potent thrombin inhibitors with the tetrapeptide being the optimal as well as the most specific thrombin inhibitor, probably due to occupation of a fourth binding site. These inhibitors also inactivate plasmin, kallikrein and urokinase but slowly and to a limited extent. Binding at secondary sites contributes to the compounds's selectivity for trypsin-like proteases. The most effective irreversible inhibitor of thrombin synthesized from this class of substrates is D-Phe-Pro-ArgCH_2Cl (PPACK or FPRCH_2Cl, Kettner et al. 1979). This compound's two first amino residues occupy the specificity pocket in a tight manner, while the arginine drops into the catalytic pocket. This allows for high specificity for thrombin as opposed to other serine proteases. Thus, D-Phe-Pro-

$ArgCH_2Cl$ has been utilized in biochemical studies of thrombin and hemostasis as well as in pharmacological studies. The toxicity of this tripeptide chloromethyl ketone is species dependent. Thus, although in mice are no toxic effects after I.V. infusions of up to 50 mg/kg, in rabbits nearly all platelets agglutinate even though clotting is prevented. The biological half life of this agent, as measured after I.V. administration to rabbits, is about 3 minutes. The agent is also absorbed in an active form subcutaneously. The anticoagulant effects of the ketone are detectable in a concentration dependent manner with the TT and APTT clotting tests as well as with the TEG whole blood clotting test. However, these effects are lost after incubation with plasma suggesting inactivation of the compound by plasma components. The antithrombotic effects of this tripeptide derivative have been examined extensively in various animal models (rats, dogs and rabbits) and in all the compound is an effective antithrombotic after both I.V. and S.C. administration.

c. Nitrile Derivatives

Tripeptide derivatives with α-nitrile groups have been synthesized as competitive inhibitors of thrombin. The peptide derivative D-Phe-Pro-Arg-CN 112 is a strong competitive inhibitor of thrombin with a K_i=0.7 μM. This agent has been studied pharmacologically in mice, rats and rabbits. The LD_{50} is 30-40 mg/kg I.V. in mice. Intravenous infusion of the agent to rats has serious blood pressure lowering effects (70-80% reduction). The bleeding effects are minimal (Stüber et al. 1988), an indication that primary hemostasis is not affected by this compound. The biological half life of the agent after I.V. injection to rabbits is around 12 minutes and it is effective after S.C.

administration as well. Biliary excretion accounts for about 30% of route of excretion of the total agent administered. This tripeptide nitrile exhibits anticoagulant effects in a concentration dependent manner as detected by the global clotting tests and the TEG whole blood clotting assay. It is also an effective antithrombotic in various rat models of thrombosis.

d. Boronic Acid Derivatives

The search for selective and potent inhibitors of thrombin has led to the development of three more classes of reversible tripeptide inhibitors: a) Trifluoromethyl ketones: the representative compound D-Phe-Pro-Arg-CF_3 (Neises et al. 1991) with a K_i in the nM range b) α-aminophosphonic acid tripeptide derivatives with K_is in the nM range. c) α-aminoboronic acid derivatives. The boronic acid derivatives were initially developed as inhibitors of elastase and chymotrypsin. In order to develop a compound that would be specific for thrombin, the arginine in the sequence D-Phe-Pro-Arg has been substituted with its boronic acid derivative. This has led to the synthesis of Ac-(D)-Phe-Pro-boroArg (K_i=41 pM), depicted in Fig. 3, Boc-(D)-Phe-Pro-boroArg (K_i=3.6 pM) and H-(D)-Phe-Pro-boroArg (K_i< 1 pM) (Kettner et al. 1990). The boroarginine derivatives have been a recent development and their pharmacologies are not complete. The Ac-(D)-Phe-Pro-boroArg appears to have a biological half life of around 15 minutes after intravenous administration to rabbits and it is an effective anticoagulant after S.C. administration. The anticoagulant effects of this compound are dose dependent and detectable with the global clotting tests after administration to rats, rabbits and baboons. This agent also exhibits antithrombotic effects as demonstrated by two thrombosis

models. The H-(D)-Phe-Pro-boroArg also exhibits antithrombotic effects in a baboon model of thrombosis and when compared to the aldehyde analog (D)-Phe-Pro-Arg-H, the boronic acid derivative achieves the same effect at a dose 20 times lower than that of the aldehyde derivative.

In attempting to develop a more specific, orally bioavailable thrombin inhibitor, the above boronic acid derivatives have been modified to yield the compound Ac-D-Phe-N-cyclopentylGly-boroArg (S18326, Verbeuren et al. 1995). Although this compound is more specific than Ac-D-Phe-Pro-boroArg and it is shown not to interfere *in vitro* with fibrinolysis, it possesses sizeable inhibitory activities against other fibrinolytic enzymes (Verbeuren et al. 1995). However, this compound has potent antithrombotic activities in rat models of arterial and venous thrombosis and is orally bioavailable (Verbeuren et al. 1995).

The above described boronic acid derivatives lack specificity for thrombin. In an attempt to overcome this, the C terminal of the tripeptides has been extended (Ketnner et al. 1990). However, the resulting compounds still lack in specificity for thrombin. Tapparelli et al. (1993) has further modified these compounds by replacing the boroArg in the third position (corresponding to the S1 pocket site of thrombin) with a neutral boron containing moiety. The compound Z-D-Phe-Pro-boromethoxypropylGly-pinendiol has a lower Ki for thrombin (8.9 nM) than its predecessors, but the specificity for thrombin is improved (Tapparelli et al. 1993). However, this compound is a weak anticoagulant in the global clotting tests and does not inhibit thrombin induced platelet aggregation *in vitro* and *in vivo*.

Another series of boronic acid derivatives has been developed as specific inhibitors of thrombin. These are Z-D-Phe-Pro-boroMpgOPin, Z-D-Phe-Pro-boroMpgOPinacol and Z-D-Phe-Pro-boroPgIOPin (TRI50, TRI50b and TRI11 respectively, Esmail et al. 1995). The most promising antithrombin agent appears to be the pinacol ester derivative, TRI50b, with a Ki of 7nM. This compound is also an effective antithrombotic in various animal model of arterial and venous thrombosis, after intravenous and intraduodenal administration (Goddard et al. 1995, Gerrard et al. 1995).

e. Other Peptide Analogues

Substituting Arg with amidinoPhe in the D-Phe-Pro-Arg prototype sequence, led to the development of a series of compounds with inhibitory activities for various enzymes. The compound N-α-(2-napthylsulfonylGly)-4-amidinoPhepiperidine (NAPAP) was found to be the most optimal thrombin inhibitor with a Ki of 6 nM (Stürzebecher et al. 1983). NAPAP is an effective antithrombotic in a series of animal models (Kaiser et al. 1985). However, in addition to adverse side effects (hypotension and respiratory depression, the half life of this compound is even shorter than most thrombin inhibitors (less than 10 min versus greater than 20 min for other thrombin inhibitors, Kaiser et al. 1985) and thus clinical development has been abandoned.

Another direct thrombin inhibitor under current development is CVS-1123 [$(CH_3CH_2CH_2)_2$-CH-CO-Asp(OCH_3)-Pro-Arg-CHO, Vlasuk et al. 1995]. This compound has an oral bioavailability of $>30\%$ when administered to cynomolgous monkeys and is an effective antithrombotic in a dog model of coronary artery thrombosis (Cousins et al. 1995).

4. Recombinant Inhibitors

a. Aptamers

Aptamers are oligonucleotides (double or single stranded DNA, or single stranded RNA) which act directly on proteins to inhibit disease processes. Thirty two such aptamers have been recently isolated as inhibitors of thrombin with binding affinities in the range of 20-200 nM (Boch et al. 1992). One of the most potent thrombin aptamers has been found to interact with thrombin's anion binding exosite, so that it competes with substrates that interact with that specific site, such as fibrinogen and thrombin platelet receptors (Macaya et al. 1993, Paborsky et al. 1993). This aptamer has been shown recently to reduce arterial platelet thrombus formation in an animal model, as well as to inhibit clot bound thrombin in an *in vitro* system (Li et al. 1994). Recently, a second pool of aptamers, with a different sequence composition than the first class, incorporating modified bases, has been isolated, which has shown promising anticoagulant activities (Latham et al. 1994). Another recent development in the area of oligonucleotide inhibitors of thrombin has been the isolation of two RNAs that bind thrombin with high affinity (Kd in the nM range). These oligonucleotides have been shown to inhibit fibrinogen clotting in an *in vitro* test (Kubik et al. 1994).

b. Hirudin and its Variants

Hirudin is the most potent family of natural thrombin inhibitors, found in the saliva of the leech *Hirudo medicinalis*. It is a single polypeptide chain of 64-66 amino acid residues, stabilized in a characteristic conformation by three disulfide bridges in the N-terminal (Petersen et al. 1976). The Tyr moiety in position 63 is sulfated and

important in imparting inhibitory potency against thrombin (Verstraete 1995). The mode of interaction of hirudin with thrombin has been elucidated from X-ray studies on the crystallized thrombin-hirudin complex (Grütter et al. 1990, Rydel et al. 1990). The amino terminus of this polypeptide interacts with the apolar binding site of thrombin (Wallace et al. 1989) while the carboxy terminus, which is highly acidic, interacts with the anion binding exosite for fibrinogen recognition (Chang et al. 1990). Thus, by binding to these two sites on thrombin, hirudin masks the catalytic site thus rendering thrombin inactive. Because hirudin recognizes two different exosites on thrombin, rather than its catalytic site, it is a highly specific inhibitor of thrombin.

Several isotypes of hirudin exist and they all have similar thrombin inhibitory potencies. These hirudin variants are named HV-1 (Dodt et al. 1993), HV-2 (Harvey et al. 1985) and HV-3 (Dodt et al. 1986) and their primary structures are shown in Fig. 4. All of these isoforms have been produced by recombinant technology (Harvey et al. 1986, Dodt et al. 1989, Scharf et al. 1989) and the expression systems include *Eschericia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. The recombinant forms differ from the natural ones in that the Tyr residue in position 63 is not sulphated in the recombinant forms. Although this lack of sulfation reduces the antithrombin activity of hirudin, the reduction is negligible since the formation of the thrombin-hirudin complex is almost irreversible (Verstraete 1995). The recombinantly produced hirudins are also named desulfatohirudins or desirudin, due to the lack of sulfation at position 63. The hybrid recombinant hirudin CX-397 has been synthesized composed of the N-terminal fragment of HV-1 and the C-terminal fragment of HV-3 (Komatsu et al. 1993). This hybridization

results in a molecule that has an improved inhibitory potency for thrombin over the natural occurring hirudins. Point mutations to develop variant forms of hirudin have been used (Scharf et al. 1989).

Binding of recombinant hirudin to dextran has been reported (Markwardt et al. 1990) in an attempt to prolong its biologic t½. More recently, polyethylene glycol coupling of recombinant hirudin, to develop longer lasting agents, has been accomplished (Kurfurst et al. 1992, Zawilska et al. 1993). This modification results in a molecular weight increase of hirudin from 7,000 to 17,000, which prevents extravasation of this agent and retards renal elimination, substantially increasing its biological half life (Rübsamen et al. 1991, Bucha et al. 1996). Furthermore, the PEG-hirudin metabolites differ from those of hirudin, suggesting different renal metabolism of the two agents (Lange et al. 1996). PEG-hirudin as well as hirudin are capable of inhibiting clot-bound thrombin when compared to heparin (Iorio et al. 1993). PEG-hirudin has also been shown to be effective in a rabbit model of DIC (Zawilska et al. 1993). Another application of PEG-hirudin is stent coating (Stemberger et al. 1996) and expansion to usage of this agent in reducing the thrombogenicity of other biomaterials, such as catheters, vascular prostheses and oxygenators is being considered.

In addition to PEG, albumin has also been linked to the carboxy terminus of hirudin to prolong its half life, without impairing its antithrombin activity (Syed and Sheffield, 1995). The albumin-hirudin product has not been tested *in vivo*.

Extensive pharmacological studies have been competed with recombinant hirudin. This compound has been repeatedly shown to be effective in various venous and arterial

thrombosis model of the rat, rabbit and the pig (Verstraete 1995). The effects are always dose-dependent at μmol/kg doses even after subcutaneous injection. Interestingly, the pharmacokinetics of desirudin reveal that it is distributed in extracellular compartments and that renal metabolism and excretion is an important component of the overall excretion profile (Verstraete 1995).

5. Inhibitors of Thrombin not Directed Against its Catalytic Site

Based on the structure of the carboxy terminus of hirudin, which interacts with the anion binding exosite of thrombin, a series of synthetic peptides cyclized via disulfide linkages were synthesized as inhibitors of thrombin (Krstenansky et al. 1988). These inhibitors block the anion binding exosite of thrombin thus preventing fibrinogen cleavage and subsequent fibrin clot formation and optimal N terminal substitution of these peptides can lead to inhibition of fibrin formation (Owen et al. 1988).

A 22 and a 27 amino acid peptide has been synthesized, modelled after the sequence of HC-II that interacts with thrombin's anion binding exosite, which prevents proteolytic cleavage of fibrinogen. The IC_{50} values for these two peptides in inhibiting thrombin are 38 and 28 μM respectively. These peptides however do not interfere with thrombin's catalytic site (Glen et al. 1989, 1990).

The same group that studied the HC-II analogues, compared these to a fibrinogen (18 residues) and a thrombomodulin (19 residues) cleavage site analog and to a peptide corresponding to the 12 terminal amino acids of hirudin. The IC_{50} values for these compounds are 130, 140 and 1.3 μM respectively.

A fibrinogen analog has also been synthesized as a thrombin inhibitor (Binnie et

al. 1991). This 24 amino acid peptide is modelled after the sequence of fibrinogen downstream of the thrombin cleavage site (different from the one studies by Glen et al. above). It is able to prevent fibrinogen clotting with a K_i of around 190 μM (Binnie et al. 1991). This peptide also does not block the thrombin catalytic site.

A new thrombin inhibitor, triabin, is a 17,000 protein isolated and cloned from the saliva of the assassin bug *Triatoma pallidipanis* (Noeske-Jungblut et al. 1995). This agent inhibits thrombin by binding to its anion-binding exosite but does not interfere with its catalytic site. A recent report (Glusa et al. 1996) shows that this agent is potent in inhibiting thrombin mediated platelet aggregation and smooth muscle contraction.

6. Chimeric Thrombin Inhibitors

Coupling of peptides that mimic the carboxy terminal of hirudin to peptides that are specific for inhibition of the catalytic site of thrombin (D-Phe-Pro-Arg) has led to the development of a series of chimeric molecules termed hirulogs, in which the amino terminus consists of the catalytic site-directed peptides, while the carboxy terminus consists of the 12 terminal residues of hirudin. The two moieties are linked together by a bridge of glycine residues of variable length (DiMaio et al. 1990, Maraganore et al. 1990). Thus, hirulogs inhibit thrombin by binding to both its catalytic site and its anion binding exosite, thus conferring specificity to these molecules for thrombin. Hirulog-1 has been developed aggressively for several cardiovascular indications (Topol et al. 1993, Cannon et al. 1993, Théroux and Lidon 1994). However, its superiority over heparin has not been established.

Recently, there has been a report of a novel synthetic thrombin inhibitor,

CVS#995 (Vlasuk et al. 1994), comprised of 19 amino acids, in which recognition sequences for the catalytic and primary exosite binding domains of thrombin have been linked by a transition state analog (Vlasuk et al. 1994). The Ki value for thrombin is in the pM range for this slow and tight binding thrombin inhibitor. When compared to hirulog-1, this agent is claimed to be superior at inhibiting platelet aggregation and venous thrombosis in a rat model (Vlasuk et al. 1994, Biemond et al. 1995).

An interesting chimeric molecule has been developed, in which the 12 terminal residues of hirudin are coupled to the tripeptide Arg-Gly-Asp (Church et al. 1991). This tripeptide is a sequence found in thrombin and the adhesive proteins fibrinogen, fibronectin, vitronectin and von Willebrand factor and is recognized by the platelet surface receptor GPIIb/IIIa which mediates platelet cell adhesion and aggregation (Ruoslahti et al. 1987, Phillips et al. 1988, Plow et al. 1989). The chimeric molecule is able to inhibit platelet adhesion to surfaces as well as inactivate thrombin. The implications of this type of structuring are that thrombin inactivation can be targeted to specific cells trapped in thrombi. Based on this hybrid molecule and the crystal structure of the hirudin-thrombin complex, a new class of recombinant hirudin variants has been developed, called hirudisins (Knapp et al. 1992) These recombinant hirudins have residues 32-35 replaced by the sequence Arg-Gly-Asp-Ser or Lys-Gly-Asp-Ser to yield molecules with characteristics similar to those of the Arg-Gly-Asp-C-terminal of hirudin chimera.

In a novel approach to target a thrombin inhibitor to the surface of a clot, hirudin was covalently linked to a fibrin specific monoclonal antibody (Bode et al. 1996). This

chimeric agent was shown to be more effective than hirudin alone in preventing platelet deposition and clot formation *in vitro* and in an *in vivo* baboon arteriovenous shunt model.

7. Thrombin Directed Antibodies

Acquired antithrombin autoantibodies are rare and have been poorly characterized. In one reported case (Sie et al. 1991) the patient had mild bleeding symptoms and markedly prolonged clotting times and eventually died of cerebral hemorrhage. The antibody was found to be an immunoglobulin that recognized at least in part the apolar binding site of thrombin, adjacent to the catalytic site. In another case, the patient developed antibodies against thrombin and factor Xa after exposure to topical treatment with bovine thrombin (Zehnder and Leung, 1990). Polyclonal antibodies against thrombin have been raised by using the human α thrombin B chain (Noe et al. 1988). These antibodies were found to inhibit the functions of thrombin that require involvement of its anion binding site, such as activation of protein C when bound the thrombomodulin, cleavage of fibrinogen and binding to hirudin. On the other hand, the catalytic site of thrombin was not hindered and thus the enzyme retained its activity to activate protein C and interact with small chromophores (directed against thrombin's catalytic site). These antibodies are useful tools in capturing complexes of thrombin (Lackman and Geczy, 1991, Lackman et al. 1991) as well as in studying requirements of thrombin sites in other interactions (Chang and Detwiler, 1991, Noe et al. 1988).

F. Clinical Applications of Thrombin Inhibitors

Ever since their introduction, most of the clinical indications for these new anticoagulants have been in the interventional cardiovascular areas (Herrman and Serruys, 1994). Initial focus has been on the prevention of abrupt closure during coronary angioplasty (Topol et al. 1993, Topol 1995). These agents have also been tried for the post-PTCA prevention of both the early and late reocclusion (Suzuki et al. 1995). In addition, some of the thrombin inhibitors have been used for the treatment of unstable angina (Topol 1995). More recently, some of these agents have been tested for their efficacy in stenting (van Beusekom et al. 1994, Buchwald et al. 1993, Stemberger et al. 1996). Although several reports on the experimental use of these agents have been made available on their use in cardiovascular surgery in animal models, only isolated reports in human studies are available (Riess et al. 1995, Edmunds 1995). Concerns over the use of these agents have been expressed (Edmunds 1995).

Thrombin inhibitors are attractive to both clinicians and surgeons as substitute anticoagulants in heparin compromised patients in particular. For patients requiring anticoagulation, exhibiting heparin induced thrombocytopenia, several trials are currently in progress to test the efficacy of new antithrombin agents (Edmunds 1995, Chamberlin et al. 1995).

There is a growing interest in the use of these agents in the prevention and treatment of DVT (Bridey et al. 1995). Eriksson (1995) reported on the successful use of recombinant hirudin in the prophylaxis of post-orthopaedic surgery. In this study he compared unfractionated heparin (5000 IU s.c. t.i.d.) with hirudin (15 mg s.c. b.i.d.)

and found hirudin to be superior. Several additional trials are currently being conducted on different antithrombin agents.

1. Antithrombin

The major application of antithrombin is in disorders associated with antithrombin deficiency, acquired or hereditary. Antithrombin replacement therapy in patients with hereditary antithrombin deficiency is in phase I and Phase II clinical studies. Supplementation of antithrombin to these patients before and after undergoing various surgical procedures with or without other treatments has proven effective (Menache 1991). The combination of antithrombin/heparin is effective in prophylaxis of venous thrombosis following total hip and total knee replacement surgery (Franscis et al. 1991). The role of antithrombin replacement therapy in prophylaxis of thrombotic episodes during pregnancy remains controversial (Owen 1991). From a study by Demers et al. (1992), it appears that replacement therapy of antithrombin in people who are deficient should be a mode of prophylaxis only during periods of high risk for thrombotic phenomena. In addition to these uses, antithrombin has been evaluated in clinical trials in patients with septic shock and DIC (Maki et al. 1987, Blauhut et al, 1985, Fourrier et al. 1993). The outcomes of these trials proved that antithrombin is effective in attenuating and correcting DIC early, but the mortality rate is not affected.

2. Argatroban

Although argatroban has been studied in wide range of animal models and has been used clinically in Japan for some years (it is approved for treatment of peripheral arterial

occlusive disease), only recently has the European and American community started to evaluate its clinical usefulness.

In an initial study in the U.S., argatroban was evaluated in patients with unstable angina (Gold et al. 1993). Even though myocardial ischaemia did not occur during the argatroban infusion, some patients had recurrent angina within 6 hours after discontinuing the infusion. However, the outcomes may be improved by changing the infusion protocol. This recurrent angina was correlated with an increase in the thrombin-antithrombin complex and was described as a rebound phenomenon. This observation was explained by either the premature cessation of argatroban infusion which resulted in inhibition of thrombin but was not enough to inhibit the generation of thrombin Gold et al. 1993), or by the heparin withdrawal prior to argatroban infusion, which itself results in recurrent angina (Willerson and Casscells, 1993). Furthermore, heparin alone promotes antithrombin clearance, so that antithrombin levels may have been artificially low prior to argatroban infusion (Willerson and Casscells, 1993).

There are currently two phase II trials in North and South America, in which Argatroban is being evaluated as an adjunct to thrombolytic agents and aspirin. The Argatroban in Myocardial Infarction (AMI) study is evaluating two doses of argatroban in conjunction with streptokinase and aspirin in patients with acute myocardial infarction (P. Théroux, M.D., Montreal Heart Institute, Principal Investigator). The Myocardial Infarction with Novastan and tPA (MINT) study is comparing two doses of argatroban to heparin in patients diagnosed with acute myocardial infarction and receiving tPA and aspirin (I.K. Jang, M.D., Harvard Medical School, Massachusetts General Hospital,

Principal Investigator). Argatroban is also evaluated in two phase II trials (ARG-911, B. Lewis, M.D., Loyola University Medical Center, Principal Investigator and ARG-912, G. Pineo, M.D. and R. Hull, M.D., University of Calgary, Principal Investigators) in patients with heparin induced thrombocytopenia. In the ARG-912 study, argatroban is being compared to ancrod (a defibrinating enzyme). The results of these trial are expected to be available in early 1997. Argatroban is also proposed as an anticoagulant for cardiovascular bypass surgery. (ARG-240, R. Pifarré, Loyola University Medical Center, Principal Investigator).

3. Efegatran

The tripeptide aldehyde D-MePhe-Pro-Arg-H is currently marketed under the generic name efegtran sulfate by Eli Lily, U.S.A. Although efegatran has been examined in various animal models and has been in studied experimentally for several years with promising results, only in the last two years has it been considered for clinical usage. Five clinical studies have been performed with efegatran in normal male volunteers, to assess safety and pharmacokinetics/ pharmacodynamics. Interestingly, some of the subjects experienced short-lived headaches, postural dizziness and vasovagal episodes when moving from a lying to a sitting position following I.V. infusion of efegatran. It is currently being studied in a phase II trial, the Prevention of Reocclusion by Inhibition of thrombin during Myocardial Events (PRIME) study, in patients with acute myocardial infarction. This trial is comparing adjunct usage of tPA and aspirin with efegatran at different doses versus adjunct usage of tPA and aspirin with heparin. The results of this study are expected in the beginning of 1996.

4. Inogatran

While inogatran is a new competitive thrombin inhibitor and limited information on its pharmacology is available, phase I studies on this thrombin inhibitor are already completed (Teger-Nilsson, 1995). It is shown that inogatran is an effective anticoagulant with a biological half life of about 1 hour, which is longer than the other thrombin inhibitors currently under clinical evaluation. There appear to be not metabolites of this agent and it is excreted evenly between kidneys and faeces. However, this thrombin inhibitor prolongs the capillary bleeding time in some healthy human subjects. Currently, inogatran is evaluated in a phase II trial in patients with unstable angina.

5. Napsagatran

As with inogatran, napsagatran is a newly developed reversible thrombin inhibitor. It is currently evaluated in phase II clinical trials for the prevention of post-operative thrombosis and in treating established venous thrombosis.

6. Hirudin

The antithrombotic and anticoagulant effects of recombinant hirudin have been well established in experimental animal models. In the clinical setting, hirudin has been studied in various clinical trials for a range of indications. Hirudin was compared with heparin in the Thrombolysis in Myocardial Infarction (TIMI) 5 Trial, in acute myocardial infarction patients treated with tPA and aspirin (Cannon et al. 1995). Although there was an improvement in clinical endpoints with hirudin as compared with heparin, there was no hirudin dose-dependence on the outcomes. The TIMI 6 Trial compared hirudin with

heparin in acute myocardial infarction patients receiving streptokinase and aspirin (Lee et al. 1995) and there was a trend for an improved outcome with the higher dose of hirudin compared to the lower hirudin dose and to heparin. However, the subsequent larger phase 3 Hirudin for Improvement of Thrombolysis (HIT III) trial, that compared hirudin to heparin in acute myocardial infarction patients treated with tPA and aspirin (Neuhaus et al. 1994), was stopped prematurely due to excessive intracranial bleeding in the hirudin group. Another large phase 3 trial stopped prematurely due to excessive intracerebral bleeding was the Global Use of Strategies to Open Occluded Arteries (GUSTO) II trial (GUSTO IIa Investigators, 1994). In this trial, hirudin was again compared to heparin, in patients with acute coronary syndromes (with unstable angina or myocardial infraction). It was observed that the incidence of hemorrhagic strokes increased in patients receiving concomitant thrombolytic therapy. Another interesting finding of this study is that hirudin, irrespective of dose, is less effective in inhibiting thrombin generation when compared to heparin (Zoldelhyi et al. 1995). The Thrombolysis and Thrombin Inhibition in Myocardial Infarction (TIMI) 9A trial, designed to compare heparin and hirudin as adjuncts to thrombolytic therapy, was also prematurely stopped because of increased incidence of hemorrhagic strokes in patients receiving either heparin or hirudin (Antman and TIMI 9A Investigators, 1994). Both the GUSTO II and TIMI 9A trials were re-initiated with lower doses of both hirudin and heparin under the acronyms GUSTO IIb and TIMI 9B (Verstraete and Zoldheyi, 1995). In the GUSTO-IIb, over 12,000 patients from 373 hospital sites in 13 countries were included to compare intravenous recombinant hirudin and heparin. Dr. Topol (45th

Annual Scientific Session of the American College of Cardiology Meeting in Orlanso FL, oral presentation) described this study as validating the direct thrombin inhibition hypothesis where hirudin at adjusted dosage exhibited comparable anticoagulant actions to heparin. However, even at this dose, the hirudin treated group showed slightly increased bleeding events. The HIT-SK trial, designed to compare hirudin and heparin as adjunct treatments to streptokinase in patients with acute myocardial infarction (Molhoek et al. 1996) was recently completed. The results of this study also showed that no clear dose-response relationship was evident and furthermore major bleeding events were also increased.

Hirudin has also been evaluated in the indication of coronary angioplasty, in the phase III, Hirudin in a European Restenosis Prevention Trial Versus Heparin in Treatment of PTCA Patients (HELVETICA, Serruys et al. 1994). When compared to heparin, the hirudin group exhibited an improvement of clinical outcomes (fewer myocardial infarctions, bypass surgeries and ischemic events) in the early stages of treatment, but in the 7 month follow-up there was no difference in the rate of restenosis between the two groups.

In the indication of unstable angina, hirudin has been compared with heparin and found to decrease the rate for subsequent myocardial infarction (OASIS, Organization to Assess Strategies for Ischemic Syndromes 1995). However, there was no dose-response relationship in the hirudin group. Another problem emerging from treatment of unstable angina with hirudin or heparin is that there is an increase of ischemic events after hirudin or heparin is stopped. A recent study on coagulation markers in unstable angina patients

treated with heparin or hirudin in conjunction with aspirin showed that although coagulation was suppressed during treatment with these agents, coagulation was reactivated when the treatment was stopped (Flather et al. 1995). Therefore, treatment should be continued for longer time periods (>72 hours).

Hirudin has been compared to heparin in patients undergoing elective total hip replacement in small phase 2 trials (Eriksson et al. 1994a,b, Ekman et al. 1996). In a recent report (Eriksson et al. 1996) a fixed dose of 15 or 20 mg hirudin pre-operatively and administered S.C. twice daily for at least 9 days, was shown to be superior to heparin and safe prophylactic regimen for thromboembolic complications in patients undergoing total hip replacement surgery. However, such a study should have compared hirudin to the standard treatment, which is currently low molecular weight heparin S.C. b.i.d. Thus, there is a need for additional clinical trials for hirudin in this indication.

A recent report describes the usage of hirudin in ten patients with severe venous thromboembolism (Parent et al. 1993, Schiele et al. 1994). It was concluded that thrombin generation was only partial and the dose of hirudin should be increased in subsequent studies and should be compared to heparin or low molecular weight heparin.

Novel and successful applications of hirudin have been reported recently in two case studies, where the patients involved developed heparin induced thrombocytopenia. In one case, the patient successfully underwent cardiopulmonary bypass surgery by using hirudin instead of heparin for anticoagulation during this procedure (Pötzsch et al. 1994). In the second case, the another patient with heparin induced thrombocytopenia successfully underwent cardiopulmonary bypass surgery with an aortic valve replacement,

after being anticoagulated with hirudin in place of heparin (Riess et al. 1995). Since these two isolated cases, an additional 11 patients with heparin-induced thrombocytopenia have been successfully treated with hirudin during cardiopulmonary bypass for cardiac surgery (Pötzsch et al. 1996). However, concerns regarding replacement of heparin with hirudin during cardiopulmonary bypass surgery include the absence of an antidote to hirudin and the questionable ability of hirudin to suppress thrombin generation (Edmunds 1995).

Another novel application of hirudin was in a patient with diabetic nephropathy and heparin induced thrombocytopenia (Nowak et al. 1996). The continual usage of hirudin in this patient in conjunction with hirudin-impermeable dialyzers proved to be safe and efficacious.

While hirudin has been regarded as a weakly immunogenic agent, a recent study reports on the detection of IgG anti-hirudin antibodies in 38 out of 82 patients with heparin induced thrombocytopenia that were treated successfully for more than 6 days with hirudin (Eichler and Greinacher 1996, Greinacher et al. 1996). Even though no allergic reactions were induced in any of the patients, the effects of these antibodies on the pharmacologic responses of hirudin are not clear at this time.

The recombinant hirudin CX-397, a chimeric molecule between HV-1 and HV-3, has been compared with heparin in two groups of 5 patients receiving aspirin, prior to coronary angiography (Ffrench et al. 1995). This agent is shown to be a strong anti-coagulant that warrants further investigations in patients undergoing coronary angioplasty.

The interactions of PEG-hirudin with aspirin were studied recently in 9 healthy human volunteers (Breddin et al. 1996). It was shown that PEG-hirudin in combination

with aspirin may be associated with a higher risk of bleeding. These bleeding additive effects were not correlated with any of the platelet function tests nor any of the coagulation parameters. To avoid bleeding complications, it was suggested that the PEG-hirudin dose should be decreased or that aspirin should be given after PEG-hirudin treatment was completed.

Phase I clinical studies on PEG-hirudin have shown that this agent is a safe thrombin inhibitor in healthy human volunteers after I.V. or S.C. injection (Esslinger et al. 1995). Due to its prolonged t½ when compared to hirudin, PEG-hirudin appeared to be from these studies a promising agent for prophylactic development as a once daily S.C. injection. The rapid clearance of PEG-hirudin from the central compartment, following I.V. injection also indicates that this agent may be suitable for low-dose continuous infusions.

7. Hirulog

Hirulog peptides have been characterized biochemically extensively. They are specific thrombin inhibitors and in the *in vitro* clotting studies they appear to be potent anticoagulants (Ofosu et al. 1992, Witting et al. 1992, Maraganore et al. 1990). Hirulog has been compared to heparin as an adjunct to streptokinase and aspirin in patients with acute myocardial infarction (Lidon et al. 1994). Based on the favorable findings for hirulog, a larger trial is now underway for the same indication (Weitz et al. 1995).

A large phase 3 trial, Hirulog Angioplasty Study, compared hirulog to heparin in patients undergoing coronary angioplasty for unstable angina or for ongoing ischaemia after myocardial infarction (Strony et al. 1995). Hirulog was found to be superior in

high-risk patients undergoing angioplasty after myocardial infarction, although there was no difference in the rate of primary outcome events (mortality, myocardial infarction, emergency bypass) overall. The same study also revealed that hirulog does not prevent restenosis after coronary angioplasty (Burchenal et al. 1995), even though the thrombin rebound effect is more suppressed with hirulog than with heparin (Strony et al. 1995).

In the TIMI 7 trial, the effects of hirulog were examined in patients with unstable angina receiving aspirin. Although death and myocardial infarction were reduced at the higher doses of hirulog, there were no heparin controls included in this study.

Hirulog also has been evaluated in prophylaxis of venous thrombosis in high-risk patients undergoing major orthopaedic surgery. The highest hirulog dose used proved to be as effective as other forms of prophylaxis, including low molecular weight heparin (Ginsberg et al. 1994).

G. Drug Interactions

Polypharmacology is necessary for an effective approach in the management of thrombotic disorders, ranging from disseminated intravascular coagulation (DIC) to deep venous thrombosis (DVT) to acute myocardial infarction (AMI). While different classes of drugs currently used adjunctly have different targets, all of which contribute to the ultimate safety and efficacy outcome, little has been done to study the direct interactions of the antithrombotic agents with other drugs. Because of the complex nature of thrombotic disorders, the impact of drug interactions in various disorders has to be carefully assessed.

The primary agents used in management of thrombotic disorders include either

antiplatelet agents or inhibitors of thrombin and/or factor Xa. Fibrinolytics (thrombolytic drugs) are also used in many of these disorders, often in conjunction with anticoagulants. Since thrombolytic drugs are believed to target clots, relatively little attention has been given to interactions of these agents with anticoagulant drugs such as heparin and newly developed antithrombin agents. Antithrombin drug-induced impairment of physiologic fibrinolysis may result in a hypercoagulable state, whereas impairment of pharmacologic thrombolysis could lead to a compromised outcome, despite a strong systemic anticoagulant state.

Although the above described thrombin inhibitors have been studied in terms of their antithrombotic activity, little work has been completed in the area of fibrinolysis. So far, only D-Phe-Pro-Arg-H, D-Me-Phe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H, argatroban and D-Phe-Pro-ArgCH_2Cl have been studied *in vitro*, for effects in fibrinolytic mechanisms. A fibrin plate assay and thrombelastography were used to compare the three arginals (Barabas et al. 1993) and it was concluded that the first two arginals were more selective than third one for thrombin in comparison to fibrinolytic enzymes such as plasmin, urokinase and streptokinase. In addition, the first two arginals were found to promote fibrinolysis (Barabas et al. 1993). The effects of argatroban on the clot lysis of human and rabbit plasma demonstrated that argatroban shortens the time to clot lysis in these systems when fibrin is cross-linked (Tamao et al. 1986). In contrast, argatroban has no effect in clot lysis when fibrin is not cross-linked (Tamao et al. 1986), indicating that argatroban prevents thrombin from activating factor XIII which is the fibrin cross-linking enzyme. Furthermore, the same group demonstrated that argatroban had weak

inhibitory activities against tissue and urokinase type plasminogen activators. D-Phe-Pro-ArgCH_2Cl was found to inhibit tPA and plasmin (Gilboa et al. 1988), suggesting this agent to be an anti-fibrinolytic agent.

Only a few animal models of thrombolysis have been studied for *in vivo* effects of antithrombin agents on fibrinolytic processes. The thrombin inhibitors examined in these model have been argatroban, D-Me-Phe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H, D-Phe-Pro-ArgCH_2Cl, hirulog-1 and hirudin. Argatroban has been shown to either decrease time to reperfusion and/or increase time to reocclusion when administered in conjunction with a fibrinolytic agent in dog and rabbit models of thrombolysis. In a dog model of coronary arterial thrombosis, occlusive thrombi are produced by mechanical injury to the vessel's endothelium (Martin et al. 1993). Thus the clots formed are platelet-rich and the amount of fibrin is minimal. Argatroban was shown to improve reperfusion in this model. In another model of dog femoral arterial thrombosis, the occlusive thrombus was formed with thrombin (Yasuda et al. 1990). Thus, this clot structure was more fibrin-rich. In this model, it was found that although time to reperfusion was not affected, time to reocclusion was prolonged by argatroban. In a dog model of femoral arterial thrombosis, occlusive thrombi were again produced by mechanical injury to the vessel's endothelium and thus platelet-rich clots are formed (Mellot et al. 1990). In these studies, argatroban enhanced time to thrombolysis. In the rabbit model of carotid artery occlusion, the damage to the endothelium was chemically induced and the thrombi thus generated were platelet aggregates (Tamao et al. 1986). Here, argatroban enhanced thrombolysis. In a model of femoral artery thrombosis model, the occlusive clot was

generated by thrombin and thus was a fibrin structure (Schneider 1991, Jang et al. 1990). In this model, argatroban was also found to increase time to reocclusion and decrease time to reperfusion in conjunction with a fibrinolytic agent.

The D-Me-Phe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H were studied for their effects on time to reperfusion in a dog model of coronary artery thrombosis, where the occlusive thrombus was induced electrochemically, thus producing a platelet-rich clot (Jackson et al. 1993). It was found that while the D-Me-Phe-Pro-Arg-H increased the time to reocclusion when used with tPA, the time to reperfusion was not influenced. Furthermore, Boc-D-Phe-Pro-Arg-H increased the time to reperfusion when used adjunctly with tPA. This finding indicates a fibrinolytic compromise induced by this thrombin inhibitor, which in contrast to the first arginal is less specific for thrombin.

D-Phe-Pro-ArgCH_2Cl, hirulog-I and hirudin were compared in a rat model of abdominal aortic thrombolysis (Klement et al. 1992). The occluding thrombus was produced by a combination of endothelial injury and blood flow stasis. In these studies it was found that all three agents prolonged the time to reocclusion and the number of reocclusions. Interestingly enough, while D-Phe-Pro-ArgCH_2Cl and hirulog-I decreased the time to thrombolysis, hirudin did not, suggesting that hirudin does not access clot-bound thrombin as effectively as the other two agents. However, the doses of these agents were based on their prolongation of activated partial thromboplastin time (APTT) to similar extents. The usage of this global clotting test to decipher equivalent doses of thrombin inhibitors with distinct mechanisms of thrombin inhibition is arguable. Hirudin was also studied in a dog model of coronary artery occlusion (Sitko et al. 1992, Martin

et al. 1992, Lunch et al. 1994). Here, the occluding thrombus was induced by electrical damage to the vessel and hirudin was found to decrease the time to reperfusion and increase the time to reocclusion.

The above observations, both *in vitro* and *in vivo*, indicate that the effects of thrombin inhibitors on fibrinolytic processes are complex and depend not only on the specificity of the inhibitors for thrombin in comparison to other enzymes, but also on the structure and type of the clot used to study the effects, the fibrinolytic agent used to lyse the clot and the endpoints measured.

Drug interactions play an important role in the overall safety and efficacy of anticoagulant agents such as thrombin inhibitors. In many indications, antithrombin agents are administered in conjunctions with other drugs. Many of the patients are often on other medications. A systematic study on the interactions of antithrombin agents with other drugs is not available. However, it wold be feasible to develop a testing system for thrombin inhibitors before this interaction with other drugs can be studied.

Hirudin, unlike other peptide or peptidomimetic antithrombin agents, because of its high specificity for thrombin does not exhibit any complex interactions (Callas et al. 1995, Fareed et al. 1995). Its actions are readily predictable because of the known pharmacologic profile. However, dosage optimization in the presence of other anticoagulant/ antithrombotic drugs is important. Hirudin and the other thrombin inhibitors are expected to exhibit augmentation of the anticoagulant actions of oral anticoagulant actions of oral anticoagulant drugs since these agents suppress the formation of functional forms of factors II, VII, IX and X. In addition, these agents also augment

the functional forms of protein C and S. In this situation, hirudin and the low molecular weight thrombin inhibitors are capable of inhibiting thrombomodulin-bound thrombin, which is needed for the activation of protein C. The consequences of this process are not known. While there is no preclinical or clinical data available, the *in vitro* studies suggest that thrombin inhibitors may produce other additive or synergistic actions of the anticoagulant effects of these agents. Dosage adjustments may be required depending on the dose of heparins, to optimize the therapeutic index of the direct antithrombin agents.

Most of the cardiovascular patients are on aspirin for prophylactic or therapeutic reasons. Since thrombin plays a key role in the activation of platelets, thrombin inhibitors are expected to produce augmentation or synergism of the antiplatelet actions of aspirin. This may be a complex situation, however, it would result in compromised hemostasis. The bleeding complications observed in the TIMI 9A and GUSTO II trials may be attributable to the use of aspirin by the patients included in this study (Antman and TIMI 9A Investigators 1994, GUSTO II Investigators 1994). This is supported by a recent report (Breddin et al. 1996), the interaction of hirudin and aspirin on normal healthy human volunteers resulted in prolongation of the bleeding time which was not correlated with any of the platelet function tests nor any of the coagulation parameters. On the other hand, the interaction may also be of clinical benefit to the patients if the dose of those agents are optimized. Other platelet interactions such as the IIb/IIIa inhibitors and ADP antagonists are expected to have similar interactions with hirudin.

Unlike many direct thrombin inhibitors, hirudin does not produce any direct inhibition of such fibrinolytic enzymes as plasmin and plasminogen activators. However,

hirudin is capable of inhibiting thrombomodulin-bound thrombin which would result in inhibition of the anticoagulant action of thrombin (activation of protein C) and thus precipitate a prothrombotic situation at higher doses. Furthermore, hirudin does augment the anticoagulant actions of the fibrinogen/fibrin degradation products in various experimental settings. Thus, hirudin is expected to produce an augmentation of the thrombolytic efficacy and may prevent rethrombosis (reocclusion). The net effect of hirudin can be validated only in clinical trials.

H. Synopsis

There is a continual influx of both the basic and clinical data in the area of thrombin inhibitors. Ever since the introduction of commercially feasible antithrombin agents such as hirudin and other synthetic inhibitors of thrombin, a strong interest in the development of these drugs is eminent. It should also be noted that thrombin plays an important role in several biological process such as mitogenesis, cellular proliferation, vascular modulation and the modulation of ion channeling processes. The focus of this review is primarily on the thrombogenic effects of this enzyme (thrombin), in context with the pharmacology of various thrombin inhibitors included in this dissertation. It is conceivable that many of the thrombin inhibitors discussed in this review also modulate non-clotting/thrombogenic functions of thrombin. Additional information on some of these actions of thrombin have been extensively reviewed. At the present time, additional information on the basic mechanism of thrombin as a procoagulant and the behavior of thrombin inhibitors as anticoagulant/ antithrombotic drugs is far from complete. A periodic update on the subject is therefore essential.

CHAPTER III

MATERIALS AND METHODS

A. Synthesis and Characterization of Serine Protease Inhibitors

The tripeptide aldehydes D-Phe-Pro-Arg-H · H_2SO_4 (GYKI 14166), D-MePhe-Pro-Arg-H · H_2SO_4 (GYKI 14766) and Boc-D-Phe-Pro-Arg-H · ½H_2SO_4 (GYKI 14451) were synthesized and were obtained from the Institute for Drug Research, Budapest, Hungary. All of these agents were provided in dried powder forms and were kept in refrigerated desiccators. The procedures for the synthesis and the chemical characterization of these compounds has been described (Bajusz et al. 1975, 1978, 1983, 1984, 1987, 1990). Because the free tripeptide aldehyde D-Phe-Pro-Arg-H was found to be an unstable compound and its functional inactivation was associated with the formation of a less polar heterocyclic derivative, N substitution was employed to synthesize more stable analogues. While the N-Boc derivative (Boc-D-Phe-Pro-Arg-H) was more stable than the free tripeptide aldehyde, it was not as specific as the other agents. On the other hand, N-alkyl substitution resulted in synthesis of stable D-Phe-Pro-Arg-H analogues, such as D-MePhe-Pro-Arg-OH, that retained the basic amino terminus characteristic which confers specificity for thrombin. The structures of these peptide aldehydes are depicted in Fig. 3. The formula molecular weights of each of these peptides is D-Phe-Pro-Arg-H · H_2SO_4 [500.6], D-MePhe-Pro-Arg-H · H_2SO_4 [514.6] and Boc-D-Phe-Pro-Arg-H · ½H_2SO_4

[551.6] respectively. Stock solutions of these agents were made in appropriate buffers and saline prior to use. The saline stock solutions were frozen at -70°C and thawed only once prior to use. The same buffer and saline were used to make serial dilutions of these agents.

The compound Ac-(D)Phe-Pro-boroArg-OH · HCl (DuP 714) was obtained from DuPont Merck Pharmaceutical Company, Wilmington, DE, in a powder form. The procedure for the synthesis and chemical characterization of this compound has been published (Kettner et al. 1990). The structure of this compound is depicted in Fig. 3. The formula molecular weight for this compound is 496.8. Stock and working solutions of this agent were also made prior to use in the appropriate buffers or saline. As with the peptide aldehydes, the saline stock solutions of this agent were frozen at -70°C and thawed once prior to usage.

Argatroban, (2R,4R)-4-methyl-1-(N^2-((3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidine carboxylic acid, consists of a mixture of the diastereoisomers 21-(R)- and 21-(S)- around the 2- and 4- positions of the piperidine ring, in a ratio of approximately 65:35. The method for the synthesis of this compound was described by Okamoto and Hijikata (1981). Argatroban was obtained from Texas Biotechnology Corp., Houston, TX. This compound was provided in a 0.5 mg/ml solution. Light resistant vials containing 10 mg of argatroban in 20 ml sterile isotonic solution were provided and kept at room temperature. The structure of this compound is depicted in Fig. 3. The formula molecular weight of this compound was 532. Further dilutions of argatroban were made also in saline and appropriate buffers prior to use.

Recombinant hirudin variant 1 (rHV-1) was obtained from Knoll AG, Ludwigshafen, Germany in lyophilized form in vial containing 20 mg, 17,000 ATU/mg, (Batch No: 00300AL). This recombinant material was produced in *Escherichia coli* and was a single 65 amino acid polypeptide chain. The primary structure of this hirudin is given in Fig. 4. The purity was >95% with 0.5% moisture. The additives were succinate and sodium chloride. The molecular weight for this compound is 6,963. The specification data provided with this material are found in Appendix 1. Stock and working solutions of his agent were also made in saline and appropriate buffers. The saline stock solutions were frozen at -70°C and thawed once prior to use.

Unfractionated porcine mucosal heparin (PMH, lot no. RB 21055) was obtained from Sanofi, Choay Institut, Paris, France. It was provided in a white powder form as a sodium salt. The molecular weight for this compound is 10,700. The specific activity of this agent was found to be 160 U/mg.

Aprotinin is broad spectrum Kunitz-type serine protease inhibitor. Aprotinin was obtained in powder form from Pentapharm AG, Basel, Switzerland (6,120 KIU[1]/mg). This compound is a Kunitz type of inhibitor, consisting of a single 58 amino acid polypeptide chain, with a molecular weight of 6,512, extracted from bovine lungs (Fritz and Wunderer, 1983). The primary structure of aprotinin is depicted in Fig. 4. This polypeptide chain is cross-linked by 3 disulfide bridges (Fritz and Wunderer, 1983).

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[1]1 Kallikrein Inactivator Unit (KIU) = amount of aprotinin that reduces the biologic activity of 2U of kallikrein by 50%

B. Analytical Profile of Peptide Thrombin Inhibitors

Due to the instability of the D-Phe-Pro-Arg-H compound, methods for determining the purity and stability of all tripeptide thrombin inhibitors studied in these experiments were utilized. The HPLC system utilized for these studies was a Waters A-45 system (2 Waters 510 HPLC pumps, Waters 712 Waters Intelligent Sample Processor, Waters 484 Tunable Absorbance Detector). The column used was a reversed phase analytical column (RAININ Microsorb-MV HPLC Column, C18, 5 μm, 100 Å, reversed-phase column, 4.6 mm ID x 25 cm). The detection was at 214 nm. The injection volume was 25 μl of 1 mg/ml of each peptide.

The eluent systems and gradients for determining the purity of the tripeptide aldehydes (D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H) were suggested and described in detail by Dr. Sandor Bajusz (Institute for Drug Research, Budapest, Hungary) and also published by Bajusz et al. (1975, 1978). All peptides eluted in 3 peaks corresponding to equilibrium structures, as was described by Bajusz et al. 1990. All other additional peaks were due to impurities (side products, epimers, racemized derivatives etc.). The amount of each peptide was determined by summing the area under the three equilibrium peaks and subtracting it from the area under all background-adjusted peaks.

The peptide Ac-(D)Phe-Pro-boroArg-OH was also profiled by an HPLC method, utilizing the same equipment and parameters as with the peptide aldehydes. The eluent system and gradients for determining the purity of Ac-(D)Phe-Pro-boroArg-OH, were suggested by Dr. Charles Kettner (DuPont-Merck Pharmaceuticals, Wilmington, DE).

1. D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H Purity Determination

The purity and concentration of the tripeptide aldehyde thrombin inhibitors was determined by standard HPLC methods, using the above described system. The specification data provided with these materials are provided in Appendix 2. The two eluent systems, for pump A and pump B respectively, were distilled water with 0.1% trifluoroacetic acid (in which the peptides are dissolved at concentrations of 1 mg/ml) and acetonitrile with 0.1% trifluoroacetic acid (as the gradient). For D-Phe-Pro-Arg-H · H_2SO_4 and D-MePhe-Pro-Arg-H · H_2SO_4, the flow rate was 1.2 ml/min and the gradient was 0-5 min: 0 → 8% pump B

5-30 min: 8% pump B (isocratic)

30-45 min: 8 → 60% pump B

45-50 min: 60% pump B (isocratic)

50-55 min: 60 → 0% pump B

55-60 min: 0% pump B

For the Boc-D-Phe-Pro-Arg-H · ½H_2SO_4 the flow rate was 1 ml/min and the gradient was 0-10 min: 0 → 30% pump B

10-25 min: 30% pump B (isocratic)

25-40 min: 30 → 60% pump B

40-45 min: 60% pump B (isocratic)

45-50 min: 60 → 0% pump B

50-55 min: 0% pump B.

2. Ac-(D)Phe-Pro-boroArg-OH Purity Determination

The purity and concentration of the tripeptide boronic acid thrombin inhibitor was determined by using the above described system, as described by Dr. Kettner. The specification data provide with this material are in Appendix 3. The two eluent systems, for pump A and pump B respectively, were distilled water with 10% methanol and 0.005 M octane sulfonate sodium salt (in which the boronic acid derivative was dissolved at a concentration of 1 mg/ml), and distilled water with 90% methanol and 0.005 M octane sulfonate sodium salt. The flow rate was 1.5 ml/min and the gradient was 54% pump A and 46% pump B. The total run time was 20 min.

C. Analytical Profile of the Unfractionated Heparin

Unfractionated heparins are poly-component agents and depending on the source and method of purification they vary in composition. The unfractionated porcine mucosal heparin used in these studies was characterized by using a published GPC method (Ahsan et al. 1994). The GPC-HPLC hardware used in this study was a Waters A-45 system (2 Waters 510 HPLC pumps, Waters 712 Waters Intelligent Sample Processor, Waters 484 Tunable Absorbance Detector and a differential refractometer joined in series with the outlet end of the columns attached to the tunable absorbance detector), equipped with Expert Ease software designed for polymer analysis. The columns used were a tandem column system (TSK 2000/TSK 3000, Beckman, San Ramon, CA). The mobile phase was 0.5 M Na_2SO_4, the flow rate was 0.5 ml/min and the injection volume was 20 μl. The run time was 65 min and the UV detection was at 234 nm. A simultaneous recording of the heparin profile was also made using the

refractive index detector. The calibration used in this system was described in detail in the publication by Ahsan et al. 1994 and comprised of 19 narrow range calibrators. The heparin used in this study was provided as a white-powder sodium salt and was dissolved in 0.5 M Na_2SO_4 to a concentration of 10 mg/ml for the GPC profiling.

D. Biochemical Assays

1. Thrombin Titration Assay

An amidolytic method was used to determine the direct antithrombin actions of various thrombin inhibitors. This method was developed by Iyer and Fareed (1995), as an assay for the determination of the specific activity of recombinant hirudin and as a tool for quality assurance and for the determination of batch to batch variation. The objective of these studies was to determine and compare the antithrombin potencies of direct thrombin inhibitors in terms of antithrombin units (ATU), utilizing a thrombin titration method. Since the definition of ATU is the amount of thrombin inhibitor that inhibits 1 NIH unit of thrombin, this assay was developed so that the final concentration of thrombin in the system was 1 NIH unit/ml. Therefore, the amount of thrombin inhibitor that produced 100% inhibition of thrombin's activity was designated as the potency of a thrombin inhibitor in ATU/mg. α-thrombin (E.C. # 3.4.21.5, lot α375, specific activity 3,369 U/mg, protein concentration 0.68 mg/ml) was obtained from Dr. John Fenton II, New York State Dept. of Health, Albany, NY. This thrombin was diluted in buffer to a working concentration of 23.5 U/ml. Each of the thrombin inhibitors were diluted to various working concentrations in sterile saline solution (Baxter Healthcare Corp., Deerfield, IL). The amidolytic activity of thrombin was detected with

the chromogenic substrate Spectrozyme®TH (H-D-HHTyr-Ala-Arg-pNA · 2AcOH, American Diagnostica, Greenwich, CT), at a working concentration of 1.5 mM (diluted in deionized water). Cleavage of this substrate by thrombin results in release of the yellow chromophore pNA (para-nitroaniline).

The buffer used to dilute the α thrombin to 23.5 U/ml was composed of Tris (0.05 M), NaCl (0.2 M) and polyethylene glycol 6000 (0.1%), adjusted to pH 7.9. The buffer used for the assay described below was composed of triethanolamine HCl (0.1 M) and NaCl (0.2 M), adjusted to pH 8.4.

The method used was carried out as follows utilizing quartz cuvettes (path length = 1 cm, Beckman Instruments, Inc., Irvine, CA) and a spectrophotometer (DU-7®, Beckman Instruments, Inc., Fullerton, CA): 1975 μl of buffer were mixed and incubated for 2 min at 37°C with 25 μl of the thrombin inhibitor at a particular concentration. 100 μl of thrombin were added to this mixture and allowed to incubate for 1 min at 37°C. 250 μl of Spectrozyme®TH were added and the rate of change in absorbance was measured for the next min at 405 nm. Sterile saline solution was used in place of the thrombin inhibitor as a control.

Residual thrombin activity, remaining after incubation with a thrombin inhibitor was expressed as a percentage of the saline control by dividing the two respective rates of change in absorbance. The final concentration of the thrombin inhibitor was plotted against the residual thrombin activity.

The order of the regression analysis utilized was the lowest that yielded regression coefficients >0.99. The thrombin inhibitor concentration that produced 50% residual

thrombin activity was the concentration that inhibited 0.5 NIH U/ml thrombin and therefore the thrombin inhibitor's ATU was double of that concentration. From these values, the relative antithrombin activity of each agent is calculated in terms of ATU/mg or ATU/nmole. Each concentration-response curve was repeated in triplicate on separate runs.

2. Thrombin Time (TT) in a Fibrinogen Based System

The fibrinogen based TT is a biochemically defined functional method for measuring the antithrombin effects of thrombin inhibitors, using human fibrinogen as the substrate for thrombin. While the thrombin titration method, described in the previous section, provides a measurement of the antithrombin activity of thrombin inhibitors utilizing thrombin's amidolytic activity, the fibrinogen based TT provides a measurement of the antithrombin activity of thrombin inhibitors utilizing thrombin's biologic activity , where soluble fibrinogen is transformed into fibrin. To perform the TT assay, a known amount of preformed human thrombin is added to the fibrinogen sample (containing varying amounts of a thrombin inhibitor) and the time required for clot formation measures the rate at which fibrin is formed. TT measurements were carried out using a solution of human fibrinogen (IMCO, Stockholm, Sweden, $>97\%$ clottable fibrinogen, each vial containing 0.25g fibrinogen, 0.1g sodium chloride and 0.25g sodium citrate, 3 μg plasminogen/mg fibrinogen and <5 μg plasmin/g fibrinogen) at 100 mg/dL, made in Owren's Veronal Buffer (Baxter Dade, Miami, FL). Each of the thrombin inhibitors was diluted at various concentrations in the buffered fibrinogen solution. Fibrindex™ brand of human thrombin (lyophilized, 50 U/ampule, Ortho Diagnostic Systems, Raritan,

NJ) at a concentration of 10 NIH U/ml in 0.025 M $CaCl_2$, or in sterile saline solution (Baxter Healthcare Corp., Deerfield, IL) was used to initiate fibrinogen conversion to fibrin. In this procedure 100 μl of 10 U/ml human thrombin prewarmed at 37°C were added to 200 μl of fibrinogen prewarmed at 37°C for 3 min, and the clotting time was measured. Each concentration-response curve was repeated in triplicate on separate days.

3. Antithrombin Assays

The antithrombin assays provided a measurement of thrombin inhibitor concentration, utilizing the inhibitory effect against the amidolytic activity of thrombin (Fibrindex™), quantitated by a chromogenic substrate for thrombin (Spectrozyme®TH). This method was initially described by Walenga et al. 1983. Calibration curves (*in vitro* supplementation of thrombin inhibitors at various concentrations) were constructed with both normal human plasma and normal rabbit pool plasma. Due to the differences in blood clotting factor levels between human and rabbit plasma, the antithrombin amidolytic assay method varied, depending on the source of the plasma.

Regardless of the species the plasma was obtained from and the procedure followed, the thrombin inhibition by each agent at each concentration or dose was calculated by the following formula: % thrombin inhibition by agent = [1 - agent ΔA_{405} / saline ΔA_{405}] x 100

The agent concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each agent. Since the molecular weights of these agents are known, all data were converted to molar concentrations or doses. Each concentration-response curve was repeated in triplicate on separate days.

a. Preparation of Normal Human Plasma (NHP)

Normal human plasma was obtained from the Loyola Blood Bank and was certified to be free of viral contamination (tested for Hepatitis B surface antigen, Hepatitis B antibodies, Hepatitis C antibodies, Hepatitis core antibodies, HIV 1 and 2 antibodies, HTLV 1 antibodies, STS antibodies). The blood was collected from normal healthy human volunteers in bags containing CPDA-1 mixture (26.3 g/L trisodium citrate, 3.27 g/L citric acid, 31.9 g/L dextrose, 2.22 g/L monobasic sodium phosphate, and 0.275 g/L adenine), so that 450 ml of blood were mixed with 63 ml of CPDA-1 anticoagulant-preservative. Within 8 hours of collection, this blood was centrifuged at 1,200xg for 30 min at 4°C. The separated plasma was then frozen at -70°C. Routinely, four or five frozen plasma bags were obtained from the blood bank and these were thawed and the plasmas were mixed to obtain a pool. This pool was then aliquoted and re-frozen at -70°C. The aliquots were thawed only once and used as needed. The unused pool plasma was appropriately disposed.

b. Antithrombin Amidolytic Assay with NHP

Each of the thrombin inhibitors was diluted in NHP to obtain various concentrations. When human plasma samples were evaluated, the assay was carried out in a fast kinetic centrifugal analyzer (ACL 300 plus, Instrumentation Laboratory, Lexington, MA). The procedure for the antithrombin amidolytic assay was as follows: 10 μl of a sample containing thrombin inhibitor were incubated with 100 μl of 5 U

thrombin (diluted in Tris/EDTA buffer[2]) for 60 sec and then 40 μl of the thrombin substrate (1 mM in deionized H_2O) were added and the optical density was measured over a period of 30 sec.

c. Preparation of Normal Rabbit Plasma (NRP)

To study the concentration-dependent effects of thrombin inhibitors in the various plasma based assays and to prepare calibration curves, pools of rabbit plasma were prepared. To obtain these pools, 10 to 15 white male New Zealand rabbits weighing 2.5 to 3.5 kg were obtained from LSR Industries, Union Grove, WI and housed individually. The animals were kept on standard rabbit chow diet and had access to water *ad libitum.* Each rabbit was lightly anesthetized with ketamine hydrochloride (Ketaset®, 100 mg/ml, Aveco Co. Inc., Fort Dodge, IA) at a dose of 50 mg/kg I.M. in combination with xylazine (Rompun®, 100 mg/ml, Mobay Corporation, Shawnee, KS) at a dose of 25 mg/kg I.M. Blood was collected through the major ear artery of each rabbit. The first 3 ml were discarded, while the subsequent 9 ml were collected in 3.8% sodium citrate (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ). The rabbits were then monitored for 20 min to ensure hemostasis and then returned to their cages. Subsequently, the blood was centrifuged (Beckman GPR Centrifuge, Beckman Instruments Inc., Irvine CA) at 1200xg at 4°C for 20 min to achieve platelet poor plasma which was pooled, mixed and frozen in aliquots at -70 °C. Aliquots were thawed and used immediately as needed (never re-frozen).

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[2]Tris/EDTA buffer:50mM Trizma Base, 175mM NaCl, 7.5mM $Na_2EDTA \cdot 2H_2O$,pH 8.4.

d. Antithrombin Amidolytic Assay with NRP

Each of the thrombin inhibitors was supplemented at various concentration to NRP. When rabbit plasma samples were tested, the antithrombin assay was carried out in a spectrophotometer (DU-7®), utilizing quartz cuvettes. The procedure for the antithrombin amidolytic assay was as follows: 25 μl of a sample containing thrombin inhibitor were incubated with 400 μl of Tris/EDTA buffer[2] at 37°C for 1 min. Then, 25 μl of 10 U thrombin (diluted in sterile saline solution) were added and incubated for 1 min at 37°C. At the end of the incubation period, 50 μl of the thrombin substrate (1 mM in deionized H_2O) were added and the rate of change of optical density was measured over a period of 1 min at 405 nm.

4. Anti-Xa Assays

The anti-Xa assay provided a measurement of the relative inactivating actions of various agents, by measuring their inhibitory effects against the amidolytic activity of factor Xa. Human factor Xa (E.C. # 3.4.21.6, 0.65 ng/ml, Enzyme Research, South Bend, IN), was analyzed using a chromogenic substrate for factor Xa (Spectrozyme®FXa, MeO-CO-D-CHG-Gly-Gly-Arg-pNA · AcOH, American Diagnostica, Greenwich, CT). This method was described by Walenga et al. (1983). Calibration curves (*in vitro* supplementation of thrombin inhibitors at various concentrations) were constructed with both normal human plasma and normal rabbit plasma. Due to the differences in factor concentrations between human and rabbit plasma, the anti-Xa amidolytic assay method differed, depending on the source of the plasma.

Regardless of the species the plasma was obtained from and the procedure followed, the factor Xa inhibition by each agent at each concentration or dose was calculated by the following formula: % factor Xa inhibition by agent = [1 - agent ΔA_{405} / saline ΔA_{405}] x 100

The agent concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each agent. Since the molecular weights of these agents are known, all data were converted to molar concentrations or doses. Each concentration-response curve was repeated in triplicate on separate days.

a. Anti-Xa Amidolytic Assay with NHP

Each of the thrombin inhibitors was diluted to various concentrations in NHP. When human plasma samples were evaluated, the assay was carried out in a fast kinetic centrifugal analyzer (ACL 300 plus). The procedure for the anti-Xa amidolytic assay was as follows: 10 μl of a sample containing thrombin inhibitor were incubated with 100 μl of factor Xa (6.6 pg/ml, diluted in Tris/EDTA buffer[2]) for 300 sec and then 75 μl of the factor Xa substrate (2.5 mM in deionized H_2O) were added and the optical density was measured over a period of 30 sec.

b. Anti-Xa Amidolytic Assay with NRP

Each of the thrombin inhibitors was supplemented at various concentration to NRP. When rabbit plasma samples were tested, whether *in vitro* supplemented with a thrombin inhibitor or obtained during the rabbit stasis thrombosis model for *ex vivo* analyses, the anti-Xa assay was carried out in a spectrophotometer (DU-7®) utilizing

quartz cuvettes. The procedure for the anti-Xa amidolytic assay was as follows: 25 μl of a sample containing thrombin inhibitor were incubated with 375 μl of Tris/EDTA buffer[2] at 37°C for 2 min. Then, 50 μl of factor Xa (5 nkat/ml, diluted in Tris/EDTA buffer[2]) were added and incubated for 2 min at 37°C. At the end of the incubation period, 50 μl of the factor Xa substrate (2.5 mM in deionized H_2O) were added and the rate of change of optical density was measured over a period of 1 min at 405 nm.

5. Inhibitory Actions of Various Thrombin Inhibitors on Specific Serine Proteases

Although some thrombin inhibitors are designed to inhibit the catalytic site of thrombin, because of the similarity between the catalytic sites of all serine proteases, it is possible that the agents that inhibit the catalytic site of thrombin, also inhibit the catalytic site of other serine proteases. The following assays were designed to examine the inhibitory effects that thrombin inhibitors may have against other serine proteases, utilizing the amidolytic activity of these enzymes, in purified systems. The general procedure followed in the following assays was conducted on a fast kinetic centrifugal analyzer (ACL 300 plus). Generally, each thrombin inhibitor was diluted in sterile saline solution at various concentrations. Ten μl of the thrombin inhibitor were incubated with 100 μl a known amount of the specific enzyme for 1 min. At the end of the incubation period, 40 μl a chromogenic substrate specific for the enzyme was added and the optical density was measured over a period of 999 sec. The serine protease inhibition by each thrombin inhibitor at each concentration was calculated by the following formula:

% protease inhibition = [1 - inhibitor ΔA_{405} / saline ΔA_{405}] x 100

The thrombin inhibitor concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each agent. Since heparin is devois of any direct serpin activities and its actions are mediated primarily through potentiation of antithrombin, the stock solution of heparin was made as 10 mg/ml in 1 U/ml antithrombin (Kabi Vitrum, Stockholm, Sweden). Further dilutions of this mixture were made in saline, as were for the other thrombin inhibitors. In addition to the thrombin inhibitors, aprotinin was also tested in these system as a positive control for the evaluation of the thrombolytic enzymes. Since the molecular weights of these agents are known, all data were converted to molar concentrations. Each concentration-response curve was repeated in triplicate on separate days.

a. Inhibition of Chymotrypsin

Chymotrypsin (E.C. # 3.4.21.1, Zolyse®, from ox pancreas, 750 U.S.P. units/vial) was obtained from Ben Venue Laboratories, Inc., Bedford, OH. Chymotrypsin was used at a final assay concentration of 6.67 U/ml. The chromogenic substrate used to detect the activity of chymotrypsin was S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA · HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden), at a final assay concentration of 0.27 mM.

b. Inhibition of Trypsin

Porcine pancreatic trypsin (E.C. # 3.4.21.4, type II, crude, trypsin activity: 1740 BAEE units[3]/mg, chymotrypsin activity: 1150 ATEE units[4]/mg) was obtained from

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[3]1 BAEE unit = ΔA_{253} of 0.001/min in 3.2 ml at pH 7.6 at 25°C

Sigma Chemical Company, St, Louis, MO. Trypsin was utilized at a final assay concentration of 66.67 μg/ml . The chromogenic substrate used to detect the activity of trypsin was S-2423 (CH_3-CO-Ile-Glu-Gly-Arg-pNA · HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden), at a final assay concentration of 0.27 mM.

c. Inhibition of Activated Protein C (APC)

APC (E.C. # 3.4.21.69) was obtained from American Red Cross, Holland Laboratories, Rockville, MD, lyophilized, 5 mg/vial. The final assay concentration of APC was 62.5 μg/ml. S-2288 (H-D-Ile-Pro-Arg-pNA · 2HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden) was the chromogenic substrate used to detect the activity of APC, at a final assay concentration of 0.27 mM.

d. Inhibition of Glandular Kallikrein

Pancreatic kallikrein (E.C.# 3.4.21.35, 4.7 units[5]/mg solid, 4.8 units/mg protein) was obtained from Sigma Chemical Company, St, Louis, MO. kallikrein was used at a final assay concentration of 0.67 mg/ml. The chromogenic substrate used to detect the activity of kallikrein was S-2302 (H-D-Pro-Phe-Arg-pNA · 2HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden), at a final assay concentration of 0.27 mM.

e. Inhibition of Tissue Plasminogen Activator (tPA)

tPA (E.C. # 3.4.21.68) was obtained from Genentech, South San Francisco, CA

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[4]1 ATEE unit = ΔA_{237} of 0.001/min in 3 ml at pH 7.0 at 25°C

[5]1 unit hydrolyzes 1.0 μM of α-N-Benzoyl-Arg-Ethyl-Ester (BAEE) to α-N-Benzoyl-L-Arg and EtOH/min at pH 8.7 at 25°C

(Activase®, Alteplase, recombinant, 50 mg or 29 million IU/vial). The final assay concentration of t-PA was 0.67 mg/ml. Spectrozyme®TH was the chromogenic substrate used to detect the t-PA activity, at a final assay concentration of 0.27 mM.

f. Inhibition of Urokinase

Urokinase (E.C. # 3.4.21.73, Abbokinase®, 250,000 I.U./vial, from human kidney cells by tissue culture, low molecular weight form) was obtained from Abbott Laboratories, North Chicago, IL. The final assay concentration of urokinase was 6,667 U/ml. The chromogenic substrate used to detect the urokinase activity was S-2444 (<Glu-Gly-Arg-pNA · HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden) at a final assay concentration of 0.27 mM.

g. Inhibition of Plasmin

Plasmin (E.C. # 3.4.21.7, 6 nKat/vial) was obtained from Diagnostica Stago, Asnieres-Sur-Seine, France. Plasmin was utilized at a final assay concentration of 1.2 nKat/ml. The chromogenic substrate used to detect the activity of plasmin was S-2251 (H-D-Val-Leu-Lys-pNA · 2HCl, Kabi Diagnostica, KabiVitrum, Stockholm, Sweden), at a final assay concentration of 0.27 mM.

6. Inhibition of Plasminogenolysis

While the direct effects of thrombin inhibitors on serine proteases were studied utilizing the above described assay systems, the relevance of these effects to the process of activation of plasminogen to plasmin, or plasminogenolysis, was not addressed. Plasminogenolysis after activation by streptokinase or t-PA was measured in the systems

described below. The source of the plasminogen was either purified plasminogen (25 CU[6], 15 CU/mg, AB KABI, Stockholm, Sweden), or pooled human blood bank plasma (Loyola Blood Bank).

In all plasminogenolysis assays, the inhibition of plasminogenolysis by each thrombin inhibitor and aprotinin at each concentration was calculated by the following formula: % inhibition = [1 - inhibitor ΔA_{405} / saline ΔA_{405}] x 100

The inhibitor concentration versus % inhibition response curves provided the basis for the calculation of the IC_{50} and IC_{25} for each agent. Since the molecular weights of these agents are known, all data were converted to molar concentrations. Each concentration-response curve was repeated in triplicate.

a. Pure Plasminogen System

In these assays, each thrombin inhibitor and aprotinin was diluted at various concentration in a TRIS/EDTA buffer[2]. Pure plasminogen at a final assay concentration of 0.97U/ml was lysed by either streptokinase (E.C. # 3.4.22.10, purified streptokinase from Group C streptococci, 600,000 I.U./vial, AB KABI, Stockholm, Sweden) at a final assay concentration of 2,500 U/ml, or t-PA (Activase®) at a final assay concentration of 5,000 U/ml. The generated plasmin was detected with a substrate specific for plasmin (Spectrozyme®PL, H-D-Nle-HHTyr-Lys-pNA · 2AcOH, American Diagnostica, Greenwich, CT), at a final assay concentration of 0.75 mM. The effects of thrombin inhibitors and of aprotinin on the process of plasminogenolysis were studied on a

[6]CU = casein unit (Sgouris et.al, 1960)

spectrophotometer (model 35, Beckman Instruments, Inc., Fullerton, CA) and quartz cuvettes. 750 μl of a thrombin inhibitor were incubated for 1 min at 37°C. At the end of this incubation period, 100 μl of streptokinase or t-PA and 25 μl of plasminogen were added and the mixture was incubated for 3 min at 37°C. At the end of this incubation period, 100 μl of Spectrozyme®PL were added and the rate of change of optical density was measured over a period of 1 min.

b. NHP System

In these assays, each thrombin inhibitor and aprotinin was diluted at various concentration in a TRIS/EDTA buffer[2]. NHP at a final assay dilution of 1:10, was used as the source of human plasminogen. The plasminogen contained within the NHP was lysed by either streptokinase (AB KABI) at a final assay concentration of 2,500 U/ml, or t-PA (Activase®) at a final assay concentration of 5,000 U/ml. The generated plasmin was detected with a substrate specific for plasmin (Spectrozyme®PL) at a final assay concentration of 0.75 mM. The effects of thrombin inhibitors and of aprotinin on the process of plasminogenolysis were studied on a spectrophotometer (model 35) and quartz cuvettes. 750 μl of a thrombin inhibitor were incubated for 1 min at 37°C. At the end of this incubation period, 100 μl of streptokinase or t-PA and 50 μl of NHP were added and the mixture was incubated for 3 min at 37°C. At the end of this incubation period, 100 μl of Spectrozyme®PL were added and the optical density was measured over a period of 1 min.

7. Inhibition of Extrinsic Activation Systems in Fibrinogen Deficient Human Plasma by Thrombin Inhibitors

While the antithrombin and anti-Xa assays are useful in determining the direct antithrombin or anti-Xa effects of thrombin inhibitors, they do not provide information on the effects of these inhibitors on the overall process of coagulation, or on the generation of thrombin and factor Xa. These plasmatic methods were developed by Kaiser et al. 1992, and were used to determine the thrombin and factor Xa generation inhibitory actions of various thrombin inhibitors, following extrinsic activation of the coagulation cascade. In these assays the materials used were: chromogenic substrate specific for thrombin (Spectrozyme®TH), chromogenic substrate specific for factor Xa (Spectrozyme®FXa), fibrinogen deficient plasma (fibrinogen < 15mg/dL, George King Biomedical, Overland Park, KS) and extrinsic activator (lyophilized acetone-dehydrated, rabbit brain Thromboplastin C, Dade, Miami, FL). The instrument used was a fast kinetic centrifugal analyzer (ACL 300 plus).

The extrinsic thrombin and factor Xa generation inhibition by each thrombin inhibitor at each concentration was calculated by the following formula:

% thrombin or factor Xa generation inhibition by agent = [1 - agent ΔA_{405} / saline ΔA_{405}] x 100

The thrombin inhibitor concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each thrombin inhibitor. Since the molecular weights of these agents are known, all data were converted to molar concentrations. Each concentration-response curve was repeated in triplicate on separate days.

a. Inhibition of Extrinsic Thrombin Generation

The chromogenic substrate for thrombin was reconstituted to 1 mM with 0.025 M $CaCl_2$ and subsequently mixed with thromboplastin C (1:6). The fibrinogen deficient plasma was diluted with Tris/EDTA buffer[2] (1:8). The procedure for this amidolytic assay was as follows: 60 μl of a known gravimetric amount of thrombin inhibitor diluted in sterile saline solution were incubated with 60 μl of fibrinogen depleted plasma for 300 sec. Then 60 μl of the thrombin substrate mixed with thromboplastin C were added and the optical density was measured over a period of 999 sec.

b. Inhibition of Extrinsic Factor Xa Generation

The chromogenic substrate for factor Xa was reconstituted to 1 mM with 0.025 M $CaCl_2$ and subsequently mixed with thromboplastin C (1:6). The fibrinogen deficient plasma was diluted with Tris/EDTA buffer[2] (1:8). The procedure for this amidolytic assay was as follows: 60 μl of a known gravimetric concentration of thrombin inhibitor diluted in sterile saline solution were incubated with 60 μl of fibrinogen depleted plasma for 300 sec. Then 60 μl of the factor Xa substrate mixed with thromboplastin C were added and the optical density was measured over a period of 999 sec.

8. Inhibition of Intrinsic Activation Systems in Fibrinogen Deficient Human Plasma by Thrombin Inhibitors

While the previous two methods were used to investigate effects of inhibitory agents on the extrinsic activation of coagulation, the following plasmatic methods were used to determine the thrombin and factor Xa generation inhibitory actions of various thrombin inhibitors, following intrinsic activation of the coagulation cascade. These

assays were also developed by Kaiser et al. 1992. In these assays the materials used were: chromogenic substrate specific for thrombin (Spectrozyme®TH), chromogenic substrate specific for factor Xa (Spectrozyme®FXa), fibrinogen deficient plasma (George King Biomedical) and intrinsic activator (Actin, Dade, Miami, FL). The instrument used was a fast kinetic centrifugal analyzer (ACL 300 plus).

The intrinsic thrombin and factor Xa generation inhibition by each thrombin inhibitor at each concentration was calculated by the following formula: % thrombin or factor Xa generation inhibition by agent = [1 - agent ΔA_{405} / saline ΔA_{405}] x 100

The thrombin inhibitor concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each thrombin inhibitor. Since the molecular weights of these agents are known, all data were converted to molar concentrations. Each concentration-response curve was repeated in triplicate on separate days.

a. Inhibition of Intrinsic Thrombin Generation

The chromogenic substrate for thrombin was reconstituted to 1 mM with 0.025 M $CaCl_2$ and subsequently mixed with actin (1:2). The fibrinogen deficient plasma was diluted with Tris/EDTA buffer[2] (1:8). The procedure for this amidolytic assay was as follows: 60 μl of a known gravimetric concentration of thrombin inhibitor diluted in sterile saline solution were incubated with 60 μl of fibrinogen depleted plasma for 300 sec. Then 60 μl of the thrombin substrate mixed with actin were added and the optical density was measured over a period of 999 sec.

b. Inhibition of intrinsic Factor Xa Generation

The chromogenic substrate for factor Xa was reconstituted to 1 mM with 0.025 M $CaCl_2$ and subsequently mixed with actin (1:2). The fibrinogen deficient plasma was diluted with Tris/EDTA buffer[2] (1:8). The procedure for this amidolytic assay was as follows: 60 μl of a known gravimetric concentration of thrombin inhibitor diluted in sterile saline solution were incubated with 60 μl of fibrinogen depleted plasma for 300 sec. Then 60 μl of the factor Xa substrate mixed with actin were added and the optical density was measured over a period of 999 sec.

9. Inhibition of KONYNE® Based Systems by Thrombin Inhibitors

The extrinsic activation systems in human fibrinogen deficient plasma described above are not devoid of intrinsic activation, since the two pathways of coagulation interact at various levels, feedback loops amplify certain steps and all plasma factors with the exception of fibrinogen are present. To develop systems in which only extrinsic activation is possible without interference from the intrinsic factors, prothrombin complex concentrates extrinsically activated were used in the following described methods. These methods were initially developed by Kaiser et al. 1994. These defined methods were used to determine the factor Xa and thrombin generation inhibitory actions of various thrombin inhibitors.

In these assays the materials used were: chromogenic substrate specific for thrombin (Spectrozyme®TH), chromogenic substrate specific for factor Xa (Spectrozyme®FXa), extrinsic activator (Thromboplastin C) and the prothrombin complex KONYNE® (of human origin, lyophilized, 580 U/vial, Cutter Laboratories, Berkeley,

CA), which represents a mixture of factors II, VII, IX and X. The instrument used was a fluorescence light scattering micro centrifugal analyzer (Multistat III, Instrumentation Laboratory, Lexington, MA).

The inhibition of thrombin and factor Xa generation by each thrombin inhibitor at each concentration was calculated by the following formula: % inhibition of thrombin or factor Xa generation = [1 - agent ΔA_{405} at 660 sec / saline ΔA_{405} at 660 sec] x 100

The thrombin inhibitor concentration versus % inhibition curves provided the basis for the calculation of the IC_{50} for each agent. Since the molecular weights of these agents were known, all data were converted to molar concentrations.

a. Inhibition of thrombin Generation from KONYNE®

The buffer used in this procedure was composed of 1 mM Tris/HCl and 6 mM $CaCl_2$, adjusted to pH 8.1. The procedure for this amidolytic assay was as follows: 165 μl of the antithrombin agent diluted in buffer were incubated with 25 μl of the thrombin substrate (2.5 mM in deionized water) and 25 μl of Thromboplastin C (diluted 1:10 in 0.025 M $CaCl_2$) for 30 sec. Then 25 μl of KONYNE® at 10 U/ml working concentration (diluted with sterile saline solution), and 10 μl of buffer were added and the optical density was measured every 60 sec for 12 min.

b. Inhibition of Factor Xa Generation from KONYNE®

The buffer used in this procedure was composed of 1 mM Tris/HCl and 6 mM $CaCl_2$, adjusted to pH 8.1. The procedure for this amidolytic assay was as follows: 165 μl of the antithrombin agent diluted in buffer were incubated with 25 μl of the factor Xa

substrate (2.5 mM in deionized water) and 25 μl of Thromboplastin C (dilute 1:10 in 0.025 M $CaCl_2$) for 30 sec. Then 25 μl of KONYNE® at 10 U/ml working concentration (diluted with sterile saline solution) and 10 μl of buffer were added and the optical density was measured every 60 sec for 12 min.

10. Inhibition of FEIBA® Based Systems by Thrombin Inhibitors

These systems, like the KONYNE® based systems, also determine the inhibitory activities of various agents on the extrinsic activation of the coagulation system. The prothrombin complex KONYNE® used in the above described assays contains factors in their pro-enzyme form only. The prothrombin complex FEIBA® IMMUNO (anti-inhibitor coagulant complex, of human origin, lyophilized, 490 U/vial, Österreichisches Institut für Haemoderivate GES.M.B.H., subsidiary of Immuno Ag, Vienna, Austria) differs from KONYNE® in that in addition to factors II, VII, IX and X, it also contains trace amounts of factor VIIa. In these assays the materials used were: chromogenic substrate specific for thrombin (Spectrozyme®TH), chromogenic substrate specific for factor Xa (Spectrozyme®FXa), extrinsic activator (Thromboplastin C) and the prothrombin complex FEIBA® IMMUNO. The instrument used was a fluorescence light scattering micro centrifugal analyzer (Multistat III).

The inhibition of thrombin and factor Xa generation by each thrombin inhibitor at each concentration was calculated by the following formula: % inhibition of thrombin or factor Xa generation = [1 - agent ΔA_{405} at 660 sec / saline ΔA_{405} at 660 sec] x 100

The thrombin inhibitor concentration versus % inhibition graphs provided the basis for the calculation of the IC_{50} for each agent. Since the molecular weights of these

agents were known, all data were also converted into molar concentrations.

a. Inhibition of Thrombin Generation from FEIBA®

The buffer used in this procedure was composed of 1 mM Tris/HCl and 6 mM $CaCl_2$, adjusted to pH 8.1. The procedure for this amidolytic assay was as follows: 165 μl of the antithrombin agent diluted in buffer were incubated with 25 μl of the thrombin substrate (2.5 mM in deionized water) and 25 μl of Thromboplastin C (diluted 1:10 in 0.025 mM $CaCl_2$) for 30 sec. Then 25 μl of FEIBA® at 10 U/ml working concentration (diluted with sterile saline solution) and 10 μl of buffer were added and the optical density was measured every 60 sec for 12 min.

b. Inhibition of Factor Xa Generation from FEIBA®

The buffer used in this procedure was composed of 1 mM Tris/HCl and 6 mM $CaCl_2$, adjusted to pH 8.1. The procedure for this amidolytic assay was as follows: 165 μl of the antithrombin agent diluted in buffer were incubated with 25 μl of the factor Xa substrate (2.5 mM in deionized water) and 25 μl of Thromboplastin C (diluted 1:10 in 0.025 mM $CaCl_2$) for 30 sec. Then 25 μl of FEIBA® at 10 U/ml working concentration (diluted with sterile saline solution) and 10 μl of buffer were added and the optical density was measured every 60 sec for 12 min.

E. Anticoagulant Effects of Thrombin Inhibitors in NHP

Previously described amidolytic methods based on the intrinsic and extrinsic activation of plasma, provide information on the effects of agents on the generation of thrombin and factor Xa, utilizing the amidolytic activities of these factors. To study the

functional inhibitory effects on clot formation, or the anticoagulant effects of thrombin inhibitors and which pathways are inhibited most effectively, each of these agents was *in vitro* supplemented to NHP and tested in the global clotting tests described below. The following clotting time tests were performed on a fibrometer (Becton, Dickinson and Company, Cockeysville, MD) according to the specifications set by each of the manufacturers. NHP was obtained from the Loyola Blood Bank. Each concentration-response curve was repeated in triplicate on separate days.

1. Prothrombin Time (PT)

This method is based on the method originally described by Quick et al. (1935). The PT reveals defects in the extrinsic system (factor VII) and in the factors common to the extrinsic and intrinsic systems (factors V and X, prothrombin and fibrinogen). Thromboplastin is added to a known amount of plasma and it activates factor VII to factor VIIa, thus activating the extrinsic pathway of coagulation. PT was measured using Thromboplastin C. In this procedure 100 μl of plasma were incubated for 3 min at 37°C. At the end of this incubation time 200 μl of Thromboplastin C prewarmed at 37°C were added and the clotting time was measured.

2. Activated Partial Thromboplastin Time (APTT)

The APTT is based on the originally described method be Proctor and Rapaport (1961). This method measures the intrinsic coagulation factors (factors VIII, IX, XI and XII), the factors common to both extrinsic and intrinsic systems (factors V, X and prothrombin) and the conversion of fibrinogen to fibrin. Prolonged clotting times reflect

deficiency or inhibition of one or more of the above factors. The time to clot formation after citrated plasma has been activated by a platelet substitute (a rabbit brain phospholipid with micronized silica as activator) and Ca^{++} is measured. APTT was measured using the APTT reagent from Organon Teknika Corporation, Durham, SC. In this procedure 100 μl of plasma were incubated for 5 min with 100 μl of APTT reagent (platelet substitute). At the end of this incubation time 100 μl of 0.025 M $CaCl_2$ prewarmed at 37°C were added and the clotting time was measured.

3. Thrombin Time (TT)

The TT method is based on the method described by Bonsnes and Sweeney (1955). In the determination of citrated plasma TT, a known amount of preformed human thrombin is added to plasma and the time required for clot formation measures the rate at which fibrin is formed. TT was carried out using Fibrindex™ human thrombin at a concentration of 10 NIH U/ml in 0.025 M $CaCl_2$. In this procedure 100 μl of 10 U/ml human thrombin prewarmed at 37°C were added to 200 μl of plasma prewarmed at 37°C for 3 min, and the clotting time was measured.

4. Ecarin Clotting Time (ECT)

ECT is a new plasma-based clotting method, based on the original description by Nowak and Bucha (1993). This method is similar to the TT described above with the exception that an enzyme (ecarin) is used to activate the pre-existing prothrombin in the citrated plasma to a procoagulant form, whereas in the TT, exogenous preformed thrombin is added. Ecarin is an enzyme purified from the *Echis carinatus* snake venom.

Ecarin activates prothrombin to meizothrombin and other intermediates, rather than fully functional thrombin. However, meizothrombin has also procoagulant activities, although weaker than thrombin. This clotting time assay was carried out on a fast kinetic centrifugal analyzer (ACL 300 plus). In this assay, 100 μl of citrated plasma were incubated for 2 min at 37°C. At the end of the incubation period, 100 μl of ecarin (Knoll AG, Ludwigshafen, Germany) were added and the time to clotting was measured.

5. Heptest

The Heptest assay, originally developed by Yin et al. (1973), measures the ability of an agent to inhibit exogenous factor Xa. Heptest clotting time is measured using the Heptest reagents (Haemachem, St. Louis, MO): bovine factor Xa and Recalmix™ ($CaCl_2$ and brain cephalin in a bovine plasma fraction). In this procedure 100 μl of plasma were incubated with 100 μl of bovine factor Xa reagent for 2 min at 37°C. At the end of this incubation time 100 μl of Recalmix™ were added and the clotting time was measured.At the final stage of this assay, thrombin is generated and results in the formation of a detectable clot.

F. Anticoagulant Effects of Thrombin Inhibitors in Native Human Whole Blood

While thrombin inhibitors can be effective anticoagulants when studied in the plasma based clotting assays described above, the same agents may behave differently when supplemented to whole blood, since plasma is only one component of whole blood. The following thrombin inhibitors were studied in these assays: D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, argatroban, hirudin and heparin. To determine whether

these thrombin inhibitors were effective anticoagulants after *in vitro* supplementation at various concentrations to native whole blood and to compare these whole blood anticoagulant effects to the plasmatic anticoagulant effects, each thrombin inhibitor was studied in the two following assays.

Whole blood was collected from normal healthy human volunteers, after written consent (blood donation consent form in Appendix 4), via venipuncture. The first 3 ml of blood drawn were discarded. Each thrombin inhibitor concentration was repeated in triplicate in normal human whole blood collected from three separate donors.

1. Activated Clotting Time (ACT)

ACT is an assay that also measures whole blood clotting time and was originally described by Hattersley in 1966 (Hattersley 1984). Because native whole blood is used, the clotting time depends on both coagulation and platelet functionality. In determining the ACT of freshly drawn blood samples, special pre-filled glass tubes containing celite (an intrinsic system activator) were used. The activated clotting time was measured utilizing a Hemochron coagulation timing instrument (International Techidyne Corporation, Edison, NJ). The procedure for determining the ACT was carried out as specified by the manufacturer. Briefly, 2 ml of freshly drawn whole blood were added to celite pre-filled glass tube (International Techidyne Corporation, Edison, NJ) and the tube was immediately inserted into the Hemochron coagulation timing instrument and the timer was started.

2. Thrombelastographic analysis

The Thrombelastograph® Coagulation Analyzer (Haemoscope Corp., Skokie, IL) automatically records the viscoelastic changes in a sample of whole blood as the sample clots, retracts and/or lyses. The resulting profile is a measure of the kinetics of clot formation and dissolution and of clot quality. Thrombelastographic measurements were made on freshly drawn whole blood samples, pre-incubated at 37°C in a glass tube for 3 min using an Hellige thrombelastograph (Haemoscope Corp., Skokie, IL.). The thrombelastograms (TEG) generated in this manner were analyzed using the standard procedure as described by Fiedel and Ku (1986): the length from the beginning of the recording until the point where the graph reaches a 2 mm divergence was recorded as the r value; the length from the beginning of the thrombelastogram until the point where the graph reaches a 2 cm divergence was recorded as the rk value; the difference between the r and rk values was the k value; the angle defined by the center line transversing the length of the graph and the tangent from the initial point of divergence and the first inflection point of the tracing, was the α angle.

G. Anticoagulant Effects of Thrombin Inhibitors in Normal Rabbit Plasma (NRP)

To study the anticoagulant effects of thrombin inhibitors and which pathways are inhibited effectively, and to construct calibration curves, each of the following agents was supplemented to NRP *in vitro* and tested in the PT, APTT, TT and Heptest, as described earlier for NHP: D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin. The clotting time tests were performed on a fibrometer according to the

specifications set by each of the manufacturers and described earlier. NRP was obtained from rabbits as described previously. Each concentration-response curve was repeated in triplicate on separate days.

H. Anticoagulant Effects of Thrombin Inhibitors in Normal Rabbit Whole Blood

To determine the anticoagulant effects of thrombin inhibitors on normal rabbit whole blood, the following thrombin inhibitors were supplemented *in vitro* in freshly drawn rabbit whole blood: D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin. The rabbit whole blood was obtained from White male New Zealand rabbits weighing 2.5 to 3.5 kg. Each rabbit was lightly anesthetized with ketamine hydrochloride at a dose of 50 mg/kg I.M. in combination with xylazine at a dose of 25 mg/kg I.M. Blood was drawn through the major ear artery. After the first 3 ml were discarded, the subsequently drawn blood was immediately supplemented with each of the thrombin inhibitors and tested in the ACT and TEG assays, as described above with the normal human whole blood. The only modification was in the glass tubes used in the ACT: when rabbit whole blood was tested, saline pre-filled glass tubes (International Techidyne Corporation, Edison, NJ) were used instead of the celite pre-filled glass tubes. Each thrombin inhibitor concentration was repeated in triplicate in normal rabbit whole blood collected from three separate rabbits.

I. Antithrombotic Effects of Thrombin Inhibitors as Studied in the Rabbit Stasis Thrombosis Model

Although thrombin inhibitors are effective anticoagulants in various *in vitro* systems, their anticoagulant effects do not always correlate with *in vivo* antithrombotic

effects. Furthermore, the degree of anticoagulation as measured by clotting tests does not always correspond to the degree of antithrombotic effects. To study the comparative antithrombotic effects of various thrombin inhibitors, four representative anticoagulants from the series studied *in vitro* were selected: D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin. A modified Wessler stasis thrombosis model (Fareed et al. 1985) was used for the determination of the *in vivo* antithrombotic effects of these four agents. The principle of this model is that clot formation in the jugular segments of the rabbit, in response to a thrombogenic trigger, can be prevented by administration of antithrombotic agents prior to administration of the thrombogenic trigger. The thrombogenic trigger used varies from serum to factor concentrates to snake venoms such as the Russel's viper venom, and the resulting clots differ morphometrically accordingly (Callas et al. 1995). In this series of experiments, recombinant tissue factor (1.38 μM r-TF in 2.57 mM phospholipid, biosynthesized and reconstituted as described in Bach et al. 1986, provided by Dr. Yale Nemerson, Mount Sinai School of Medicine, New York, NY) was used as the trigger for clot formation in the jugular segments. This thrombogenic trigger represented a defined approach in which the r-TF activated the clotting process through the extrinsic pathway.

1. Standardization of the Rabbit Stasis Thrombosis Model

The dose of r-TF to be used for comparing the antithrombotic effects of the antithrombin agents was selected based on a series of experiments where the dose-thrombogenic response relationship was established in this model. The r-TF was thawed and reconstituted in sterile saline to a final concentration of 5.5 nM and re-frozen in

aliquots. Immediately prior to the experiment, an aliquot was thawed and diluted in sterile saline. In order to keep the volume of r-TF injected consistent independent of dose, a dilution was made such that the injection volume was always 0.25 ml/kg in every rabbit. Therefore, the r-TF was diluted to concentrations corresponding to quadruple the doses that were used, eg. 5.41 pmol/ml for dosing at 1.35 pmol/kg. The r-TF doses that were tested were 1.35, 0.68, 0.34, 0.17 and 0.04 pmol/kg. Groups of six rabbits were tested at each r-TF dose.

The stasis thrombosis procedure was carried out as follows: White male New Zealand rabbits weighing 2.5 to 3.5 kg were obtained from LSR Industries and housed individually. The animals were kept on standard rabbit chow diet and had access to water *ad libitum*. Ketamine hydrochloride at a dose of 50 mg/kg I.M. in combination with xylazine at a dose of 25 mg/kg I.M. was used to anesthetize each rabbit. Both jugular veins and the right carotid were carefully isolated. The right carotid was cannulated for blood collection utilized in *ex vivo* analyses. The left marginal ear vein was also cannulated for I.V. administration of the thrombogenic and antithrombotic agents or saline. After a baseline blood draw was performed, the sterile saline solution was injected at 1 ml/kg, through the cannulated marginal ear vein. After 5 min of the saline injection, a second blood draw was performed. Immediately following the second blood draw, r-TF was injected through the cannulated marginal ear vein as the thrombogenic challenge. Twenty sec after the I.V. administration of r-TF, the jugular segments were tied.

a. Clot Score Analyses

Ten min after the injection of r-TF the right segment was dissected in a petri dish and the contained thrombi were graded. Twenty min after the administration of r-TF the same procedure was repeated on the left segment. The grading system for the thrombi was on a scale of 0 to +4, as previously established (Fareed et al. 1985). The animal was euthanized with an I.V. injection of 0.5 ml sodium pentobarbital and phenytoin sodium solution (Beuthanasia®-D, Steris Laboratories, Phoenix, AZ).

The r-TF dose that consistently gave a clot score of at least +3 was determined to be 0.68 pmol/kg and therefore was selected as the dose used in determining the antithrombotic effects of the thrombin inhibitors, as described in the following sections.

In the following sections, each thrombin inhibitor was diluted in sterile saline to various concentrations, so that the agent concentration matched the dose (eg. 1 mg/ml thrombin inhibitor for dosing at 1mg/kg). Thus, the volume injected in each rabbit (either I.V. or S.C.) remained at a 1ml/kg animal weight.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Each time a blood draw was performed (at baseline and 5 min post saline injection as described in the previous section), the first 3 ml were discarded, the next 3 ml were tested in the ACT and saline TEG assays, as described in section H. Anticoagulant Effects of Thrombin Inhibitors in Normal Rabbit Whole Blood, and the next 9 ml were collected in 3.8% sodium citrate and processed as described in section D.3.c. Preparation of Normal Rabbit Plasma and frozen in aliquots at -70°C. At the conclusion of the rabbit stasis thrombosis model experiments, these aliquots were thawed

in batches and the following amidolytic and clotting tests were performed as described earlier: antithrombin assay (section D.3.d. Antithrombin Amidolytic Assay with NRP), anti-Xa assay (section D.4.b. Anti-Xa Amidolytic Assay with NRP), PT, APTT, TT and Heptest (section G. Anticoagulant Effects of Thrombin Inhibitors in NRP).

2. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration at Fixed Time Points

To determine the dose-dependent antithrombotic effects of the thrombin inhibitors after I.V. administration in the above described rabbit stasis thrombosis model, each thrombin inhibitor was injected through the marginal ear vein of the left ear, 5 min prior to injecting r-TF, in place of saline, as described above. Each thrombin inhibitor was injected at various doses, to obtain a dose-response curve. The doses of each thrombin inhibitor studied were for D-MePhe-Pro-Arg-H, 0.097, 0.194, 0.485, 0.971 and 1.942 μmol/kg; for Ac-(D)Phe-Pro-boroArg-OH, 0.010, 0.025, 0.050 and 0.101 μmol/kg; for hirudin, 0.001, 0.004, 0.007, 0.014 and 0.036 μmol/kg and for heparin 0.002, 0.005, 0.009 and 0.023 μmol/kg. Groups of six rabbits were tested at each dose. Blood samples were collected at baseline and 5 min post antithrombin agent injection.

a. Clot Score Analyses

The jugular vein segments were isolated in sequence and the clots contained within were graded, as described earlier in section I.1.a Clot Score Analyses.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Blood samples drawn at baseline and 5 min post thrombin inhibitor injection were

processed and analyzed in exactly the same manner as described earlier in section I.1.b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples.

3. Duration of Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration at a Fixed Dose

To determine the time-dependance of the antithrombotic effects of the thrombin inhibitors studied in this rabbit stasis thrombosis model after I.V. administration, the dose of each agent that produced sub-maximal antithrombotic effects in the dose-dependent I.V. studies was selected and studied at different circulation times, prior to injecting r-TF as described above. The dose of each thrombin inhibitor studied was: D-MePhe-Pro-Arg-H at 1.942 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, hirudin at 0.036 μmol/kg and heparin at 0.023 μmol/kg. The circulation time points selected for studying the antithrombotic effects of these thrombin inhibitors at these doses were 5, 30, 60 and 90 min (prior to r-TF injection). Groups of six rabbits were tested at each time point for each thrombin inhibitor. Blood draws were performed at baseline and prior to r-TF injection (5 or 30 or 60 or 90 min post thrombin inhibitor injection).

a. Clot Score Analyses

The jugular vein segments were isolated in sequence and the clots contained within were graded, as described earlier in section I.1.a Clot Score Analyses.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Blood draws were performed at baseline and prior to r-TF injection (5 or 30 or 60 or 90 min post thrombin inhibitor injection). These samples were processed analyzed

in exactly the same manner as described earlier in section I.1.b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples.

4. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration at Fixed Time Points

To determine the dose-dependent antithrombotic effects of the thrombin inhibitors after S.C. administration in the above described rabbit stasis thrombosis model, each thrombin inhibitor was injected subcutaneously 45 min prior to injecting r-TF, in place of saline, as described above. Each thrombin inhibitor was injected at various doses, to obtain a dose-response curve. The doses of each thrombin inhibitor studied were for D-MePhe-Pro-Arg-H, 0.194, 0.485, 0.971, 1.942 and 4.854 μmol/kg; for Ac-(D)Phe-Pro-boroArg-OH, 0.050, 0.097, 0.201 and 0.503 μmol/kg; for hirudin, 0.004, 0.007, 0.014, 0.036, 0.072 and 0.144 μmol/kg and for heparin 0.046, 0.091, 0.250 and 0.455 μmol/kg. Groups of six rabbits were tested at each dose. Blood samples were collected at baseline and 45 min post thrombin inhibitor injection (prior to r-TF injection).

a. Clot Score Analyses

The jugular vein segments were isolated in sequence and the clots contained within were graded, as described earlier in section I.1.a Clot Score Analyses.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Blood draws were performed at baseline and 45 min post thrombin inhibitor injection. These samples were processed analyzed in exactly the same manner as described earlier in section I.1.b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples.

5. Duration of Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration at a Fixed Dose

To determine the time-dependance of the antithrombotic effects of the thrombin inhibitors studied in this rabbit stasis thrombosis model after S.C. administration, the dose of each agent that produced sub-maximal antithrombotic effects in the dose-dependent S.C. studies was selected and studied at different circulation times, prior to injecting r-TF as described above. The dose of each thrombin inhibitor studied was: D-MePhe-Pro-Arg-H at 4.854 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.503 μmol/kg, hirudin at 0.144 μmol/kg and heparin at 0.455 μmol/kg. The circulation time points selected for studying the antithrombotic effects of these thrombin inhibitors at these doses were 15, 45, and 90 min (prior to r-TF injection). Groups of six rabbits were tested at each time point for each thrombin inhibitor. Blood samples were collected at baseline and prior to r-TF injection (15, 30, 45, 60 or 90 min post antithrombin agent injection).

a. Clot Score Analyses

The jugular vein segments were isolated in sequence and the clots contained within were graded, as described earlier in section I.1.a Clot Score Analyses.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Each time a blood draw was performed, at baseline and prior to r-TF injection (15, 30, 45, 60 or 90 min post thrombin inhibitor injection). These samples were processed analyzed in exactly the same manner as described earlier in section I.1.b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples.

J. Antithrombotic Effects of Thrombin Inhibitors as Studied in the Rat Model of Laser-Induced Thrombosis

The physiologic responses in the arterial and venous systems involve different mechanisms which include differences in blood flow conditions and clot compositions. Therefore, thrombin inhibitors may have different effects on arteries when compared to veins. Since the rabbit stasis thrombosis model simulated venous thrombosis, a rat model of laser-induced thrombosis was selected to study the antithrombotic effects of the thrombin inhibitors in the arterial system, as described in detail by Weichert et al. 1988. In this procedure the rat (Wistar, male, 300-350 g, LSR Industries, Inc., Union Grove, WI) was anesthetized with sodium pentobarbital, 100 mg/kg I.P. (Butler Co., Colombus, OH). An intestinal loop was exposed through a hypogastric incision and continuously irrigated with sterile saline solution, spread on a stage mounted on a microscope table (Nikon, inverted microscope, Diaphot-TMD, Nippon Kogaku K.K., Tokyo, Japan). Vascular lesions were induced with an argon laser system (INNOVA® 70-2 Argon Laser, Coherent, Inc., Santa Clara, CA). The laser beam was directed through the optical path of the microscope on small mesenteric arterioles (10-15 μm I.D.) of the fat-free portion of the mesentery. The laser beam pathway was controlled by means of a camera shutter (Uniblitz® VS1452Z0, Vincent Associates, Rochester, NY) and driver (Uniblitz® T132, Vincent Associates, Rochester, NY). The effective constant energy was 50 mW as measured with a power meter on the microscope stage (model 200/10W, Coherent, Inc., Santa Clara, CA). The exposure time of the laser lesions was 150 msec. The time interval between laser shots was 1 min. Results were evaluated by direct microscopic observation of the occlusive changes in the blood vessel. The number of laser injuries

required to induce a thrombus which was of a length equal to 1.5 x I.D. of the vessel, or that produced complete occlusion was the endpoint. The rat was sacrificed at the end of the experiment by I.V. injection of sodium pentobarbital (Beuthanasia®, 0.5 ml/kg).

Each thrombin inhibitor was diluted in sterile saline to various concentrations, so that the agent concentration corresponded to the dose (eg. 1 mg/ml thrombin inhibitor for dosing at 1mg/kg). Thus, the volume injected in the rat (either I.V. or S.C.) was always 1ml/kg animal weight. After the rat was prepared and placed on the microscope stage and a suitable vessel was located, each thrombin inhibitor was injected I.V. through one of the rat tail veins, at various concentrations. Each thrombin inhibitor was allowed to circulate for 5 min prior to the initiation of the laser-induced vascular lesion. The doses of each thrombin inhibitor used in this model were as follows: D-MePhe-Pro-Arg-H at 0.97, 1.94, 3.40 and 4.86 μmol/kg; Ac-(D)Phe-Pro-boroArg-OH at 0.10, 0.15 and 0.20 μmol/kg; hirudin at 0.07, 0.14 and 0.72 μmol/kg; heparin at 0.05, 0.09 and 0.46 μg/kg. Groups of six rats were tested at each dose for each thrombin inhibitor.

K. Hemorrhagic Effects of Thrombin Inhibitors as Studied in the Rabbit Ear Bleeding Model

While thrombin inhibitors can be potent antithrombotic agents *in vivo* due to their inhibitory actions on the coagulation cascade, they can also produce hemorrhagic effects since coagulation is involved in hemostasis and platelet activation. To study the hemorrhagic effects of thrombin inhibitors, a rabbit model of ear blood loss as described by Cade et al. 1975 was used. In this procedure, white male New Zealand rabbits weighing 2.5 to 3.5 kg were obtained from LSR Industries. The animals were kept on

standard rabbit chow diet and had access to water *ad libitum*. Ketamine at a dose of 50 mg/kg I.M. was injected in combination with xylazine at a dose of 25 mg/kg I.M. to induce anesthesia. The marginal ear vein was cannulated for I.V. administration of antithrombin agents at various doses. After a constant circulation time, the right ear was immersed in a 950 ml, 37°C saline bath for a few seconds and then 5 uniform, full thickness incisions were made in the ear in areas free of major vascularization, and returned to the saline bath for 10 min. The saline was collected and the red blood cells were counted using a hemacytometer (Brightline, Improved Neubauer, Hausser Scientific 0.100 mm deep). The same procedure was repeated on the other ear 15 min after the procedure was initiated on the first ear. At the end of the experiment, hemostasis was ensured and the animal was returned to its cage.

In the following sections, each thrombin inhibitor was diluted in sterile saline (Baxter Healthcare Corp., Deerfield, IL) to concentrations corresponding to 10-fold the dose to be used, so that the volume of the injected agent remained constant at 0.1 ml/kg (I.V. or S.C.) animal weight for every rabbit.

1. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After I.V. Administration at Fixed Time Points

To determine the dose-dependent hemorrhagic effects of the thrombin inhibitors after I.V. administration, each thrombin inhibitor was injected through the marginal ear vein of the left ear, 5 min prior to making the incisions in the right ear, as described above. The incisions in the left ear were made 20 min after the I.V. injection of the antithrombotic agent. Each thrombin inhibitor was injected at various doses, to obtain

a dose-response curve. The doses of each thrombin inhibitor studied were for D-MePhe-Pro-Arg-H, 0.97, 1.94 and 4.85 μmol/kg; for Ac-(D)Phe-Pro-boroArg-OH, 0.10, 0.25, 0.50 and 1.00 μmol/kg; for hirudin, 0.07, 0.14 and 0.36 μmol/kg and for heparin 0.05, 0.09, 0.23 and 0.46 μmol/kg. Groups of six rabbits were tested at each dose. Groups of six rabbits were used for each treatment.

2. Duration of Hemorrhagic Effects of Thrombin Inhibitors After I.V. Administration at a Fixed Dose

To determine the time-dependance of the hemorrhagic effects of the thrombin inhibitors studied in this hemorrhagic model after I.V. administration, the dose of each agent that produced sub-maximal hemorrhagic effects in the dose-dependent I.V. studies was selected and studied at different circulation times, prior to making the incisions in the ears as described above. The dose of each thrombin inhibitor studied was D-MePhe-Pro-Arg-H at 4.85 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.50 μmol/kg, hirudin at 0.36 μmol/kg and heparin at 0.46 μmol/kg. The circulation time points selected for studying the hemorrhagic effects of these thrombin inhibitor at these doses were 5, 20, 30, 45, 60 and 90 min after I.V. administration. Groups of six rabbits were tested at each time point for each thrombin inhibitor.

3. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After S.C. Administration at Fixed Time Points

To determine the dose-dependent hemorrhagic effects of the thrombin inhibitors after S.C. administration, each thrombin inhibitor was injected subcutaneously 45 min prior to making the incisions in the right ear, as described above. The incisions in the

left ear were made 60 min after the S.C. injection of the antithrombotic agent. Each thrombin inhibitor was injected at various doses, to obtain a dose-response curve. The doses of each thrombin inhibitor studied were for D-MePhe-Pro-Arg-H, 1.94, 4.84 and 9.71 μmol/kg; for Ac-(D)Phe-Pro-boroArg-OH, 0.20, 0.50, 1.00 and 2.01 μmol/kg; for hirudin, 0.07, 0.14, 0.36 and 0.77 μmol/kg and for heparin 0.46 and 0.91 μmol/kg. Groups of six rabbits were tested at each dose.

4. Duration of Hemorrhagic Effects of Thrombin Inhibitors After S.C. Administration at a Fixed Dose

To determine the time-dependance of the hemorrhagic effects of the thrombin inhibitors studied in this hemorrhagic model after S.C. administration, the dose of each agent that produced sub-maximal hemorrhagic effects in the dose-dependent S.C. studies was selected and studied at different circulation times, prior to making the incisions in the ears as described above. The dose of each thrombin inhibitor studied was D-MePhe-Pro-Arg-H at 9.71 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 2.01 μmol/kg, hirudin at 0.77 μmol/kg and heparin at 0.91 μmol/kg. The circulation time points selected for studying the hemorrhagic effects of these thrombin inhibitor at these doses were 15, 30, 45, 60 and 90 min after I.V. administration. Groups of six rabbits were tested at each time point for each thrombin inhibitor.

L. Effects of Thrombin Inhibitors on Blood Pressure After I.V. Administration to Rabbits

Since the building block for many of the synthetic thrombin inhibitors is arginine, concerns have been expressed over the potential effects of these thrombin inhibitors or

their metabolites on the vascular endothelium, independent of their actions on serine proteases. Specifically, the potential blood-pressure lowering effects of these agents may be compromising.

The effects of thrombin inhibitors on blood pressure after I.V. injection to rabbits were monitored during the procedures of the stasis thrombosis model, for the highest dose of each thrombin inhibitor used (D-MePhe-Pro-Arg-H at 1.942 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, hirudin at 0.036 μmol/kg and heparin at 0.023 μmol/kg). After isolating and cannulating the femoral artery, the mean arterial pressure was monitored via a pressure transducer (Type 4-327-C, range 0-400 mmHg, Beckman Instruments Inc., Irvine, CA) connected to a physiograph (model R511A, Beckman Instruments Inc., Irvine CA). The blood pressure was continuously monitored from before any agents were injected into the rabbit (after induction of anesthesia and cannulation of the femoral artery), until the rabbit was sacrificed at the end of the stasis thrombosis model procedure.

M. Statistical analysis

The experimental data derived from the *in vitro* assays were compiled as mean $\pm$ SD (standard deviation of the sample). The data derived from the *in vivo* and *ex vivo* studies were compiled as mean $\pm$ SEM (standard error of the mean). The sample mean was the average of the measurements within each group. The SEM was reported along with the mean to provide an indication of the precision of estimation of the population mean and to facilitate comparison between means of different groups.

The data in the figures were presented as mean $\pm$ SD or mean $\pm$ SEM,

depending on whether they were derived from *in vitro* or *in vivo/ex vivo* procedures. All figures were drawn using SigmaPlot® software. The error bars were not shown if the they were smaller than the size of the symbols in the figure.

The statistical tests performed on the data included one-way ANOVA followed by Neuman Keuls multiple comparison test when the one-way ANOVA revealed a significant difference. These statistical tests were used to evaluate the results from the all studies except for the clot scores from the stasis thrombosis model. The Kruskal-Wallis test was used to examine the differences between clot scores obtained in the rabbit stasis thrombosis model.

CHAPTER IV

RESULTS

This dissertation represents an integrated account of both the biochemical and pharmacolgic properties of heparin and several thrombin inhibitors. The results on various experimental protocols are divided into various sections. In the first section, the basic physicochemical and analytical properties of each of these agents are discussed. The available information on the individual chemical and molecular properties along with the data generated using gas permeation chromatography (GPC) is discussed. The comparative biochemical profiling included the studies on the potency evaluation, inhibitory profiling utilizing purified serine proteases and the effects of these agents on the generation of protease in both the coagulation and fibrinolytic based systems. The global anticoagulant effects of each of these inhibitors are reported in terms of their ability to prolong the clotting profile of either the normal human plasma or normal rabbit plasma. A newly developed highly specific test, namely the ecarin clotting time (ECT), is employed to determine the relative anticoagulant potency of these agents. Whole blood studies using both the normal human whole blood and normal rabbit whole blood based assays in the TEG and ACT analyses are also included. All of these results are reported in statistically defined gravimetric and molar values.

The comparative pharmacological data on various agents included in this

dissertation are presented in various sections representing the antithrombotic effects in the venous stasis thrombosis model in the I.V. and S.C. studies, where the dose-response relationship is reported. These data are also compared in terms of the ED_{50} for each individual agent in different experimental conditions. The duration of the fixed I.V. and S.C. dosage of each of the antithrombin agents studied is also reported and the t½ of each agent is provided. The *ex vivo* analysis using different assays is also compiled to compare the antithrombotic action with measurable *ex vivo* effects. The results of the comparative antithrombotic actions for each of these agents in a rat model of laser-induced thrombosis are also presented. No *ex vivo* analyses from these studies are included. The comparative hemorrhagic effects of various thrombin inhibitors and heparin are reported for both the I.V. and S.C. studies, which were carried out in a rabbit model for ear blood loss. Time-dependance of the hemorrhagic effects in this model is also reported for the fixed dosage studies in both the I.V. and S.C. experiments. The results on the effect of various thrombin inhibitors and heparin on blood pressure measurements in rabbits are also compiled for the I.V. studies.

The comparative data for both the biochemical and pharmacological studies are compiled in individual figures where the tabulated data are plotted to represent a cumulative data analysis. Similarly, individual tables on the data compiled from various experiments are tabulated in terms of means and standard deviations or standard error of the means, to represent each of the data points in either gravimetric or molar values or both. Appendix 4 represents supportive data tables for each of the tables included in the text for both biochemical and pharmacologic study results. The tables in Appendix

IV are numbered by arabic numerals corresponding to the main text tables, followed by sequential lower-case letters.

A. Analytical Profile of Peptide Thrombin Inhibitors

All of the tripeptide thrombin inhibitors employed in these studies were obtained in dry powdered form and were stored at 4°C in desiccators. The purity of these agents was evaluated by utilizing the HPLC methodology described in Chapter III. B. The elution profiles described below were compared against reference elution profiles of the medium in which each compound was dissolved in distilled H_2O. The background peaks appearing in the elution buffer elution were then subtracted from the elution profile of individual peptides. The background-adjusted peaks were then integrated by the system software and assigned to particular structures, in accordance according to reported analyzed values (Bajusz et. 1990).

1. Stability and Homogeneity Profile of D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H.

The tripeptide aldehyde D-Phe-Pro-Arg-H has been reported to be unstable in solution and is transformed into an inactive 5,6,8,9,10,10a-hexahydro-2-(3'-guanidinopropyl)-5-benzyl-6-oxoimidazo[1,2-a]pyrrolo[2,1-c]pyrazine (Bajusz et al. 1989). To determine the extent of possible racemization, a validated HPLC method was employed. The result of this profile for the compound D-Phe-Pro-Arg-H $\cdot$ H_2SO_4 is depicted in the panel A of Fig. 5. This peptide eluted between the 36 and 42 min after injection of the compound into the column. Peaks B′, A and B represented the three equilibrium structures of the D-Phe-Pro-Arg-H aldehyde, the aldehyde hydrate (peak A)

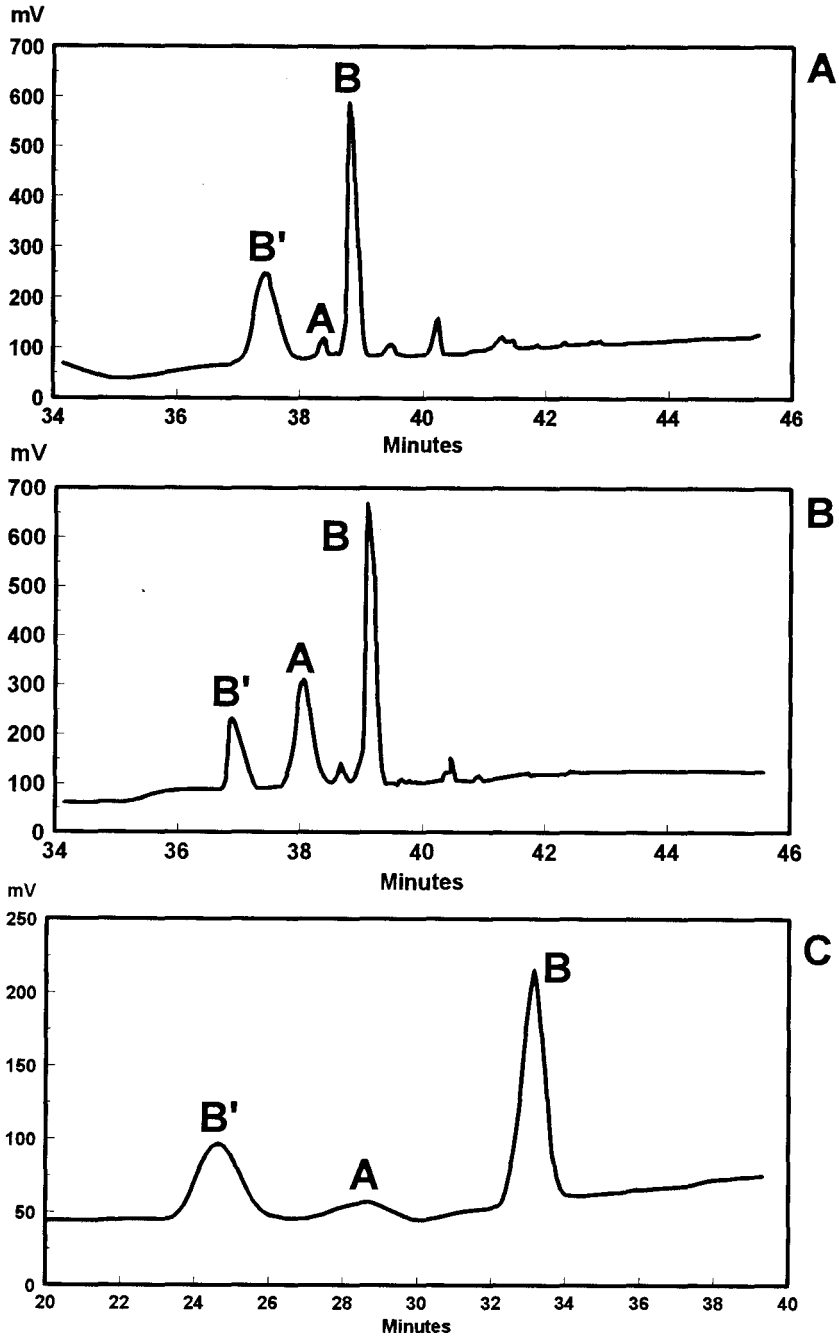


Fig. 5. Analytical profile of D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H, as detected at 214 nm. The X axis represents the time (min) at which the various components of the agent injected eluted, while the Y axis represents the intensity of the signal (mV) emitted by the substance detected. Panel A depicts the HPLC profile of D-Phe-Pro-Arg-H, represented by peaks B′ (retention time 37.5 min), A (retention time 38.5 min) and B (retention time 39.0 min). Panel B depicts the HPLC profile of D-MePhe-Pro-Arg-H, represented by peaks B′ (retention time 37.1 min), A (retention time 38.3 min) and B (retention time 39.4 min). Panel C depicts the HPLC profile of Boc-D-Phe-Pro-Arg-H represented by peaks B′ (retention time 25.2 min), A (retention time 29.2 min) and B (retention time 33.8 min).

and the two amino cyclols (peaks B and B′). The poorly resolved peak between peaks A and B was due to the additional equilibrium structure of either the aldehyde or to the impurity arising from one of the three forms of the DLD epimer (the D-Arg-H containing epimer). The three peaks following peak B were due to the other two forms of the DLD epimer and the inactive decomposition product respectively. All peaks appearing during its elution were integrated and their % area, based on the sum of all background-adjusted peaks, was calculated by the system software. The % peptide content was calculated by summing the % area under peaks B′, A and B and was found to be 89.64 %.

Table 3 -- Integration of various peaks eluted during the analysis of D-Phe-Pro-Arg-H · H_2SO_4

	Structure	Retention Time (min)	% Area
Peak B′	equilibrium structure, amino cyclol	37.5	40.73
Peak A	equilibrium structure, aldehyde hydrate	38.5	3.89
Unknown	equilibrium aldehyde or DLD epimer	38.7	0.59
Peak B	equilibrium structure, amino cyclol	39.0	45.02
Unknown	DLD epimer	39.7	2.51
Unknown	DLD epimer	40.5	4.12
Unknown	inactive decomposition product	41.6	3.14

The peptide D-MePhe-Pro-Arg-H · H_2SO_4 was also eluted in a similar manner as the D-Phe-Pro-Arg-H · H_2SO_4, as depicted in the panel B of Fig. 5. The equilibrium peaks that appeared during its elution profile were also due to the three equilibrium structures of the D-MePhe-Pro-Arg-H aldehyde, the aldehyde hydrate (peak A) and the

two amino cyclols (peaks B and B′). As in the case of D-Phe-Pro-Arg-H, the peak between peaks A and B was due to an impurity arising from one of the three forms of the DLD epimer (the D-Arg-H containing epimer). The peak following peak B was due to one of the other two forms of the DLD epimer. Unlike the D-Phe-Pro-Arg-H elution profile, D-MePhe-Pro-Arg-H did not exhibit any unstable products. All peaks appearing during this elution were integrated and their % area, based on the sum of all background-adjusted peaks appearing, was calculated by the system software. The % peptide content was calculated by summing the % area under peaks B′, A and B and was found to be 94.99 %.

Table 4 -- Integration of various peaks eluted during the analysis of D-MePhe-Pro-Arg-H · H_2SO_4

	Structure	Retention Time (min)	% Area
Peak B′	equilibrium structure, amino cyclol	37.1	17.60
Peak A	equilibrium structure, aldehyde hydrate	38.3	31.32
Unknown	DLD epimer	39.0	2.89
Peak B	equilibrium structure, amino cyclol	39.4	46.07
Unknown	DLD epimer	40.8	2.11

The compound Boc-D-Phe-Pro-Arg-H · ½H_2SO_4 also eluted in a similar manner as D-MePhe-Pro-Arg-H · H_2SO_4, as depicted in panel C of Fig. 5. The equilibrium peaks appearing in the elution of this peptide were due to the three equilibrium structures of the Boc-D-Phe-Pro-Arg-H aldehyde, the aldehyde hydrate (peak A) and the two amino

cyclols (peaks B and B′). No additional peaks due to impurities or epimers appeared during the elution of Boc-D-Phe-Pro-Arg-H. All peaks appearing in this analysis were integrated and their respective % area, based on the sum of all background-adjusted peaks appearing, was calculated by the system software. The % peptide content was calculated by summing the % area under peaks B′, A and B and was found to be 100 %.

Table 5 -- Integration of various peaks during the analysis of Boc-D-Phe-Pro-Arg-H · ½H_2SO_4

	Structure	Retention Time (min)	% Area
Peak B′	equilibrium structure, amino cyclol	25.2	34.18
Peak A	equilibrium structure, aldehyde hydrate	29.2	9.12
Peak B	equilibrium structure, amino cyclol	33.8	56.70

2. Ac-(D)Phe-Pro-boroArg-OH Purity Determination

The elution profile of Ac-(D)Phe-Pro-boroArg-OH · HCl is depicted in Fig. 6. After subtracting the background peaks this agent eluted in 4 distinct peaks. These peaks were integrated and the respective % area was calculated and reported in Table 6. The elution of this peptide exhibited a rather heterogeneous polycomponent behavior. As stated, 4 distinct peaks were eluted. The first peak represented the major compound with a retention time of 8 min and a relative abundance of about 60%. Close to the first peak, the second peak eluted at 8.4 min and showed a rather sharp distribution profile with a relative abundance of 29%. Two minor peaks eluted at 9.2 and 9.7 min with baseline separation with a 1.4 and 9.4% abundance respectively.

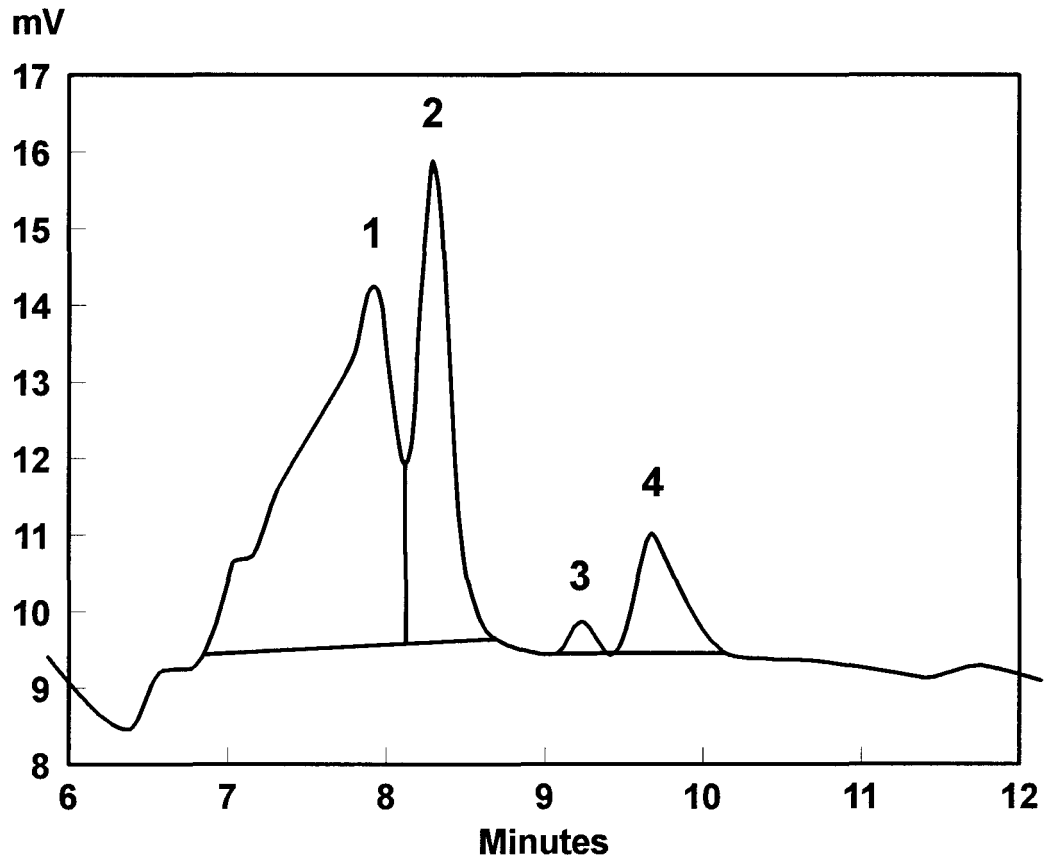


Fig. 6. Analytical profile of Ac-(D)Phe-Pro-boroArg-OH, as detected at 214 nm. The X axis represents the time (min) at which the various components of the agent injected eluted, while the Y axis represents the intensity of the signal (mV) emitted by the substance detected. The elution profile of this compound was composed of four peaks, starting at retention times of 8.0, 8.4, 9.2 and 9.7 min respectively.

Table 6 -- Integration of various peaks during the analysis of Ac-(D)Phe-Pro-boroArg-OH · HCl

	Retention Time (min)	Area %
1st Peak	8.0	60.27
2nd Peak	8.4	28.91
3rd Peak	9.2	1.44
4th Peak	9.7	9.37

B. Analytical Profile of the Unfractionated Heparin

The molecular profile of unfractionated heparin mainly depends on the source. Porcine mucosal heparin usually has a lower molecular mass distribution. The unfractionated heparin used in these studies was of porcine origin and was provided in a white powdered sodium salt form (Sanofi, Paris, France). To determine the molecular profile of this glycosaminoglycan mixture and the relative purity, this agent was analyzed using a GPC method. The elution profile of heparin is depicted in Fig. 7, with panel A being the profile detected in the UV detector at 234 nm and panel B being the profile detected in the differential refractometer (RI). In both panels, the first peak from the left is due to heparin, eluted between 23 and 45 min. The subsequent peaks represent the salt peaks. In both panels, the heparin peak was integrated by the system software and the % distribution was calculated. As described by Ahsan et al. 1994, based on narrow range calibrators, the software system also assigned molecular weights corresponding to retention time. Thus, for each heparin peak, the % heparin falling in a particular range of molecular weight was calculated. Table 7 summarizes the molecular weight

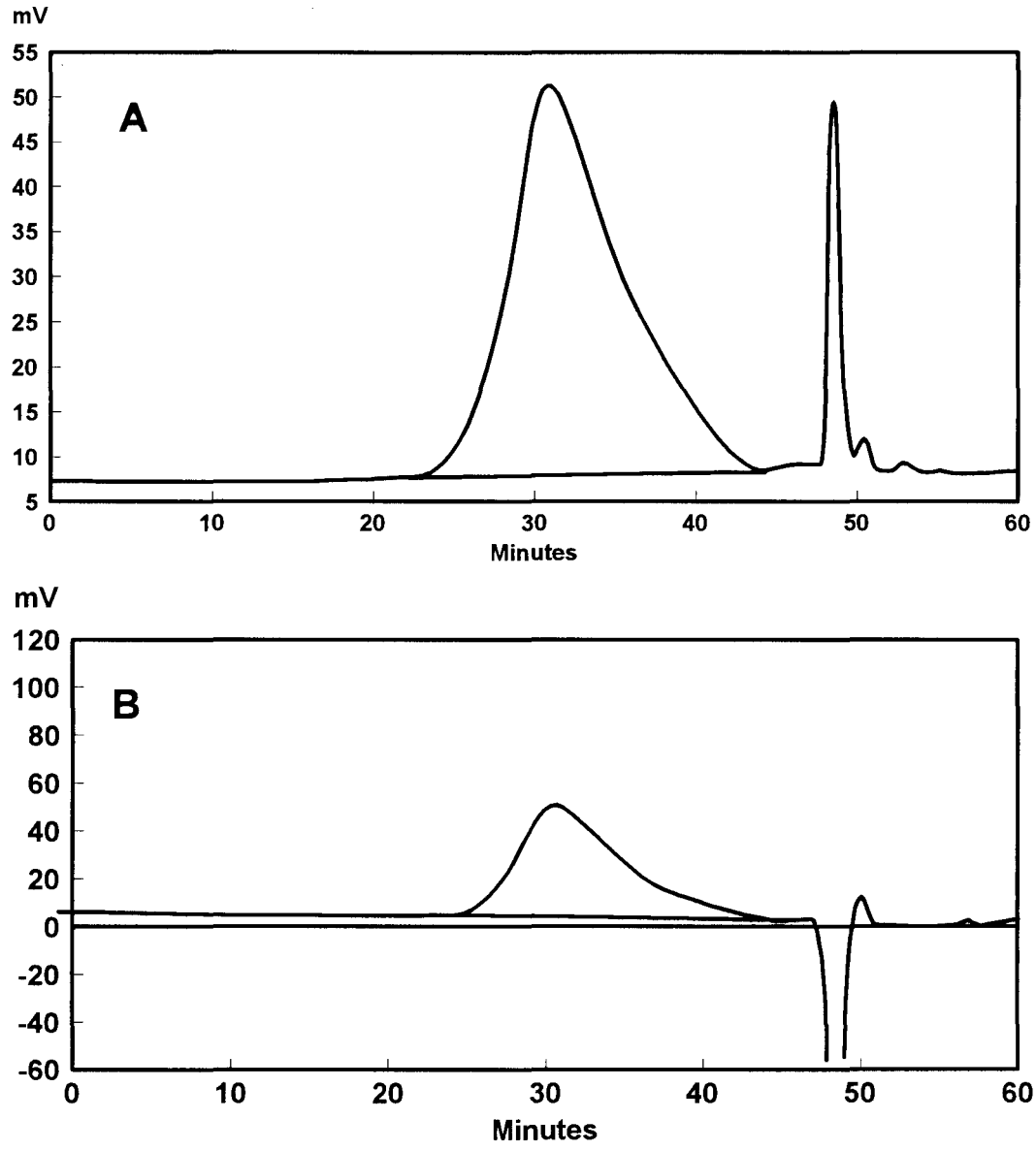


Fig. 7. Analytical profile of unfractionated porcine mucosal heparin. The X axis represents the time (min) at which the various components of the agent injected eluted, while the Y axis represents the intensity of the signal (mV) emitted by the substance detected. Panel A represents the GPC profile of heparin as detected by the UV detector at 234 nm. Panel B represents the GPC profile of heparin as detected by the RI detector. As detected by both methods, heparin eluted in a profile represented by a single peak, between retention times of 23 and 45 min. The peaks eluted at 47 min represent Na_2SO_4.

distribution in this unfractionated heparin. The results from both the UV and RI detectors were in close agreement. Nearly half of the material was found to be between 8,000-12,000. About 20% of heparin was in the 5,000-8,000 range and just as much was in the 16,000-20,000 range. Less than 10% of the total heparin was found to be greater than 16,000 or lower than 5,000. The weight average was approximately 10,700, with a peak MW of 11,100 and 11,400 for the UV and RI analyses respectively.

Table 7 -- Molecular Weight Distribution of Unfractionated Heparin

	UV (234 nm)	RI
Weight Average	10,717	10,680
Peak MW	11,121	11,445
>20,000	4.1%	3.6%
16,000-20,000	5.1%	5.3%
12,000-16,000	21.2%	20.7%
8,000-12,000	45.4%	48.3%
5,000-8,000	20.0%	16.8%
2,500-5,000	4.0%	4.3%
<2,500	0.2%	1.0%

C. Biochemical Assays

The inhibition of the activity of thrombin can be explored *in vitro* utilizing the amidolytic and the clotting activities of thrombin, by using chromogenic substrates that yield color when cleaved by thrombin, and by using fibrinogen which is converted to fibrin when digested by thrombin. The following studies were designed to explore the relative *in*

vitro inhibition of the formed thrombin by various agents, amidolytically with the thrombin titration assay and the plasma-based antithrombin assay, and functionally with thrombin time in the fibrinogen-based assay. In addition, the inhibition of the generation of thrombin in various assay systems was also explored in the fibrinogen-deficient plasma assay systems, as well as in the KONYNE® and FEIBA® assays. The effect of these thrombin inhibitors on other serine proteases, such as factor Xa, chymotrypsin, trypsin, activated protein C, tissue-type plasminogen activator, urokinase and plasmin was studied in assays based on the amidolytic activity of these enzymes. The effects of these antithrombin agents on the generation of some of these enzymes was also examined in the thrombin and factor Xa generation assay systems. Each of these inhibitors was also tested for its effects on the plasminogenolysis assays where the generation of plasmin was measured.

1. Thrombin Titration Assay

An amidolytic method was used to compare the direct antithrombin activity of thrombin inhibitors. The results of the studies of inhibition of the amidolytic activity of thrombin by specific thrombin inhibitors are depicted in Fig. 8. Panel A represents the inhibitory activity of D-Phe-Pro-Arg-H, panel B shows the effects of D-MePhe-Pro-Arg-H, panel C represents the inhibition produced by Boc-D-Phe-Pro-Arg-H. The inhibitory profile exhibited by Ac-(D)Phe-Pro-boroArg-OH is shown in Panel D, while Panel E represents the effects of argatroban and Panel F depicts the effects of hirudin. The concentration of each antithrombin agent that resulted in 50% inhibition of thrombin was the agent concentration that inhibited 0.5 thrombin units, since a total of 1 thrombin

unit/L was present in the assay. Therefore, based on this value, the amount of thrombin units inhibited by a mg or a nmol of each thrombin inhibitor was calculated. The specific activities in terms of ATU potencies for each thrombin inhibitor, based on the results depicted in Fig. 8, are reported in Table 8. On a gravimetric basis, the most potent thrombin inhibitor appeared to be Ac-D-Phe-Pro-boroArg-OH with about 200,700 ATU/mg. The aldehydes D-Phe-Pro-Arg-H and D-Me-Phe-Pro-Arg-H followed with about half of the activity of Ac-D-Phe-Pro-boroArg-OH (about 118,500 and 102,400 ATU/mg respectively), while Boc-D-Phe-Pro-Arg-H and hirudin exhibited much weaker (< 10%) activities when compared to Ac-D-Phe-Pro-boroArg-OH (around 29,600 and 22,400 ATU/mg respectively). Argatroban appeared to be the weakest thrombin inhibitor with about 1% of the activity of Ac-D-Phe-Pro-boroArg-OH (2,800 ATU/mg).

Table 8 -- Specific Activities of Various Thrombin Inhibitors as Assessed by Utilizing an Amidolytic Assay.

	ATU/mg	ATU/nmol
D-Phe-Pro-Arg-H	118,493 ± 6,868	59,365 ± 3,441
D-MePhe-Pro-Arg-H	102,422 ± 6,342	52,747 ± 3,266
Boc-D-Phe-Pro-Arg-H	29,605 ± 2,326	16,342 ± 1,284
Ac-D-Phe-Pro-boroArg-OH	200,669 ± 11,825	99,732 ± 5,877
Argatroban	2,774 ± 138	1,476 ± 73
Hirudin	22,382 ± 1,929	155,849 ± 13,432

Each value represents the mean ± 1 SD of three separate determinations. On the ATU/mg basis, all values were significantly different from each other, except for those produced by Boc-D-Phe-Pro-Arg-H and hirudin. On the ATU/nmol basis, all values were significantly different from each other except for those produced by D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H. ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test).

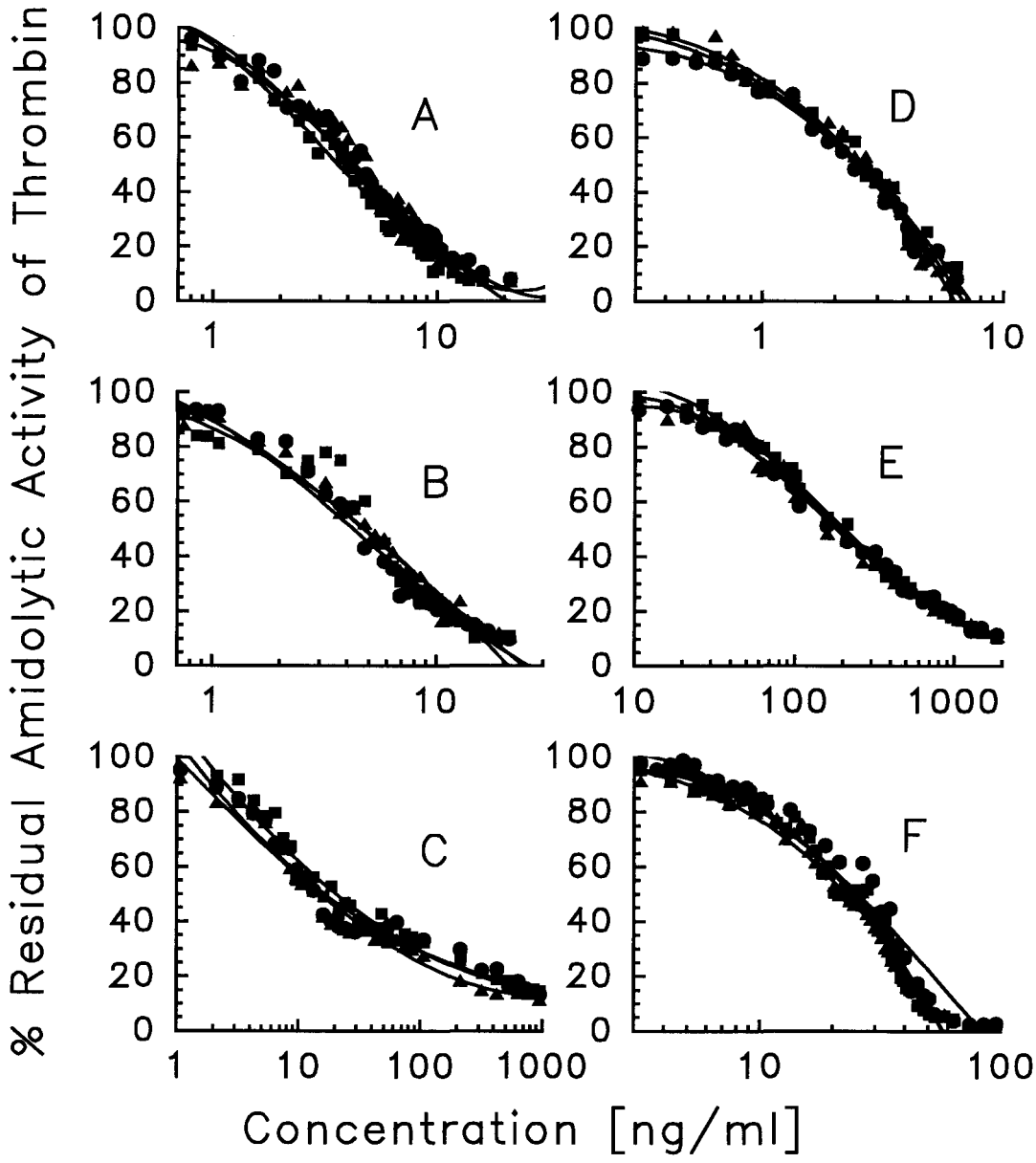


Fig. 8. Fig. 9. Concentration dependent inhibition of the amidolytic activity of thrombin by antithrombin agents. Panel A depicts the results produced by D-Phe-Pro-Arg-H. Panel B represents results produced by D-MePhe-Pro-Arg-H. Panel C represents results produced by Boc-D-Phe-Pro-Arg-H. Panel D represents results produced by Ac-(D)Phe-Pro-boroArg-OH. Panel E represents results produced by argatroban. Panel F represents results produced hirudin. Each agent was tested in triplicate. The mean % residual thrombin activity ± SD for each concentration and the gravimetric equivalent is given in Tables 8a-8f.

The order of potency for each of these thrombin inhibitors on a molar basis was found to be different from the order of potency on a gravimetric basis. On a molar basis, hirudin was found to be the strongest thrombin inhibitor with 155,800 ATU/nmol, followed by Ac-(D)Phe-Pro-boroArg-OH with about 99,700 ATU/nmol. D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H exhibited about 59,400 and 52,700 ATU/nmol, while Boc-D-Phe-Pro-Arg-H exhibited about 16,300 ATU/nmol. Argatroban was again the weakest thrombin inhibitor in this assay with 1,500 ATU/nmol. Individual % inhibitory values for each thrombin inhibitor concentration are given in Tables 8a through 8f.

2. Thrombin Time (TT) in a Fibrinogen Based System

A buffered fibrinogen based clotting assay was developed to evaluate the comparative antithrombin effects of individual inhibitors on the clotting function of thrombin in an isolated test system. Human thrombin was added to a human fibrinogen solution to mediate clotting, with no feedback and amplification processes involved (as there would be in a plasmatic medium where other factors are present). The results of these studies are depicted in Fig. 9. Panel A depicts the results from the assay where thrombin reconstituted in sterile saline was used, while panel B depicts the results from the test where thrombin was reconstituted in 0.025 M $CaCl_2$. To evaluate the comparative effects of thrombin inhibitors in these assays, an index was devised whereby the concentration of a thrombin inhibitor required to prolong the fibrinogen clotting time to 100 sec (CT_{100}) was calculated. These values are reported in Table 9. The clotting time at each concentration point for the individual inhibitors can be found in Tables 9a - 9d and Tables 9e - 9h, for the saline/thrombin and the $CaCl_2$/thrombin tests,

respectively. The order of potencies for each of the individual inhibitors was similar in both assays: hirudin was consistently found to be the strongest anticoagulant with CT_{100}s of 47 and 56 nM in the saline and $CaCl_2$ tests respectively, followed closely by Ac-(D)Phe-Pro-boroArg-OH with CT_{100} of 49 and 100 nM in the same assay systems.

Table 9 -- Antithrombin potencies of thrombin inhibitors as determined in fibrinogen clotting systems.

	Prolongation of clotting time to 100 sec [nM]	
	10 U TT	10 Ca^{++}TT
D-Phe-Pro-Arg-H	181 ± 5	512 ± 5
D-MePhe-Pro-Arg-H	134 ± 10	592 ± 12
Boc-D-Phe-Pro-Arg-H	586 ± 190	1542 ± 150
Ac-D-Phe-Pro-boroArg-OH	49 ± 1	100 ± 6
Argatroban	140 ± 24	422 ± 128
Hirudin	47 ± 3	56 ± 3
Heparin	>9,090	>9,090

Each value represents the mean ± 1 SD of three separate determinations. In the 10 U TT fibrinogen-based assay, only the value produced by Boc-D-Phe-Pro-Arg-H was significantly different from all others. In the 10 U Ca^{++}TT fibrinogen-based assay, all values were significantly different from each other except for the following pairs: D-Phe-Pro-Arg-H vs D-MePhe-Pro-Arg-H, D-Phe-Pro-Arg-H vs argatroban, D-MePhe-Pro-Arg-H vs argatroban and Ac-(D)Phe-Pro-boroArg-OH vs hirudin. ($p<0.05$, ANOVA followed by Student-Newman-Keuls test).

Heparin had no effects in these assays at concentrations as high as 9 μM. In the saline/thrombin system, D-MePhe-Pro-Arg-H with a CT_{100} of 134 nM was more potent than argatroban, D-Phe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H (CT_{100}s of 140, 181 and 586 nM respectively), while in the $CaCl_2$/thrombin assay system argatroban with a CT_{100} of 422 nM was more potent than D-Phe-Pro-Arg-OH, D-MePhe-Pro-Arg-H and Boc-D-

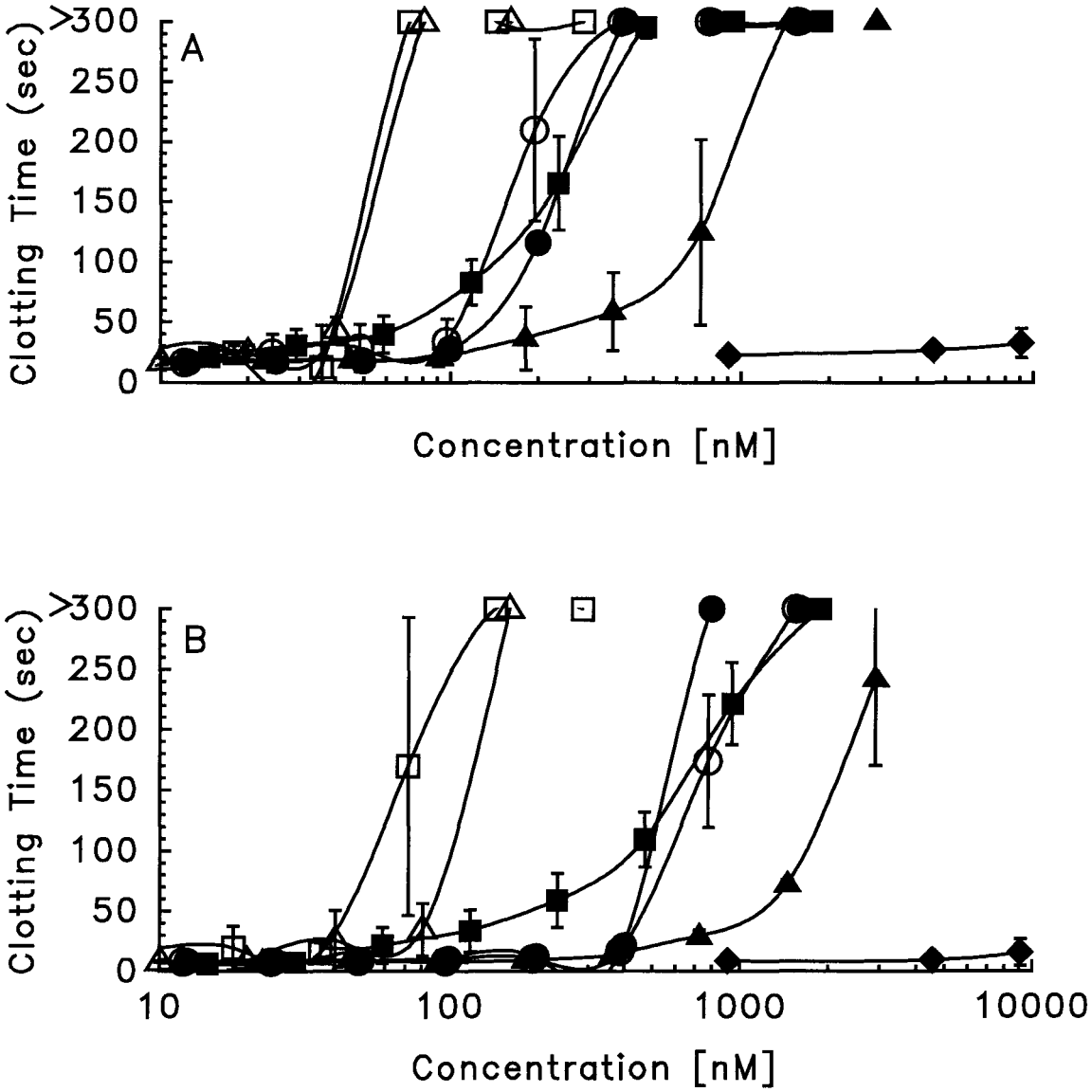


Fig. 9. Concentration dependent prolongation of the fibrinogen-based TT by various agents. Panel A depicts the effects produced by thrombin inhibitors in the fibrinogen-based TT system where thrombin was reconstituted in saline. The individual values at each concentration point and their gravimetric equivalents are given in Tables 9a-9d. Panel B represents effects produced by antithrombin agents in the fibrinogen-based TT system where thrombin was reconstituted in $CaCl_2$. The individual values at each concentration point and their gravimetric equivalents are given in Tables 9e-9h. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

Phe-Pro-Arg-H with CT_{100}s of 512, 592 and 1542 nM respectively. With the exception of heparin, all thrombin inhibitors were capable of prolonging the clotting time to >300 sec at nM concentrations. Hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-Phe-Pro-Arg-H appeared to have all-or-none anticoagulant effects, while the other thrombin inhibitors exhibited a more graded concentration-anticoagulant response relationship.

3. Antithrombin Effects of Thrombin Inhibitors

As in the thrombin titration assay, these assays are based on the ability of antithrombin agents to inhibit the amidolytic activity of thrombin, as detected by the release of pNA from a specific chromogenic substrate. In these assays the thrombin inhibitors are diluted in plasma rather than buffer, to mimic circulating plasma matrix. Thus, these assays represent a more physiologic condition in which the effects of plasmatic factors on the antithrombin agents can be readily observed. Both the NHP and NRP preparations were used to carry out these studies.

a. Antithrombin Amidolytic Assay with NHP

The results of the antithrombin activities of various agents after *in vitro* supplementation to NHP are depicted in panel A of Fig. 10. With the exception of heparin, all other thrombin inhibitors exhibited a sigmoidal concentration-response curve, that eventually reached 100% thrombin inhibition. After calculating the IC_{50} for each thrombin inhibitor, as seen in Table 10, it was apparent that even though heparin did not completely inhibit the amidolytic activity of thrombin, it appeared to be the strongest inhibitor with an IC_{50} of 9.6 nM. Hirudin, D-Phe-Pro-Arg-H and Ac-(D)Phe-Pro-

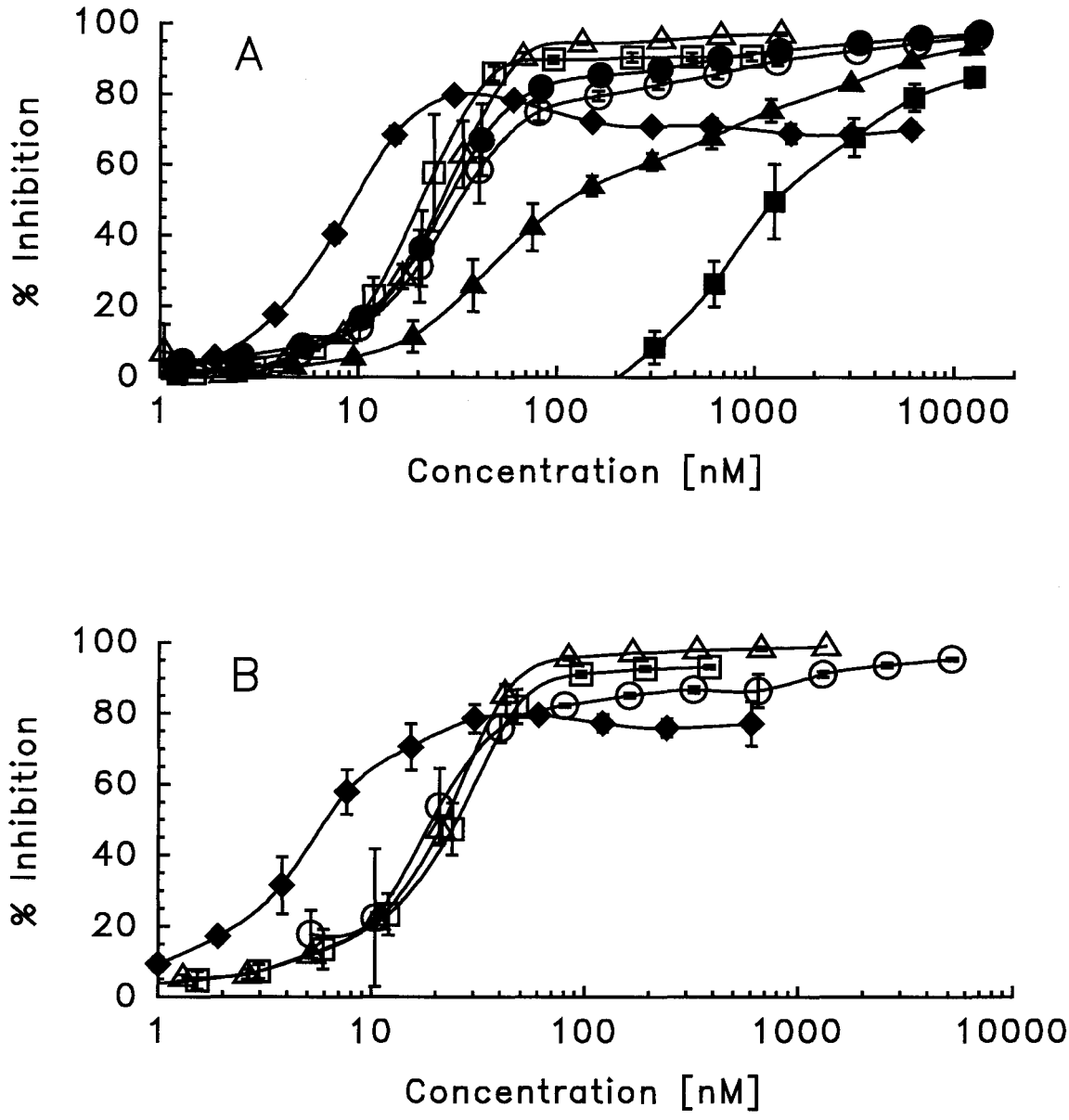


Fig. 10. Concentration dependent inhibition of the amidolytic activity of thrombin by various agents. Panel A depicts the effects produced by thrombin inhibitors in the NHP-based system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 10a-10d. Panel B represents effects produced by antithrombin agents in the NRP-based system. The individual values at each concentration point and their gravimetric equivalents are given in Table 10e. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

boroArg-OH followed closely, with IC_{50}s of 21, 28 and 32 nM respectively, while Boc-D-Phe-Pro-Arg-H was weaker with an IC_{50} of 110 nM. The weakest inhibitor in this test was found to be argatroban with an IC_{50} of 1,250 nM. While most thrombin inhibitors reached maximal thrombin inhibition in the nM range, Boc-D-Phe-Pro-Arg-H and argatroban produced its effects only in the μM range. The individual % thrombin inhibitory values for each thrombin inhibitor concentration are given in Tables 10a - 10d.

Table 10 -- Antithrombin potencies of thrombin inhibitors as determined in amidolytic systems in NHP and NRP.

	IC_{50} [nM]	
	NHP	NRP
D-Phe-Pro-Arg-H	28 ± 5	-
D-MePhe-Pro-Arg-H	32.2 ± 7	19.4 ± 4.2
Boc-D-Phe-Pro-Arg-H	110 ± 30	-
Ac-D-Phe-Pro-boroArg-OH	26.8 ± 5	21.8 ± 1.2
Argatroban	1,250 ± 140	-
Hirudin	21 ± 2	25 ± 4
Heparin	9.6 ± 0.5	6.1 ± 1.1

Each value represents the mean ± 1 SD of three separate determinations. In the NHP-based antithrombin amidolytic assay, only the value produced by argatroban was significantly different from all others. In the NRP-based antithrombin amidolytic assay, the value produced by heparin was significantly different from those produced by D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH and hirudin. ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test).

b. Antithrombin Amidolytic Assay with NRP

Fig. 10, panel B depicts the results of the antithrombin activities of D-MePhe-Pro-

Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin, after *in vitro* supplementation into NRP. All four agents produced a sigmoidal concentration-response. In this test, heparin appeared to be the most potent inhibitor of the amidolytic activity of thrombin with an IC_{50} of 6.1 nM. The activity of heparin was followed by that of D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH and hirudin with IC_{50} values of 19.4, 21.8 and 25 nM respectively. The concentration-response curves of the three other agents overlapped. Although heparin was the most potent inhibitor in this assay, its inhibitory activity plateaued around 80% inhibition, in contrast to the other antithrombin agents, which achieved maximal inhibition of thrombin at the higher concentrations used. The individual inhibitory values for every agent concentration can be found in Table 10e.

4. Anti-Xa Effects of Thrombin Inhibitors

These assays are based on the ability of thrombin inhibitors to suppress the amidolytic activity of factor Xa, as detected by a specific chromogenic substrate. As in the antithrombin assays, the thrombin inhibitors were diluted in plasma, to demonstrate any matrix effects.

a. Anti-Xa Amidolytic Assay with NHP

The results of the anti-Xa activities of thrombin inhibitors after *in vitro* supplementation to NHP are depicted in the panel A of Fig. 11. With the exception of heparin and Ac-(D)Phe-Pro-boroArg-OH, none of the thrombin inhibitors exhibited any anti-factor Xa activity. After calculating the IC_{50} for heparin and Ac-(D)Phe-Pro-boroArg-OH, as described in Table 11, it was apparent that even though heparin failed

to achieve a complete inhibition of the amidolytic activity of factor Xa, it was the most potent inhibitor with an IC_{50} value in the nM range (5.4 nM). While Ac-(D)Phe-Pro-boroArg-OH also exhibited concentration-dependent anti-Xa effects with an IC_{50} also in the nM range (390 nM), it was about 72 fold weaker than heparin. Interestingly, a gradual decrease in the inhibitory activity of heparin against factor Xa was noted at concentrations greater than 100 nM with a concomitant increase in the scatter of the data. The individual % factor Xa inhibitory values for each thrombin inhibitor concentration point are found in Tables 11a and 11b.

Table 11 -- Anti-factor Xa potencies of thrombin inhibitors as determined in amidolytic systems in NHP or NRP.

	IC_{50} [nM]	
	NHP	NRP
D-Phe-Pro-Arg-H	>11,000	-
D-MePhe-Pro-Arg-H	>10,500	>5,000
Boc-D-Phe-Pro-Arg-H	>10,000	-
Ac-D-Phe-Pro-boroArg-OH	390 ± 60	~1,000
Argatroban	>10,000	-
Hirudin	>800	>300
Heparin	5.4 ± 0.2	56

Each value represents the mean ± 1 SD of three separate determinations. In the NHP-based factor Xa amidolytic assay, the values produced by Ac-(D)Phe-Pro-boroArg-OH and heparin were significantly different. ($p<0.05$, ANOVA followed by Student-Newman-Keuls test).

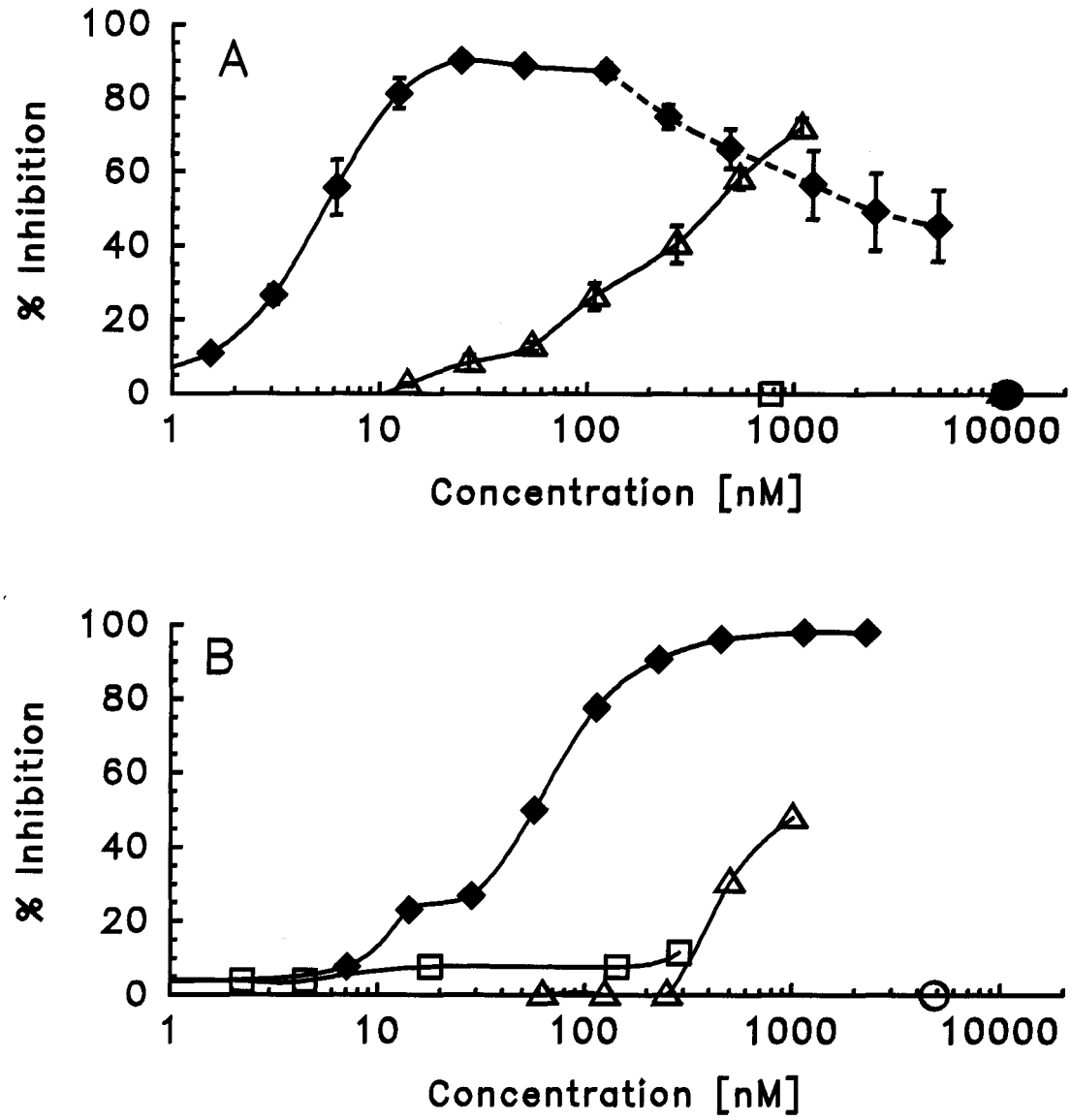


Fig. 11. Concentration dependent inhibition of the amidolytic activity of factor Xa by various agents. Panel A depicts the effects produced by thrombin inhibitors in the NHP-based system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 11a-11b. Panel B represents effects produced by antithrombin agents in the NRP-based system. The individual values at each concentration point and their gravimetric equivalents are given in Table 11c. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. The broken line denotes that results may be due to a precipitation effect mediated by heparin. Each point represents a mean ± 1 SD of three individual determinations.

b. Anti-Xa Amidolytic Assay with NRP

Fig. 11, panel B depicts the anti-Xa activities of various thrombi inhibitors after *in vitro* supplementation to NRP. While D-MePhe-Pro-Arg-H and hirudin exhibited no anti-Xa activities, heparin and Ac-(D)Phe-Pro-boroArg-OH produced concentration-dependent inhibition of the amidolytic activity of factor Xa. The IC_{50} values for these compounds were found to be 52 and ~1,000 nM, respectively. The individual values for the factor Xa inhibition produced by each agent concentration are given in Table 11c.

5. Inhibitory Actions of Various Thrombin Inhibitors on Specific Serine Proteases

To evaluate the anti-protease spectrum of thrombin inhibitors, amidolytic assays utilizing enzyme specific synthetic substrates were used. In these biochemically defined assays, only one enzyme was present in purified form, thus ensuring that inhibition of amidolytic activity was due to inhibition of the particular enzyme. Since heparin is devoid of any direct antiprotease activities and its mechanism of action is mediated primarily through antithrombin, stock solutions of heparin were made with antithrombin, and then further diluted in saline, prior to studying the effects of heparin on each one of the following serine proteases. In addition to the thrombin inhibitors, aprotinin was also included as a broad spectrum serine protease inhibitor and a positive control.

a. Inhibition of Chymotrypsin

Fig.12 depicts the inhibitory activities of various agent against the amidolytic activity of chymotrypsin. In this study, none of the thrombin inhibitors studied were capable of producing any inhibition of this amidolytic activity at high concentrations:

Table 12 -- Inhibition of serine proteases by thrombin inhibitors as determined in biochemically defined amidolytic systems.

	IC_{50} [μM]						
	Chymo-trypsin	Trypsin	APC	Glandular Kallikrein	tPA	Urokinase	Plasmin
D-Phe-Pro-Arg-H	>27	0.47±0.13	4.2±0.4	>67	33.6±2.2	>67	3.2±0.2
D-MePhe-Pro-Arg-H	>26	0.18±0.02	8.4±0.8	>65	52.7±2.9	>65	1.58±0.10
Boc-D-Phe-Pro-Arg-H	>24	0.24±0.02	14.6±1.4	>60	7.3±0.06	15.3±0.7	0.33±0.05
Ac-D-Phe-Pro-boroArg-OH	>27	0.095±0.025	0.75±0.05	14.7±0.6	6.8±0.05	1.3±0.3	0.095±0.02
Argatroban	>25	>125	>115	>115	>115	>115	>115
Hirudin	>1.9	>1.9	>9.6	>9.6	>9.6	>9.6	>9.6
Heparin	>1.2	>1.2	>62	>62	>62	>62	>62
Aprotinin	0.8	0.39±0.09	16.6±0.2	0.322±0.015	>5	>5	0.057±0.005

Each value represents the mean ± 1 SD of three separate determinations. In the trypsin assay, D-Phe-Pro-Arg-H and aprotinin were significantly different from D-MePhe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH. In the APC assay, all values (except for argatroban, hirudin and heparin) were significantly different from each other. In the kallikrein assay, the values produced by Ac-(D)Phe-Pro-boroArg-OH and aprotinin were significantly different from each. In the tPA assay, all values (except for argatroban, hirudin, heparin and aprotinin) were significantly different from each other except for the pair Boc-D-Phe-Pro-Arg-H vs Ac-(D)Phe-Pro-boroArg-OH. In the urokinase assay, the values produced by Boc-D-Phe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH were significantly different from each other. In the plasmin assay, the values produced by all thrombin inhibitors (except for argatroban, hirudin and heparin) were significantly different from each other, with the exception of the Boc-D-Phe-Pro-Arg-H vs Ac-(D)Phe-Pro-boroArg-OH comparison. ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test).

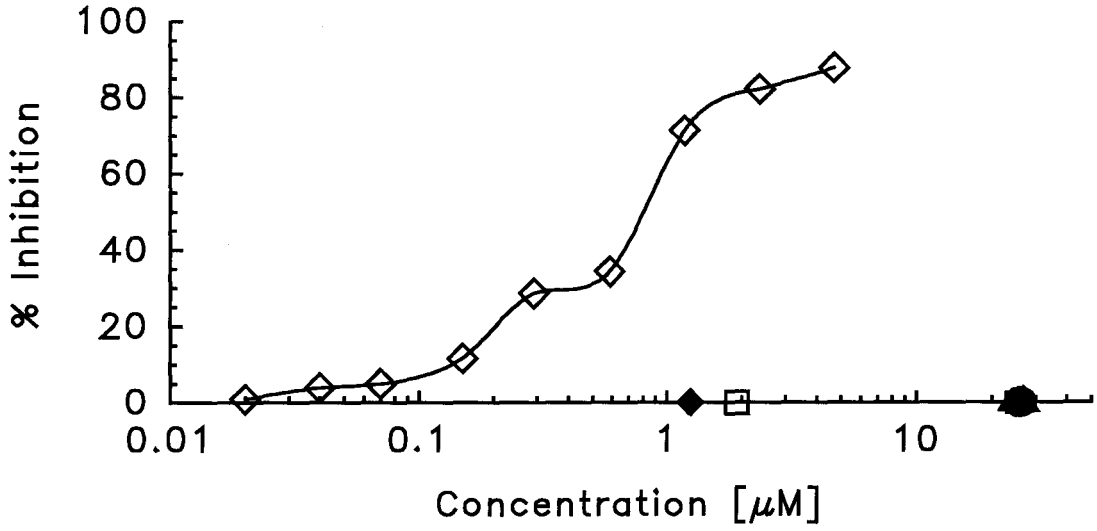


Fig. 12. Concentration dependent inhibition of the amidolytic activity of chymotrypsin by various agents. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. The individual values at each aprotinin concentration point and the gravimetric equivalents are given in Table 12a.

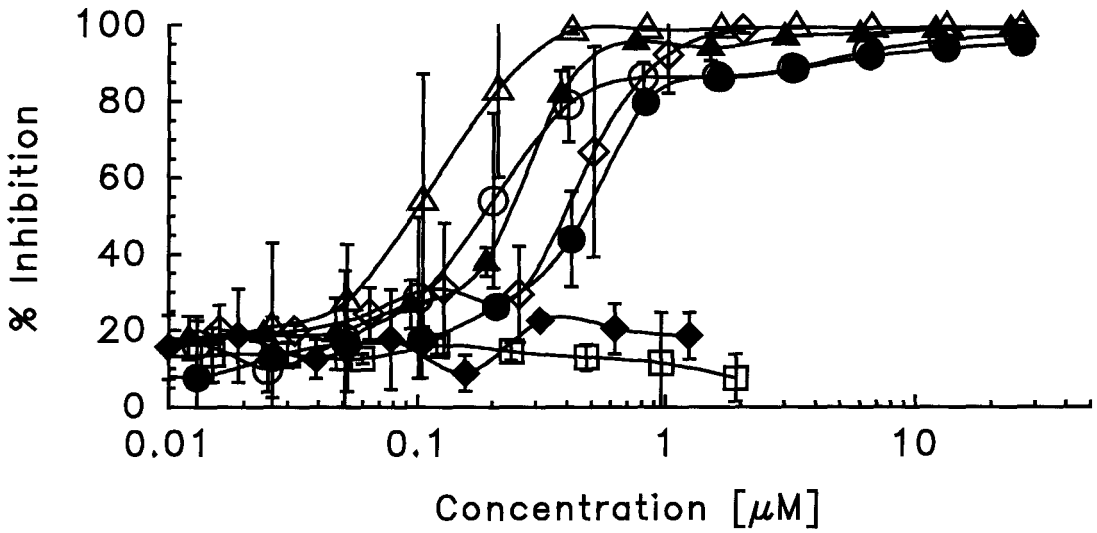


Fig. 13. Concentration dependent inhibition of the amidolytic activity of trypsin by thrombin inhibitors. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 12b.

D-Phe-Pro-Arg-H >27 μM, D-MePhe-Pro-Arg-H >26 μM, Boc-D-Phe-Pro-Arg-H >24 μM, Ac-(D)Phe-Pro-boroArg-OH >27 μM, argatroban >25 μM, hirudin >1.9 μM, heparin >1.2 μM. However, aprotinin, produced a concentration-dependent inhibition of chymotrypsin with an apparent IC_{50} of 0.8 μM, as reported on Table 12. The individual inhibitory values produced by aprotinin are given in Table 12a.

b. Inhibition of Trypsin

Fig. 13 depicts the results from the trypsin inhibitory studies by various agents. In this assay, argatroban, hirudin and heparin had relatively weaker inhibitory activities against the amidolytic activity of trypsin at concentrations higher than 125, 1.9 and 1.2 μM respectively. The other thrombin inhibitors demonstrated a concentration dependent inhibition of trypsin. According to the IC_{50} values for each thrombin inhibitor, as reported in Table 12, the most potent thrombin inhibitor in this assay was Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.095 μM), followed by D-MePhe-Pro-Arg-H (IC_{50} 0.18 μM), Boc-D-Phe-Pro-Arg-H (IC_{50} 0.24 μM) and then by D-Phe-Pro-Arg-H (IC_{50} 0.47 μM). The anti-fibrinolytic agent aprotinin, used in these studies as the positive control, also exhibited a concentration-dependent ant-trypsin effect with an IC_{50} of 0.39 μM. The inhibition produced by each antithrombin agent is found in Table 12b.

c. Inhibition of Activated Protein C (APC)

The inhibitory effects of thrombin inhibitors against the amidolytic activity of APC are depicted in Fig. 14. With the exception of heparin, hirudin and argatroban, all of the other thrombin inhibitors exhibited a concentration-dependent inhibitory effect

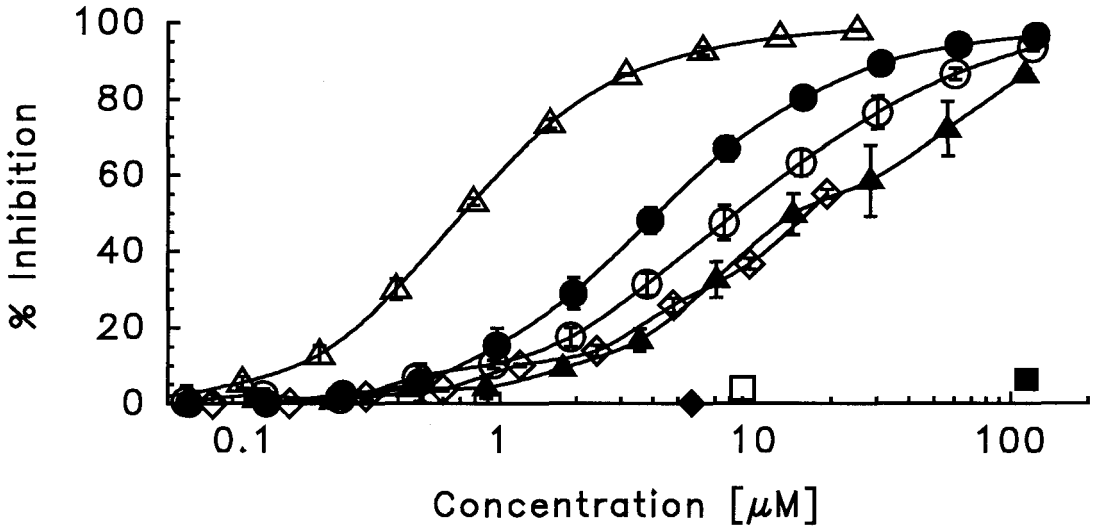


Fig. 14. Concentration dependent inhibition of the amidolytic activity of APC by various agents. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 12c.

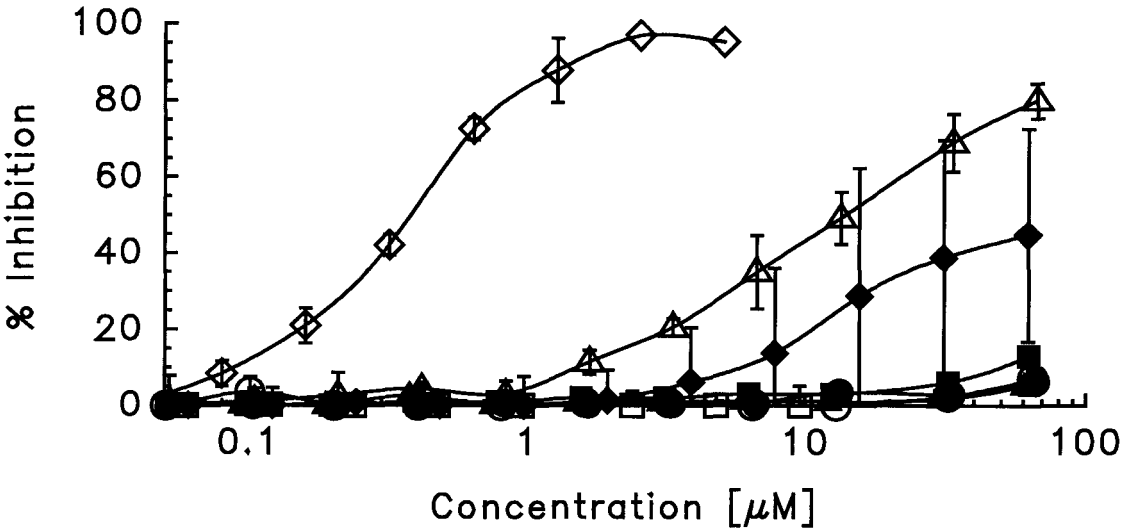


Fig. 15. Concentration dependent inhibition of the amidolytic activity of kallikrein by thrombin inhibitors. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 12d.

against APC. While heparin, hirudin and argatroban did not exhibit any inhibitory actions at high concentrations (>9, >10 and >100 μM, respectively), the other thrombin inhibitors had IC_{50} values <10 μM, as shown in Table 12. The most potent inhibitor was Ac-(D)Phe-Pro-boroArg-OH with an IC_{50} of 0.75 μM, followed by D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H with IC_{50}s of 4.2, 8.4 and 14.6 μM respectively. Aprotinin was slightly weaker than the thrombin inhibitors. However, this agent also inhibited APC in a concentration-dependent manner with an IC_{50} of 16.6 μM. All four agents were capable of completely inhibiting the APC amidolytic activity at concentrations around 100 μM. The individual % APC inhibitory values for each inhibitor concentration point are found in Table 12c.

d. Inhibition of Glandular Kallikrein

The representative data of the inhibition of glandular kallikrein by various thrombin inhibitors are depicted in Fig. 15. In this assay, only heparin and Ac-(D)Phe-Pro-boroArg-OH exhibited concentration-dependent inhibitory effects against the amidolytic action of glandular kallikrein. When compared to aprotinin (IC_{50} 0.32 μM), these thrombin inhibitors were found to be relatively weaker inhibitors, with Ac-(D)Phe-Pro-boroArg-OH exhibiting an IC_{50} of 14.7 μM, whereas for heparin it was >62 μM. Furthermore, neither of the thrombin inhibitors was capable of completely inhibiting kallikrein at concentrations higher than 60 μM. The IC_{50} values of these agents are found in Table 12, while the individual % kallikrein inhibitory values for each inhibitor concentration point are found in Table 12d.

e. Inhibition of Tissue Plasminogen Activator (tPA)

The amidolytic activity of tPA was inhibited by all of the tripeptide thrombin inhibitors, as seen in Fig. 16. As can be seen in this figure, argatroban, hirudin, heparin and aprotinin did not affect the amidolytic activity of tPA at extremely higher concentrations of up to 60, 10, 62 and 5 μM respectively. The tripeptide thrombin inhibitors exhibited a concentration-dependent tPA inhibition. The estimated IC_{50} values for these inhibitions are shown in Table 12. The most potent inhibitor was found to be Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 6.8 μM) followed closely by Boc-D-Phe-Pro-Arg-H (IC_{50} 7.3 μM). The peptides D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H also inhibited the amidolytic activity of tPA, but at higher concentrations wit IC_{50}s of 33.6 and 52.7 μM respectively. Furthermore, while Ac-(D)Phe-Pro-boroArg-OH and Boc-D-Phe-Pro-Arg-H reached 100% inhibition of tPA at concentrations under 60 μM, the other two tripeptides were not as potent at similar concentrations. The individual % tPA inhibitory values for each inhibitor concentration point are found in Table 12e.

f. Inhibition of Urokinase

Fig. 17 depicts the inhibitory activities of various agents on the amidolytic action of urokinase. Argatroban, hirudin, heparin and aprotinin did not inhibit the amidolytic activity of urokinase at high concentrations (>60, >10, >62 and >5 μM respectively). While all tripeptide thrombin inhibitors reduced the amidolytic activity of tPA in a concentration-dependent manner, only two of these were capable of inhibiting the amidolytic action of urokinase. Table 12 depicts the IC_{50} values for Ac-(D)Phe-Pro-boroArg-OH and Boc-D-Phe-Pro-Arg-H, which were 1.3 and 15.3 μM respectively. On

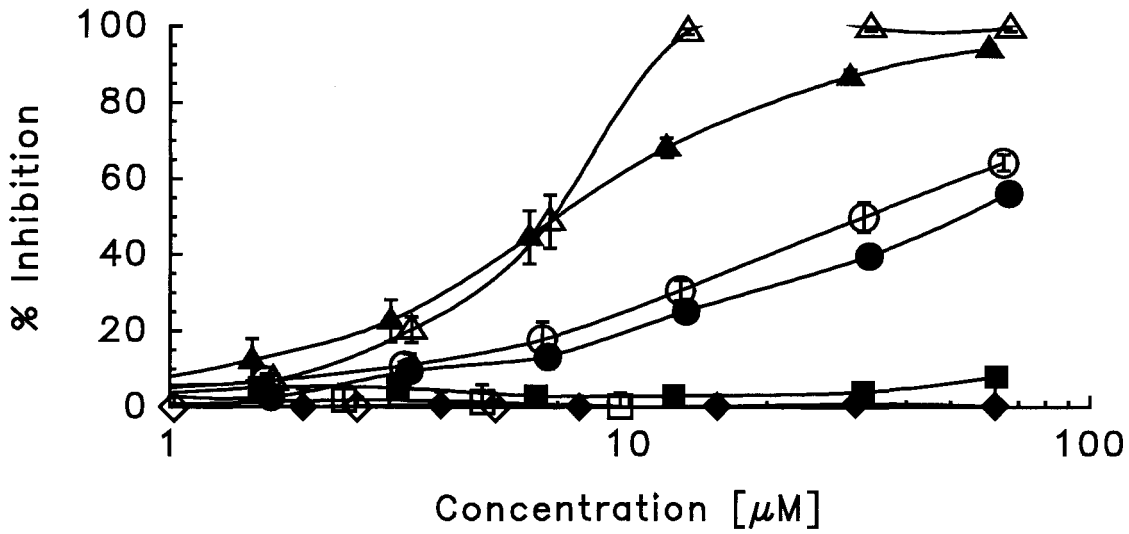


Fig. 16. Concentration dependent inhibition of the amidolytic activity of t-PA by antithrombin agents. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 12e.

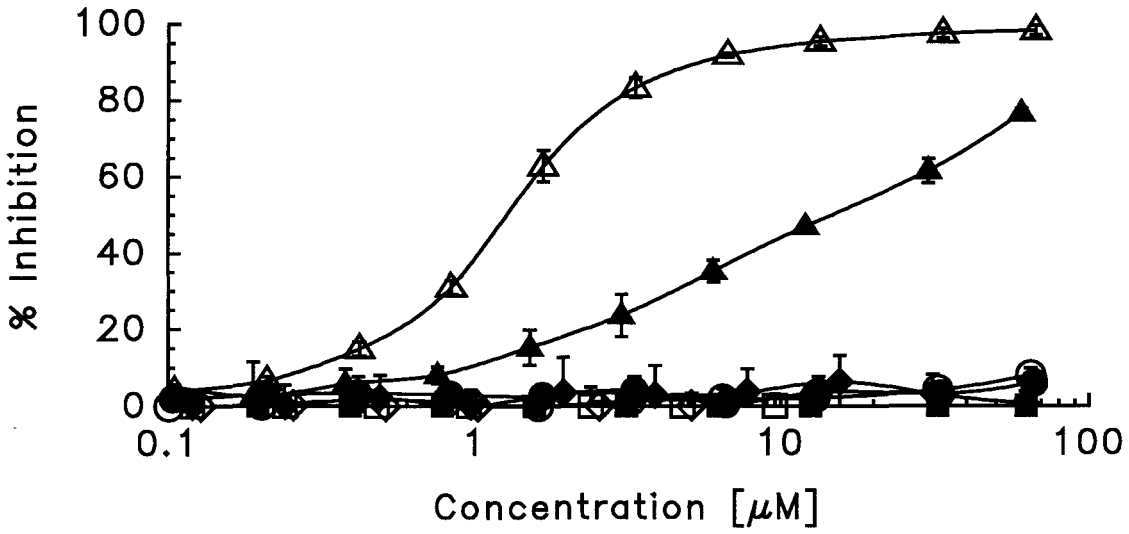


Fig. 17. Concentration dependent inhibition of the amidolytic activity of urokinase by various agents. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 12f.

the other hand, D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H did not exhibit inhibitory activities in this assay at concentrations $>60\ \mu M$. The individual % urokinase inhibitory values for each inhibitor concentration point are found in Table 12f.

g. Inhibition of Plasmin

As depicted on Fig. 18, with the exception of hirudin, the amidolytic activity of plasmin was inhibited by all thrombin inhibitors and aprotinin. Argatroban exhibited the weakest inhibitory activity and even the highest concentration (115 μM) did not achieve 50% inhibition of plasmin. Interestingly, heparin exhibited inhibitory activity against plasmin at concentrations $>10\ \mu M$ which was not concentration-dependent and plateaued around 20% inhibition. All tripeptide thrombin inhibitors and aprotinin exhibited concentration-dependent anti-plasmin activity, and all were capable of maximal plasmin inhibition at μM concentrations. After calculating the IC_{50} value for each of these inhibitors, reported in Table 12, the most potent plasmin inhibitor was aprotinin (IC_{50} 0.057 μM), followed by Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.095 μM), Boc-D-Phe-Pro-Arg-H (IC_{50} 0.33 μM), D-MePhe-Pro-Arg-H (IC_{50} 1.58 μM) and D-Phe-Pro-Arg-H (IC_{50} 3.2 μM). The individual % plasmin inhibitory values for each inhibitor concentration point are found in Tables 12g and 12h.

6. Inhibition of Plasminogenolysis

Since it was found that some of the thrombin inhibitors studied in the above described assays had inhibitory activities against serine proteases other than thrombin and because some of these proteases are involved in the regulation of fibrinolysis, more

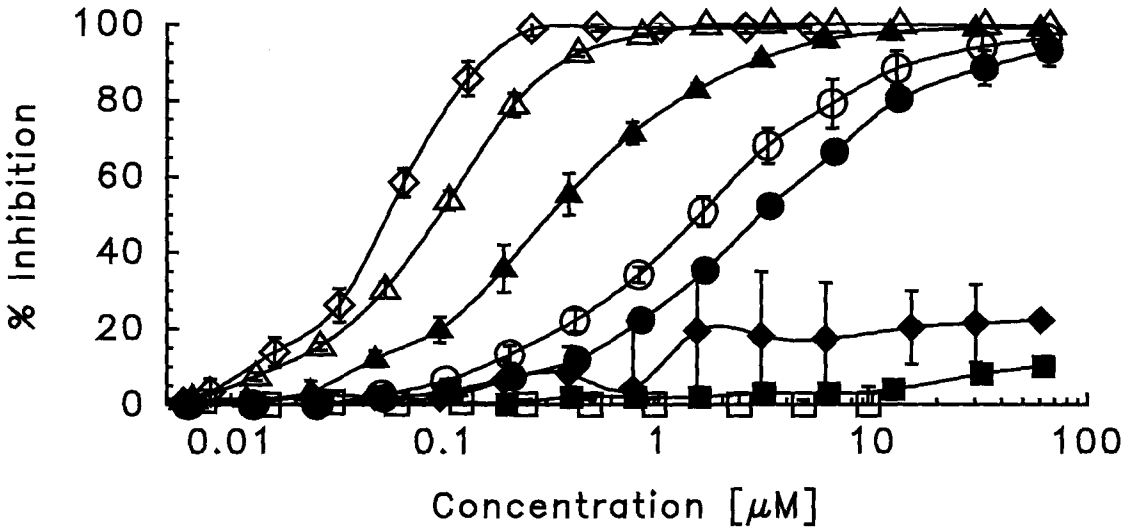


Fig. 18. Concentration dependent inhibition of the amidolytic activity of plasmin by thrombin inhibitors. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 12g-12h.

specifically, the activation of plasminogen to plasmin, the effect of these agents on the generation of plasmin was also investigated. In these assays, plasminogen was activated to plasmin by either streptokinase or tPA in buffered plasminogen and NHP-based assays.

a. Pure Plasminogen System

Fig. 19 depicts the effects of various agents on the process of plasminogenolysis in the assays where plasminogen is provided in a purified form. Except for argatroban, all thrombin inhibitors studied in these assay systems exhibited inhibitory activities in the process of plasminogenolysis, although to varying degrees, regardless of whether activation was mediated by streptokinase (Fig. 19, panel A) or tPA (Fig. 19, panel B). The inhibitory patterns that emerged from the two assays were parallel.

As depicted in Fig. 19, panel A, in the streptokinase-mediated plasminogenolysis assay, hirudin appeared to be the weakest inhibitor (IC_{50} >1.2 μM), with a maximal inhibition of around 20% at the highest concentration used (1 μM), concentration-independently. On the other hand Ac-(D)Phe-Pro-boroArg-OH was the strongest inhibitor (IC_{50} <1.97 μM) followed closely by Boc-D-Phe-Pro-Arg-H (IC_{50} 4.9 μM). While the effects of D-MePhe-Pro-Arg-H (IC_{50} 7 μM) and D-Phe-Pro-Arg-H (IC_{50} 12.6 μM) followed those of Ac-(D)Phe-Pro-boroArg-OH and Boc-D-Phe-Pro-Arg-H, aprotinin was a stronger concentration-dependent inhibitor of plasminogenolysis. The inhibitory pattern of heparin was weakly concentration-dependent in this assay (IC_{50} >0.73 μM). The IC_{50} value for each thrombin inhibitor in the streptokinase assay are reported in Table 13. The individual % plasminogenolysis inhibitory values for each inhibitor concentration point are found in Tables 13a - 13c.

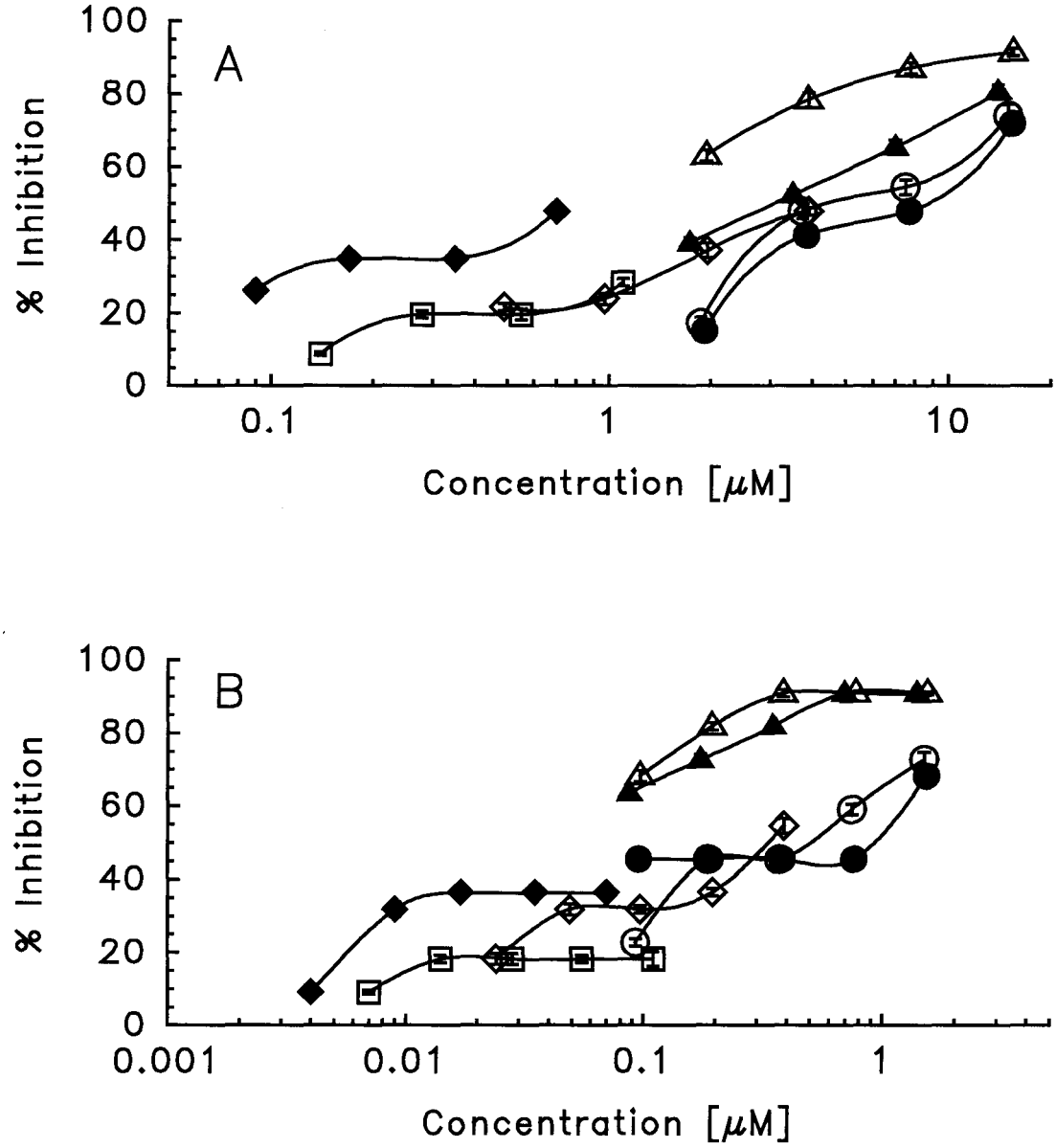


Fig. 19. Concentration dependent inhibition of the process of plasminogenolysis by antithrombin agents. Panel A depicts the effects mediated by various agents in the pure plasminogen system, activated by streptokinase. The individual values at each concentration point and their gravimetric equivalents are given in Tables 13a-13c. Panel B depicts the effects mediated by agents in the pure plasminogen system, activated by t-PA. The individual values at each concentration point and their gravimetric equivalents are given in Tables 13d-13f. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations.

Table 13 -- Inhibition of plasminogenolysis by thrombin inhibitors as determined in biochemically defined and NHP amidolytic systems.

	Pure Plasminogen Assay, IC_{50} in [μM]		NHP-Derived Plasminogen Assay, IC_{50} in [μM]	
	Streptokinase	tPA	Streptokinase	tPA
D-Phe-Pro-Arg-H	12.6 ± 1.6	1.18 ± 0.02	7.0 ± 1.6	2.5 ± 0.1
D-MePhe-Pro-Arg-H	7.0 ± 0.8	0.62 ± 0.02	5.24 ± 0.97	3.5 ± 0.1
Boc-D-Phe-Pro-Arg-H	4.9 ± 0.5	<0.089	4.3 ± 0.7	<1.77
Ac-D-Phe-Pro-boroArg-OH	<1.97	<0.098	<1.97	<1.97
Argatroban	>1.0	>1.0	>1.0	>1.0
Hirudin	>1.22	>1.22	>1.22	>1.22
Heparin	>0.73	>0.73	>0.73	>0.73
Aprotinin	>3.84	0.355 ± 0.215	>3.84	1.92 ± 0.02

Each value represents the mean ± 1 SD of three separate determinations. In the pure plasminogen lysed by streptokinase assay, the value produced by D-Phe-Pro-Arg-H was significantly different from those produced by D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H. In the pure plasminogen lysed by tPA assay, the value produced by Boc-D-Phe-Pro-Arg-H was significantly different from those produced by D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H. In the NHP-derived plasminogen lysed by streptokinase assay, non of the values were significantly different from each other. Similarly, in the NHP-derived plasminogen lysed by tPA assay, non of the values were significantly different from each other. ($p<0.05$, ANOVA followed by Student-Newman-Keuls test).

Fig. 19, panel B depicts the inhibitory effects of various agents on the plasminogenolysis mediated by tPA. Hirudin was found to be again the weakest inhibitor (IC_{50} >1.2 μM), producing a plateau at around 20% inhibition even at the highest concentration (1 μM). Ac-(D)Phe-Pro-boroArg-OH was found to be the strongest inhibitor of the tPA-induced plasminogenolysis (IC_{50} <0.1 μM), followed by Boc-D-Phe-Pro-Arg-H (IC_{50} <0.09 μM), aprotinin (IC_{50} 0.36 μM), D-MePhe-Pro-Arg-H (IC_{50} 0.6 μM) and D-Phe-Pro-Arg-H (IC_{50} 1.2 μM). Heparin produced weak inhibitory effects which plateaued at 35% inhibition at concentration in the nM range. The IC_{50} values for each of the antithrombin agents are reported in Table 13. The individual inhibitory values at each agent concentration point are given in Tables 13d - 13f.

b. NHP System

Fig. 20 represents the effects of various inhibitors on the process of plasminogenolysis in assays where the source of plasminogen is NHP. With the exception of argatroban and heparin, all thrombin inhibitors studied in these tests exhibited inhibitory activities in the process of plasminogenolysis, although to varying degrees, regardless of whether activation was mediated by streptokinase (Fig. 20, panel A) or tPA (Fig. 20, panel B). The inhibitory patterns that resulting from the two tests were parallel.

In the streptokinase-induced plasminogenolysis assay (Fig. 20, panel A), heparin appeared to be the weakest inhibitor, with a maximal inhibition of around 25% at the highest concentration used (0.73 μM), followed by hirudin with a maximal inhibition of 30% at the highest concentration studied (1 μM). On the other hand Ac-(D)Phe-Pro-

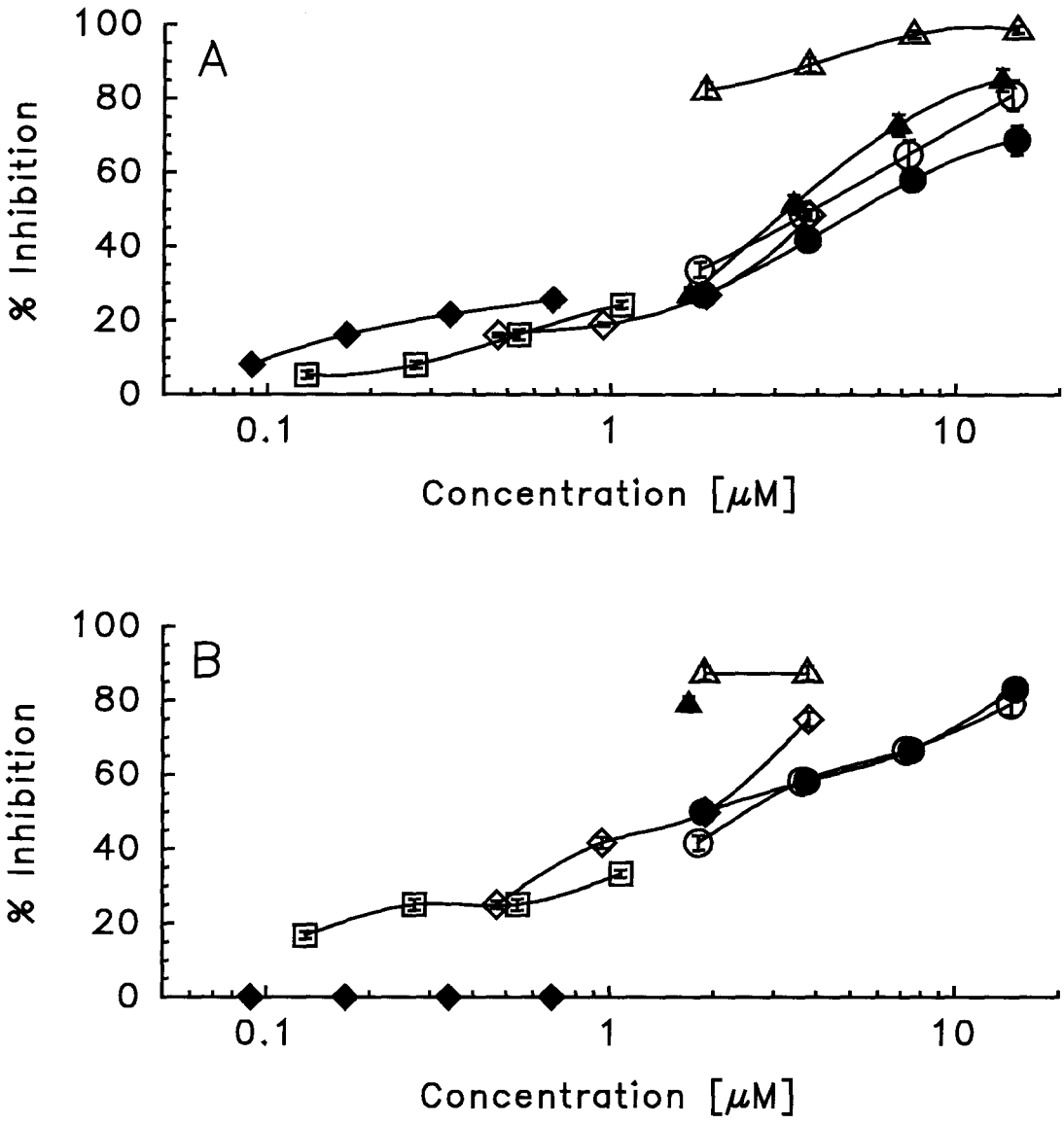


Fig. 20. Concentration dependent inhibition of process of plasminogenolysis by antithrombin agents. Panel A depicts the effects mediated by various agents in the NHP system, activated by streptokinase. The individual values at each concentration point and their gravimetric equivalents are given in Tables 13g-13i. Panel B depicts the effects mediated by agents in the NHP system, activated by t-PA. The individual values at each concentration point and their gravimetric equivalents are given in Tables 13j-13l. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (□) hirudin, (◆) heparin, (◇) aprotinin. Each point represents a mean ± 1 SD of three individual determinations.

boroArg-OH was the strongest inhibitor (IC_{50} <2.0 μM), followed closely by Boc-D-Phe-Pro-Arg-H (IC_{50} 4.3 μM). While the concentration-dependent effects of D-MePhe-Pro-Arg-H (IC_{50} 5.2 μM) and D-Phe-Pro-Arg-H (IC_{50} 7 μM) followed those of Ac-(D)Phe-Pro-boroArg-OH and Boc-D-Phe-Pro-Arg-H, they were superimposable with those of aprotinin. The IC_{50} values for each thrombin inhibitor are given in Table 13, while the values for the individual concentrations of each agent are reported in Tables 13g - 13i.

In the tPA-induced plasminogenolysis, heparin had no effects even at the highest concentration studied (0.73 μM). Hirudin was again found to be the weakest inhibitor of plasminogenolysis (IC_{50} <1 μM) and inhibited only 33% of the process. The strongest inhibitor in this assay was found to be Ac-(D)Phe-Pro-boroArg-OH (IC_{50} <2 μM), followed by Boc-D-Phe-Pro-Arg-H (IC_{50} <1.8 μM), aprotinin (IC_{50} 1.9 μM), D-Phe-Pro-Arg-H (IC_{50} 2.5 μM) and D-MePhe-Pro-Arg-H (IC_{50} 2.5 μM). Table 12 reports the IC_{50} values of each thrombin inhibitor in this assay. Tables 13j - 13l contain the inhibitory values at each concentration for the individual antithrombin agents.

7. Inhibition of Extrinsic Activation Systems in Fibrinogen Deficient Human Plasma by Thrombin Inhibitors

Although the antithrombin and anti-Xa assays provided data on the direct inhibitory activities of thrombin inhibitors against thrombin and factor Xa, their effects on protease generation were not addressed in these assays. The coagulation cascade is a complex enzyme network with feedback mechanisms and amplification loops. Thus, it is conceivable that even if a thrombin inhibitor is effective in directly inhibiting

thrombin or factor Xa, it may not inhibit the generation of various proteases and thrombin. Therefore, the inhibitory effects of thrombin inhibitors on the generation of thrombin and factor Xa were studied in the fibrinogen deficient plasma where specific activators of the extrinsic and intrinsic assay systems were used. The human plasma used in these assays was fibrinogen deficient, in which clot formation was avoided to minimize clotting interference.

a. Inhibition of Extrinsic Thrombin Generation

Fig. 21. panel A depicts the data of the inhibition of thrombin generation after extrinsic activation by various thrombin inhibitors. All agents produced a concentration-dependent inhibition of thrombin. While hirudin was the most potent inhibitor (IC_{50} 0.02μM) in this assay, the maximal inhibitory activity plateaued around 80%. With the exception of argatroban, the rest of the thrombin inhibitors were relatively weaker inhibitors but were able to completely inhibit the generation of thrombin at higher concentrations. According to the IC_{50} values for each thrombin inhibitor in this test, reported in Table 14, after hirudin, the most potent inhibitor was heparin (IC_{50} 0.2 μM), closely followed by Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.4 μM). The effects of D-Phe-Pro-Arg-H (IC_{50} 5.3 μM) and D-MePhe-Pro-Arg-H (IC_{50} 5.1 μM) were superimposable. Although Boc-D-Phe-Pro-Arg-H had a similar IC_{50} value as the other two aldehydes (5.5 μM), the slope of the response was steeper, indicating improved efficacy. Argatroban was found to be the weakest inhibitor in this test. The individual % thrombin generation inhibitory values for each inhibitor concentration point are found in Tables 14a - 14e.

Table 14 -- Inhibition of generation of thrombin and factor Xa by thrombin inhibitors as determined in fibrinogen-deficient amidolytic systems.

	Extrinsic Generation, IC_{50} [μM]		Intrinsic Generation, IC_{50} [μM]	
	Thrombin	Factor Xa	Thrombin	Factor Xa
D-Phe-Pro-Arg-H	5.3 ± 0.6	10 ± 1.5	3.8 ± 0.8	0.07 ± 0.01
D-MePhe-Pro-Arg-H	5.1 ± 0.5	13 ± 1.5	1.4 ± 0.4	0.106 ± 0.005
Boc-D-Phe-Pro-Arg-H	5.5 ± 0.5	2.2 ± 0.4	1.4 ± 0.2	0.16 ± 0.01
Ac-D-Phe-Pro-boroArg-OH	0.4 ± 0.1	0.24 ± 0.06	0.14 ± 0.04	0.036 ± 0.005
Argatroban	19 ± 3	>60	6.5 ± 0.6	0.17 ± 0.03
Hirudin	0.02 ± 0.01	>5	0.04 ± 0.005	0.018 ± 0.002
Heparin	0.2 ± 0.1	0.045 ± 0.01	0.032 ± 0.004	0.006 ± 0.002

Each value represents the mean ± 1 SD of three separate determinations. In the extrinsic generation of thrombin assay, all values were significantly different from each other except for the following pairs: D-Phe-Pro-Arg-H vs D-MePhe-Pro-Arg-H, D-Phe-Pro-Arg-H vs Boc-D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H vs Boc-D-Phe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH vs hirudin, Ac-(D)Phe-Pro-boroArg-OH vs heparin and hirudin vs heparin. In the extrinsic generation of factor Xa, the values produced by D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H were significantly different when compared to each other and when compared to Boc-D-Phe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH and heparin. In the intrinsic thrombin generation assay, all values produced by all thrombin inhibitors were significantly different from each other, with the exception of the following pairs: D-MePhe-Pro-Arg-H vs Boc-D-Phe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH vs hirudin, Ac-(D)Phe-Pro-boroArg-OH vs heparin and hirudin vs heparin. In the extrinsic factor Xa generation assay, all values were significantly different from each other, except for the comparison between Boc-D-Phe-Pro-Arg-H and argatroban. ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test).

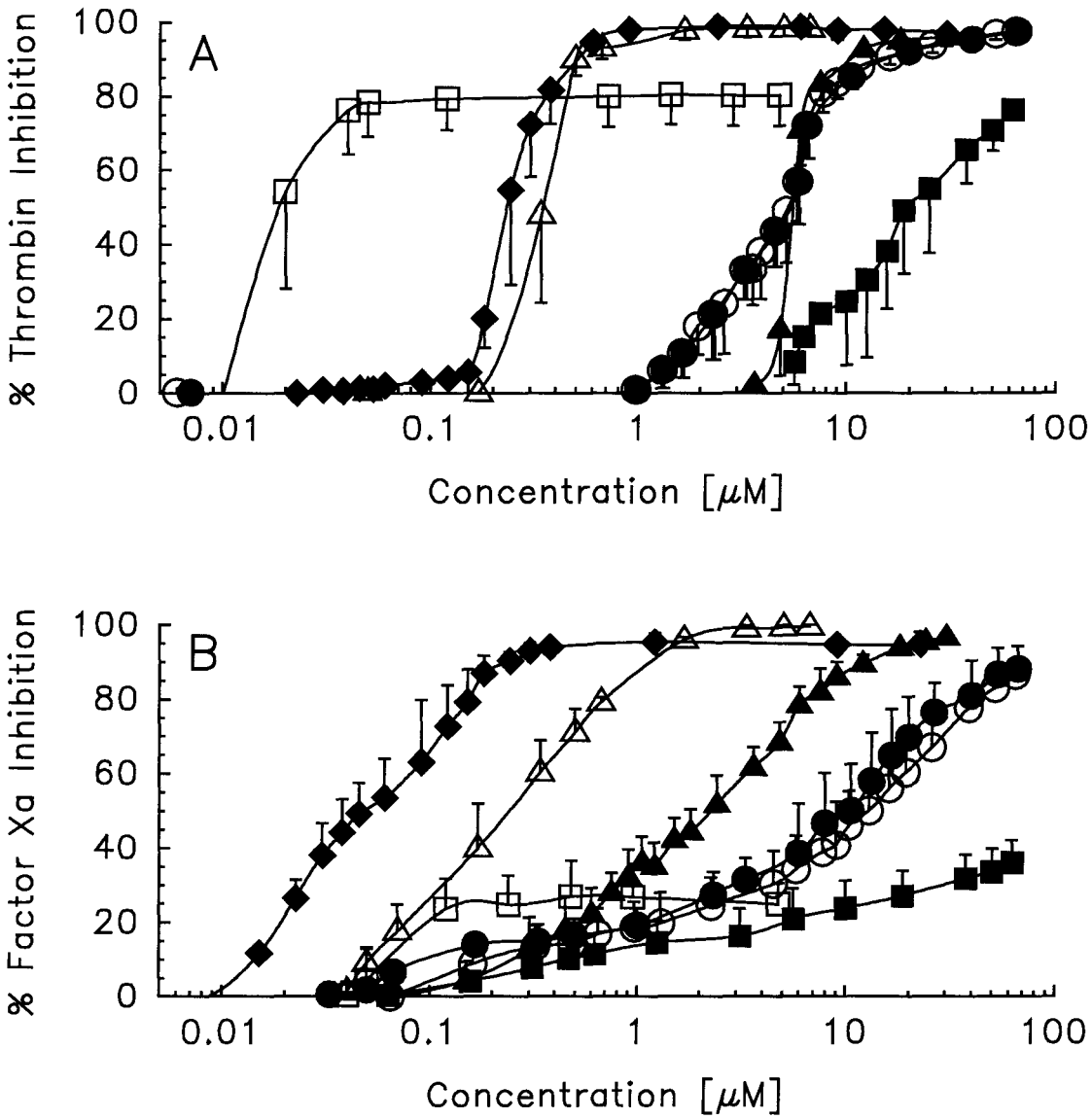


Fig. 21. Concentration-dependent inhibition of the extrinsic activation of coagulation, by antithrombin agents, in the fibrinogen-deficient system. Panel A depicts the effects mediated by various agents in the extrinsic thrombin generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 14a-14e. Panel B depicts the effects mediated by agents in the extrinsic factor Xa generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 14f-14j. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

b. Inhibition Extrinsic Factor Xa Generation

Fig. 21, panel B depicts the results on the inhibition of factor Xa generation after extrinsic activation by various agents. All inhibitors produced a concentration-dependent inhibition of factor Xa. Hirudin appeared to be a weak inhibitor of extrinsic factor Xa generation and only about 20% of factor Xa generation was maximally inhibited at concentration as high as 5 μM. With the exception of argatroban, the other thrombin inhibitors were able to completely inhibit the generation of factor Xa. According to the IC_{50} values for each antithrombin agent in this assay reported in Table 14, the most potent inhibitor was heparin (IC_{50} 0.045 μM), followed by Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.24 μM) and then by Boc-D-Phe-Pro-Arg-H (IC_{50} 2.2 μM). The effects of D-Phe-Pro-Arg-H (IC_{50} 10 μM) and D-MePhe-Pro-Arg-H (IC_{50} 13 μM) were superimposable. Argatroban was the weakest inhibitor in this assay system (IC_{50} >62 μM). The individual % factor Xa generation inhibitory values for each inhibitor concentration point are found in Tables 14f - 14j.

8. Inhibition of Intrinsic Activation Systems in Fibrinogen Deficient Human Plasma by Thrombin Inhibitors

The extrinsic and intrinsic activation of the coagulation cascade trigger different sequences of clotting factor activations. Therefore, the proportions of active factors generated, as well as the speed at which they are generated are different. To study the effects of thrombin inhibitors on the coagulation cascade after intrinsic activation, their effects on the generation of thrombin and factor Xa were studied in the following tests, where human plasma was activated intrinsically and the amount of thrombin and factor

Xa generated was detected with specific chromogenic substrates. As with the extrinsic assays described earlier, fibrinogen deficient human plasma was used in these assays.

a. Inhibition of Intrinsic Thrombin Generation

Fig. 22, panel A depicts the inhibition of thrombin generation after intrinsic activation by various thrombin inhibitors. The inhibition produced by these agents was concentration-dependent. While heparin was found to be the most potent inhibitor in this test (IC_{50} 0.032 μM), followed closely by hirudin (IC_{50} 0.04 μM), the maximal inhibitory activity of both agents plateaued around 80%. With the exception of argatroban, the other thrombin inhibitors appeared to be weaker than heparin and hirudin but were able to completely inhibit the generation of thrombin at higher concentrations. According to the IC_{50} values for each thrombin inhibitor in this assay, reported in Table 14, after hirudin and heparin, the most potent inhibitor was Ac-(D)Phe-Pro-boroArg-OH with an IC_{50} of 0.14 μM. The effects of D-MePhe-Pro-Arg-H (IC_{50} 1.4 μM) and Boc-D-Phe-Pro-Arg-H (IC_{50} 1.4 μM) were superimposable, followed by D-Phe-Pro-Arg-H (IC_{50} 3.8 μM). Argatroban was found to be the weakest inhibitor in this assay system (IC_{50} 6.5 μM) and it also appeared to plateau around 65%. The individual % thrombin generation inhibitory values for each inhibitor concentration point are found in Tables 14k - 14o.

b. Inhibition of Intrinsic Factor Xa Generation

Fig. 22, panel B, represents the inhibition of factor Xa generation after intrinsic activation by various agents. The inhibition produced by these thrombin inhibitors was concentration-dependent and all agents were capable of completely inhibiting factor Xa generation at the higher concentrations. The order of potency of these antithrombin

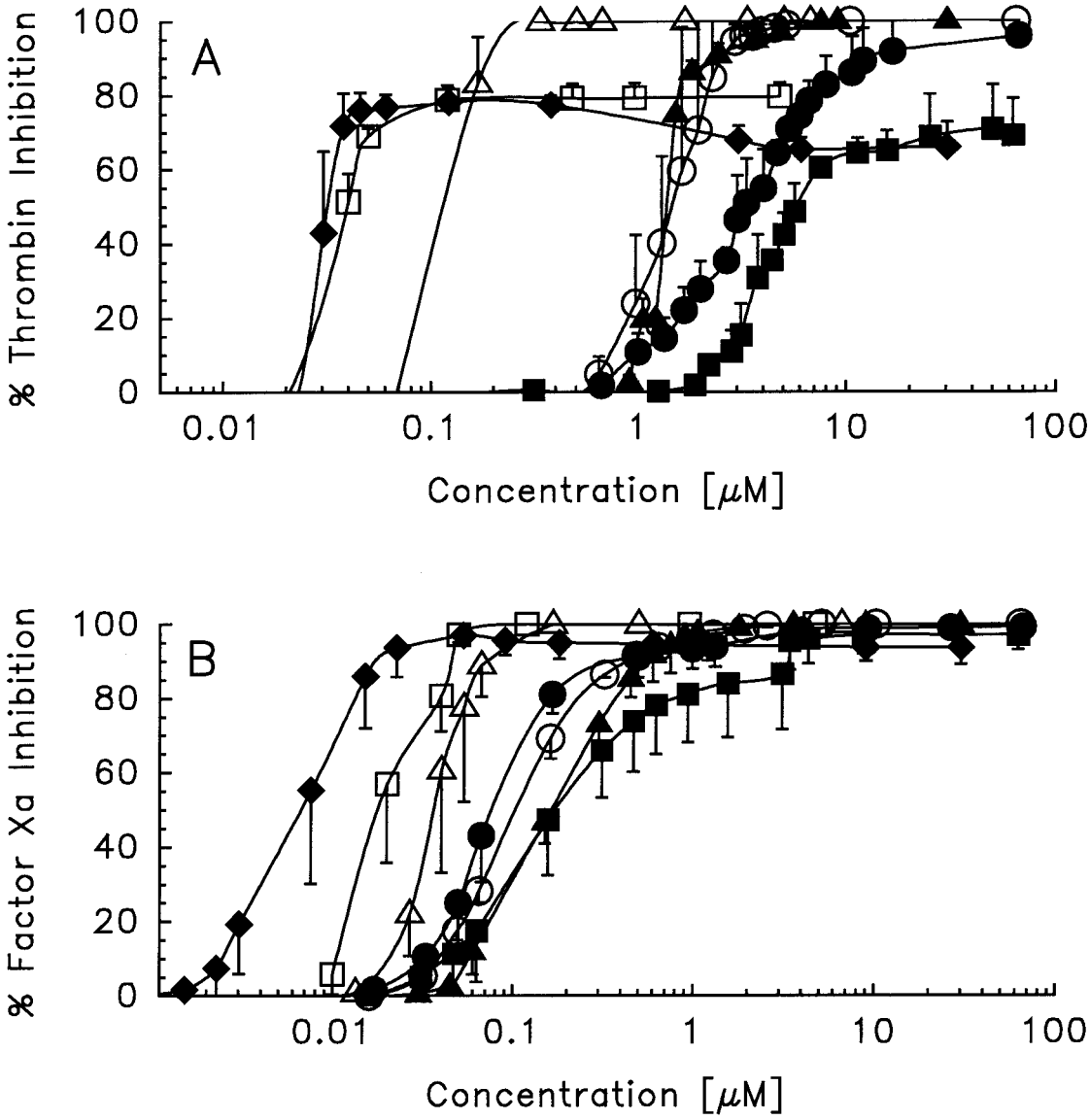


Fig. 22. Concentration dependent inhibition of the intrinsic activation of coagulation, by antithrombin agents, in the fibrinogen-deficient system. Panel A depicts the effects mediated by various agents in the intrinsic thrombin generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 14k-14o. Panel B depicts the effects mediated by agents in the intrinsic factor Xa generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 14p-14t. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

agents was heparin (IC_{50} 0.006 μM) >hirudin (IC_{50} 0.018 μM) >Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.17 μM) >D-Phe-Pro-Arg-H (IC_{50} 0.11 μM) >D-MePhe-Pro-Arg-H (IC_{50} 0.07 μM) >Boc-D-Phe-Pro-Arg-H (IC_{50} 0.16 μM) >argatroban (IC_{50} 0.17 μM), as indicated by their IC_{50} values, reported in Table 14. The individual % factor Xa generation inhibitory values for each inhibitor concentration point are found in Tables 14p - 14t.

9. Inhibition of KONYNE® Based Systems by Thrombin Inhibitors

In the extrinsic thrombin and factor Xa generation experiments described above, while the initial activation was provided extrinsically, the intrinsic pathway was also eventually activated due to the feedback loops mediated by thrombin. To devise a test where only extrinsic activation was present without interference from the intrinsic factors, a mixture of factors II, VII, IX and X purified from human plasma (KONYNE®) was utilized instead of fibrinogen deficient human plasma. Thus, with extrinsic activation only the extrinsic and common pathway were amplified. The amount of thrombin and factor Xa generated were detected based on their amidolytic activity on chromogenic substrates, specific for thrombin and factor Xa respectively.

a. Inhibition of Thrombin Generation from KONYNE®

Inhibition of thrombin generated from KONYNE® after extrinsic activation, by thrombin inhibitors was concentration-dependent, as illustrated in Fig. 23, panel A. Hirudin was the most potent inhibitor in this assay but the maximal inhibitory activity plateaued around 40%. On the other hand, the rest of the thrombin inhibitors were

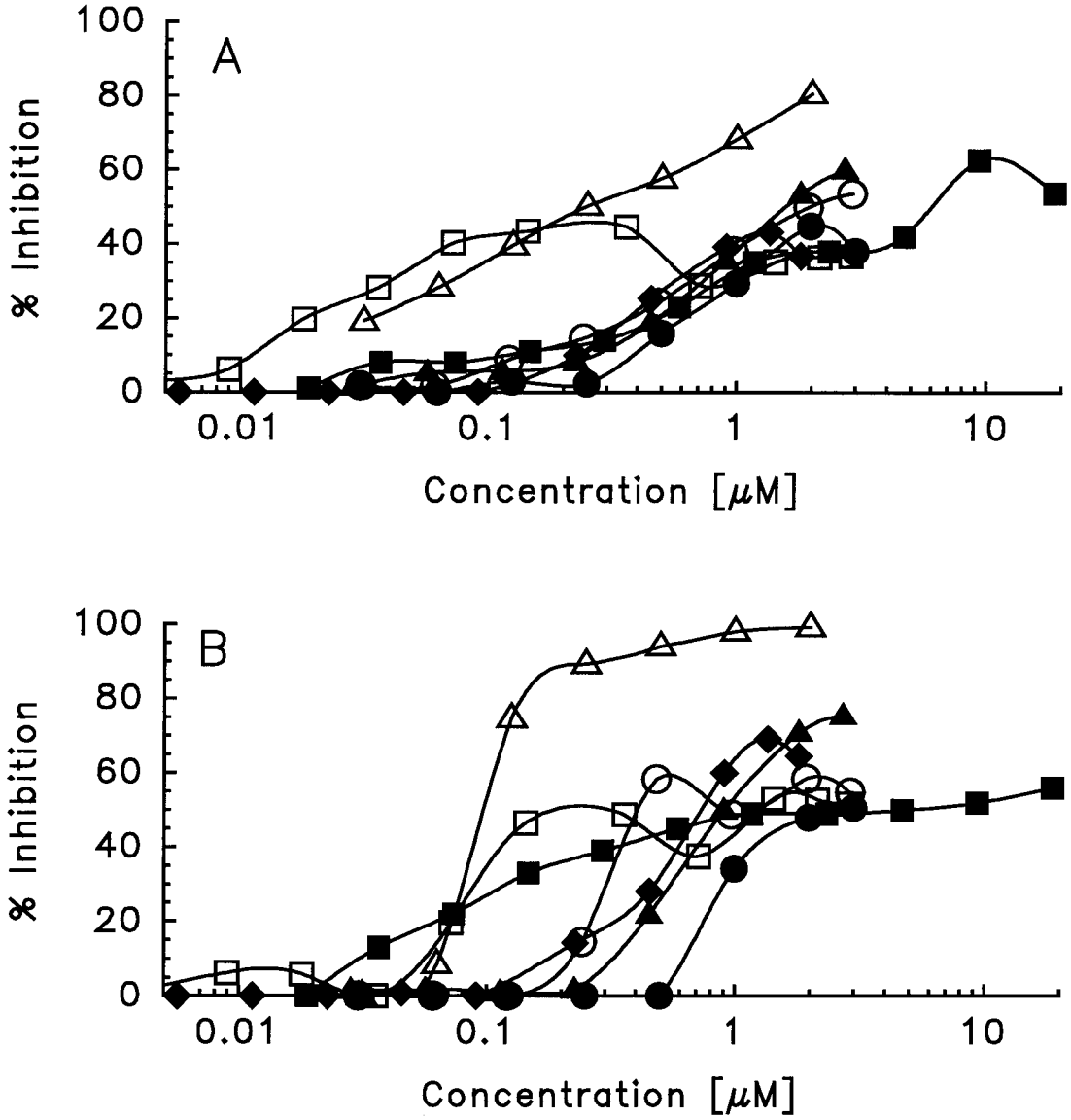


Fig. 23. Concentration-dependent inhibition of the extrinsic activation of coagulation in the KONYNE® system, by antithrombin agents. Panel A depicts the effects mediated by various agents in the extrinsic thrombin generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 15a-15c. Panel B depicts the effects mediated by agents in the extrinsic factor Xa generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 15d-15f. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

relatively weaker but were able to inhibit the generation of thrombin to a greater extent at higher concentrations. According to the IC_{50} values for each thrombin inhibitor in this assay system, reported in Table 15, the most potent inhibitor Ac-(D)Phe-Pro-boroArg-OH with an IC_{50} of 0.25 μM. The effects of D-MePhe-Pro-Arg-H (IC_{50} 1.94 μM) and Boc-D-Phe-Pro-Arg-H (IC_{50} 1.59 μM) were superimposable. As with hirudin, D-Phe-Pro-Arg-H and heparin also appeared to plateau in their inhibitory activities around 40%. Argatroban was the weakest inhibitor in this test with an IC_{50} of 10.0 μM. The individual % thrombin generation inhibitory values for each inhibitor concentration point are found in Tables 15a - 15c.

Table 15 -- Inhibition of generation of thrombin and factor Xa by thrombin inhibitors as determined in FEIBA® and KONYNE amidolytic systems.

	KONYNE® System IC_{50} [μM]		FEIBA® System IC_{50} [μM]	
	Thrombin	Factor Xa	Thrombin	Factor Xa
D-Phe-Pro-Arg-H	>3.0	2.58	2.73	2.8
D-MePhe-Pro-Arg-H	1.94	0.41	>2.9	1.68
Boc-D-Phe-Pro-Arg-H	1.59	0.91	>2.7	2.21
Ac-(D)Phe-Pro-boroArg-OH	0.25	0.10	0.66	0.36
Argatroban	10.0	10.0	>18.8	0.82
Hirudin	>2.15	1.28	0.54	0.54
Heparin	>1.8	0.75	1.66	>1.8

Each value was derived from a single determination.

b. Inhibition of Factor Xa Generation from KONYNE®

Inhibition of factor Xa generated from KONYNE®, after extrinsic activation, by thrombin inhibitors was concentration-dependent, as illustrated in Fig. 23, panel B. Hirudin appeared to be a weak inhibitor of factor Xa generation and only about 50% of factor Xa generation was maximally inhibited at concentrations around 2.9 μM. The antithrombin agents were able to inhibit the generation of factor Xa to a greater extent. According to the IC_{50} values for each thrombin inhibitor in this assay reported in Table 15, the most potent inhibitor was Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.1 μM), followed by D-MePhe-Pro-Arg-H (IC_{50} 0.9 μM), heparin (IC_{50} 0.8 μM), Boc-D-Phe-Pro-Arg-H (IC_{50} 0.9 μM), hirudin (IC_{50} 1.3 μM), D-Phe-Pro-Arg-H (IC_{50} 2.6 μM) and lastly by argatroban (IC_{50} 10 μM). The individual % factor Xa generation from KONYNE® inhibitory values for each inhibitor concentration point are found in Tables 15d - 15f.

10. Inhibition of FEIBA® Based Systems by Thrombin Inhibitors

These assays are similar to the KONYNE® based reaction mixtures as described earlier. However, FEIBA® mainly contains factor VIIa and factors II, VII, IX and X, in both the native and active forms. This product is purified from human plasma. In these studies thromboplastin C was used an activator. Thus, with this activation only the extrinsic and common pathway were activated. The amount of thrombin and factor Xa generated were detected based on their amidolytic activity on chromogenic substrates, specific for thrombin and factor Xa respectively.

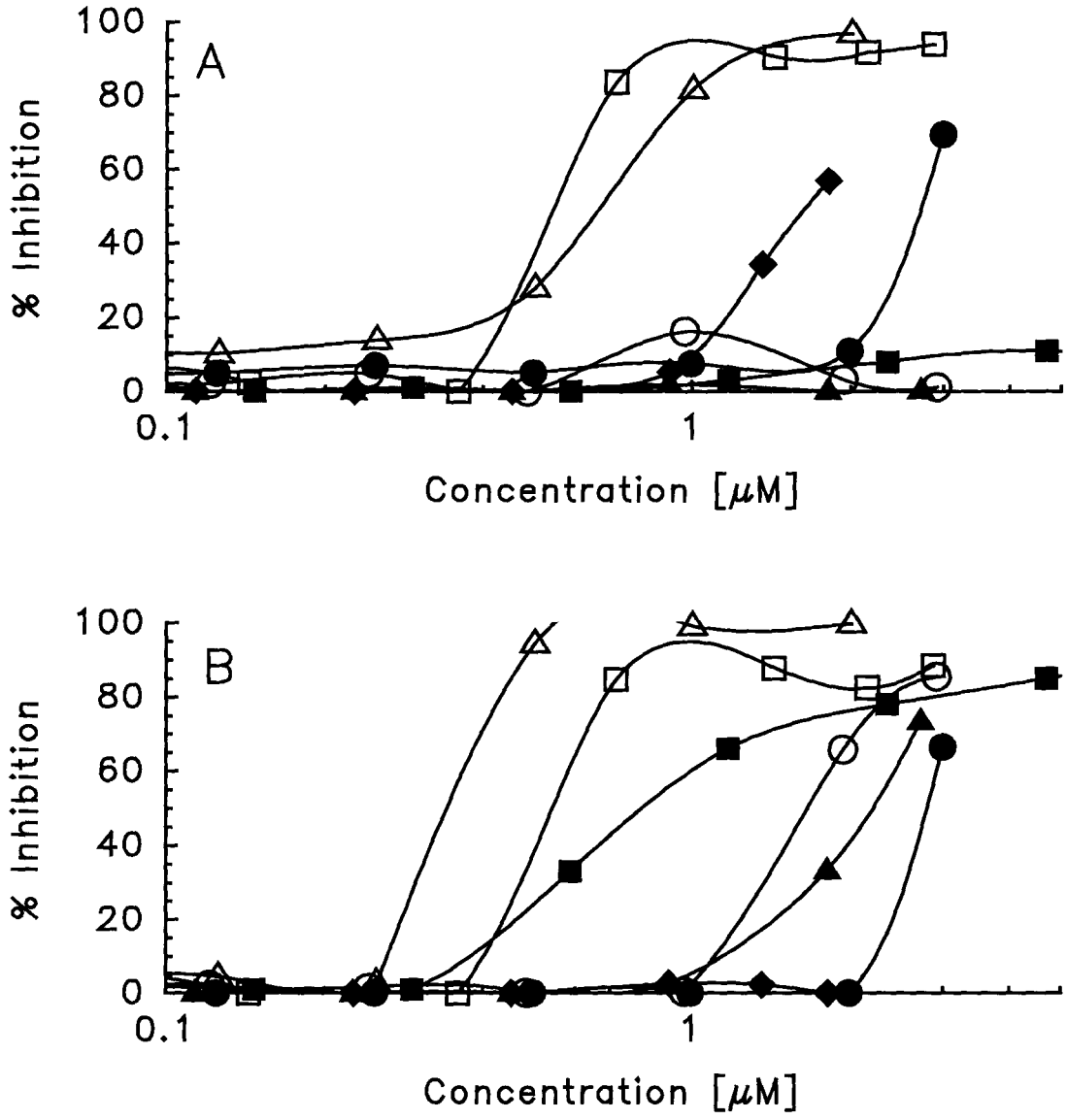


Fig. 24. Concentration-dependent inhibition of the extrinsic activation of coagulation in the FEIBA® system, by antithrombin agents. Panel A depicts the effects mediated by various agents in the extrinsic thrombin generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 15g-15i. Panel B depicts the effects mediated by agents in the extrinsic factor Xa generation system. The individual values at each concentration point and their gravimetric equivalents are given in Tables 15j-15l. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

a. Inhibition of Thrombin Generation from FEIBA®

Fig. 24, panel A depicts the inhibition of thrombin generation from FEIBA® after extrinsic activation by various thrombin inhibitors. All agents exhibited concentration-dependent inhibition of thrombin generation in this assay. Hirudin was the most potent inhibitor in this assay (IC_{50} 0.54 μM) but the maximal inhibitory activity plateaued around 90%. The effects of hirudin were closely followed by those of Ac-(D)Phe-Pro-boroArg-OH (IC_{50} 0.66 μM). While heparin (IC_{50} 1.66 μM) and D-Phe-Pro-Arg-H (2.73 μM) had weaker inhibitory activities in this test than Ac-(D)Phe-Pro-boroArg-OH, D-MePhe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H and argatroban where devoid of any effects at high concentrations (IC_{50} >2.9, 2.7 and 18.8 μM respectively). The IC_{50} values for each thrombin inhibitor in this assay system are reported in Table 15 and the individual % thrombin generation inhibitory values for each inhibitor concentration point are found in Tables 15g - 15i.

b. Inhibition of Factor Xa Generation from FEIBA®

Inhibition of factor Xa generated from FEIBA® after extrinsic activation, by thrombin inhibitors was concentration-dependent, as illustrated in Fig. 24, panel B. Ac-(D)Phe-Pro-boroArg-OH was the most potent inhibitor in this assay (IC_{50} 0.36 μM) followed by hirudin (IC_{50} 0.54 μM), argatroban (IC_{50} 0.82 μM), D-MePhe-Pro-Arg-H (IC_{50} 1.68 μM), Boc-D-Phe-Pro-Arg-H (IC_{50} 2.21 μM) and D-Phe-Pro-Arg-H (IC_{50} 2.8 μM). Heparin was devoid of any effects at high concentrations (>1.9 μM). The IC_{50} values for all agents are reported in Table 15. The individual % factor Xa generation from KONYNE® inhibitory values for each inhibitor concentration point are given in

Tables 15j - 15l.

D. Anticoagulant Effects of Thrombin Inhibitors in NHP

All of the results described in section C. Biochemical Assays, with the exception of the TT in the human fibrinogen based test, were based on the inhibition of the amidolytic activity of thrombin, factor Xa and other serine proteases. Furthermore, the TT in the fibrinogen based test was a defined assay in which no other clotting factors except for thrombin and fibrinogen were present. Therefore, the *in vitro* anticoagulant effects of thrombin inhibitors, based on the inhibition of thrombin clotting activity, were studied in the following global clotting tests, where all clotting factors were present in normal human plasma. Each assay was based on the activation of the coagulation in a distinct manner, thus providing specific data on the inhibitory actions of these antithrombin agents at various sites. To compare the relative anticoagulant effects of thrombin inhibitors in these assays, the concentrations of each inhibitor that resulted in prolongation of the clotting time to 100 sec (CT_{100}) was calculated and is given in Table 16.

1. Prothrombin Time (PT)

In the PT assay, thromboplastin was added to NHP to activate the coagulation cascade extrinsically (starting with factor VII), leading to thrombin formation which resulted in clot formation. The inhibitory activities of thrombin inhibitors in this assay system were concentration-dependent, as seen in Fig. 25. Heparin had no effects in this test at high concentrations ($>0.9\ \mu M$). When comparing the concentrations of each

Table 16 -- Comparative anticoagulant effects of thrombin inhibitors in NHP.

	Concentrations that prolong clotting time to 100 sec in [μM]				
	PT	APTT	TT	ECT	Heptest
D-Phe-Pro-Arg-H	61 ± 2	11.9 ± 0.4	5.1 ± 0.2	0.16 ± 0.04	21.4 ± 0.8
D-MePhe-Pro-Arg-H	33 ± 1	8.2 ± 0.2	2.7 ± 0.3	0.22 ± 0.05	11.2 ± 0.4
Boc-D-Phe-Pro-Arg-H	101 ± 4	7.7 ± 0.5	5.5 ± 0.2	0.89 ± 0.03	23.8 ± 0.6
Ac-D-Phe-Pro-boroArg-OH	6.2 ± 1	0.68 ± 0.05	0.60 ± 0.02	0.095 ± 0.010	2.05 ± 0.05
Argatroban	28 ± 1	6.90 ± 0.20	8.9 ± 0.2	0.21 ± 0.01	3.65 ± 0.10
Hirudin	2.3 ± 0.5	1.44 ± 0.05	0.009 ± 0.006	0.065 ± 0.015	1.3 ± 0.05
Heparin	>0.9	0.22 ± 0.03	0.155 ± 0.005	>10.0	0.30 ± 0.05

Each value represents the mean ± 1 SD of three separate determinations. In the PT assay, values produced by all thrombin inhibitors (except for heparin) were significantly different from each other. In the APTT assay, values produced by all thrombin inhibitors were significantly different from each other, with the exception of the Ac-(D)Phe-Pro-boroArg-OH vs heparin comparison. In the 10 U Ca^{++}TT assay, values produced by all thrombin inhibitors were significantly different from each other, with the exception of the hirudin vs heparin comparison. In the ECT assay, values produced by all thrombin inhibitor (except for heparin) were significantly different from each other with the exception of the following pairs: D-Phe-Pro-Arg-H vs D-MePhe-Pro-Arg-H, D-Phe-Pro-Arg-H vs argatroban, D-MePhe-Pro-Arg-H vs argatroban and Ac-(D)Phe-Pro-boroArg-OH vs hirudin. In the Heptest assay, values produced by all thrombin inhibitors were significantly different from each other. ($p<0.05$, ANOVA followed by Student-Newman-Keuls test).

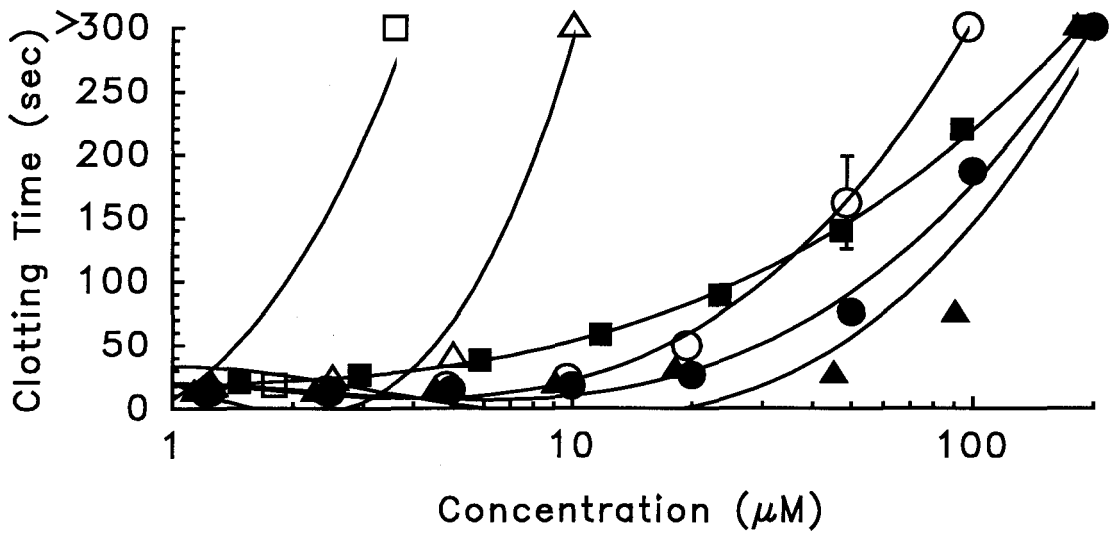


Fig. 25. Comparative anticoagulant effects of antithrombin agents, after supplementation to NHP, as studied in the PT. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 16a-16c.

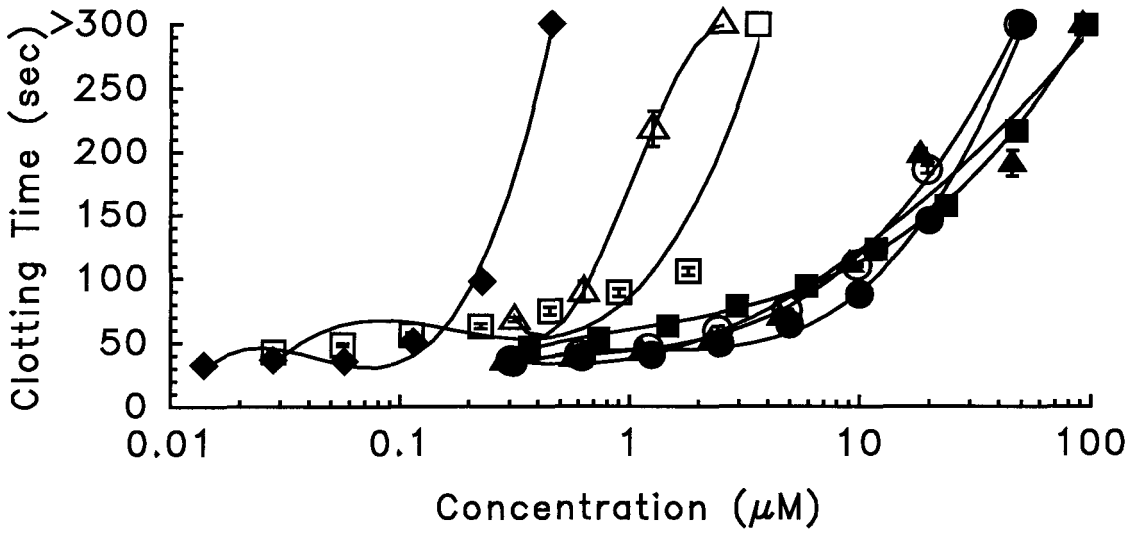


Fig. 26. Comparative anticoagulant effects of various agents, after supplementation to NHP, as studied in the APTT. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 16d-16f.

thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec, reported in Table 16, it was found that the most potent inhibitor of the PT was hirudin (CT_{100}: 2.3 μM) followed by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 6.2 μM). Both hirudin and Ac-(D)Phe-Pro-boroArg-OH appeared to have an all-or-none effects on the PT. The effects of Ac-(D)Phe-Pro-boroArg-OH were followed by argatroban (CT_{100}: 28 μM), D-MePhe-Pro-Arg-H (CT_{100}: 33 μM), D-Phe-Pro-Arg-H (CT_{100}: 61 μM) and lastly by Boc-D-Phe-Pro-Arg-H (CT_{100}: 101 μM). Even though argatroban produced a clotting time prolongation to 100 sec at a lower concentration than D-MePhe-Pro-Arg-H, the latter inhibitor prolonged the PT to over 300 sec at lower concentrations than argatroban (97 μM versus 188 μM respectively). The individual clotting times for each thrombin inhibitor concentration point are reported in Tables 16a - 16c.

2. Activated Partial Thromboplastin Time (APTT)

In the APTT assay, a contact activator was added to NHP to activate the coagulation cascade intrinsically (starting with factor XII), leading to thrombin formation which resulted in clot formation. As seen in Fig. 26, the inhibitory activities of various thrombin inhibitors in this assay were concentration-dependent. When comparing the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec as given in Table 16, it was found that the most potent inhibitor of the APTT was heparin (CT_{100}: 0.22 μM) followed by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 0.68 μM) and then closely by hirudin (CT_{100}: 1.44 μM). The effects of hirudin were followed by argatroban (CT_{100}: 6.9 μM), Boc-D-Phe-Pro-Arg-H (CT_{100}: 7.7 μM), D-MePhe-Pro-Arg-H (CT_{100}: 8.2 μM) and D-Phe-Pro-Arg-H (CT_{100}: 11.9 μM). The inhibitory profiles of

the last four thrombin inhibitors were superimposable. The individual clotting times for each thrombin inhibitor concentration point are reported in Tables 16d - 16f.

3. Thrombin Time (TT)

In the TT assay, thrombin was added to NHP to convert the existing fibrinogen to fibrin clots. Furthermore, the added thrombin activated the coagulation cascade at multiple points (extrinsically via factors VII and V, intrinsically via factor XI and common pathway via factor VIII), leading to additional thrombin formation which resulted in clot formation. The inhibitory activities of thrombin inhibitors in this assay system were concentration-dependent, as seen in Fig. 27. The concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec are given in Table 16. The most potent inhibitor of the TT was hirudin (CT_{100}: 0.009 μM), followed by heparin (CT_{100}: 0.155 μM). The effects of heparin were followed by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 0.6 μM) and then by D-MePhe-Pro-Arg-H (CT_{100}: 1.7 μM), D-Phe-Pro-Arg-H (CT_{100}: 5.1 μM), Boc-D-Phe-Pro-Arg-H (CT_{100}: 5.5 μM) and lastly by argatroban (CT_{100}: 8.9 μM). The inhibitory profiles of D-Phe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H were superimposable. The individual clotting times for each thrombin inhibitor concentration point are given in Tables 16g - 16i.

4. Ecarin Clotting Time (ECT)

In the ECT assay, ecarin was added to NHP to activate the existing prothrombin to meizothrombin and other intermediates with clotting activity, which led to fibrinogen conversion to fibrin clots. This was different from the TT, where exogenous thrombin

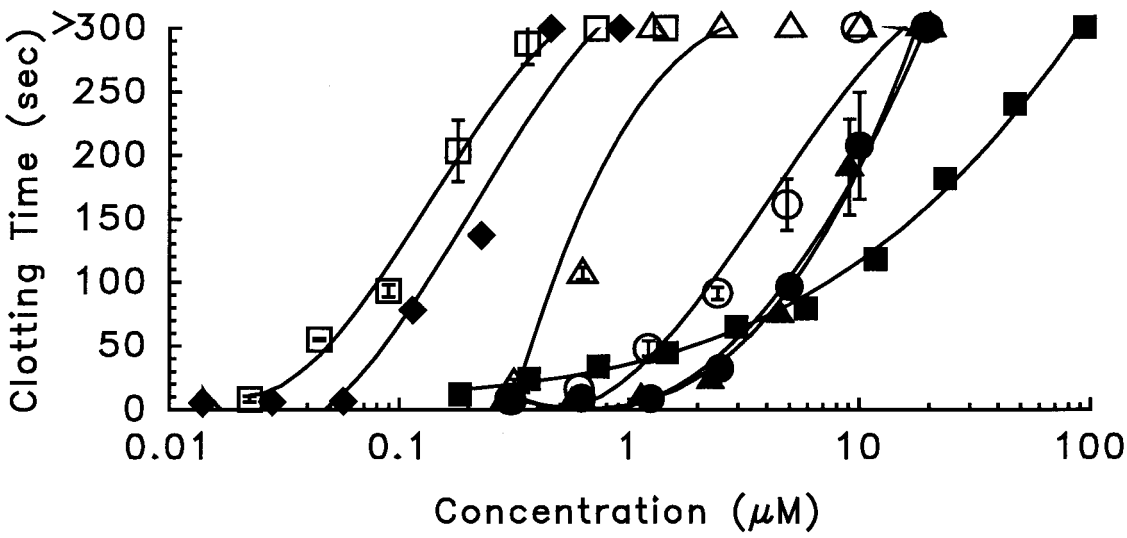


Fig. 27. Comparative anticoagulant effects of thrombin inhibitors, after supplementation to NHP, as studied in the TT. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 16g-16i.

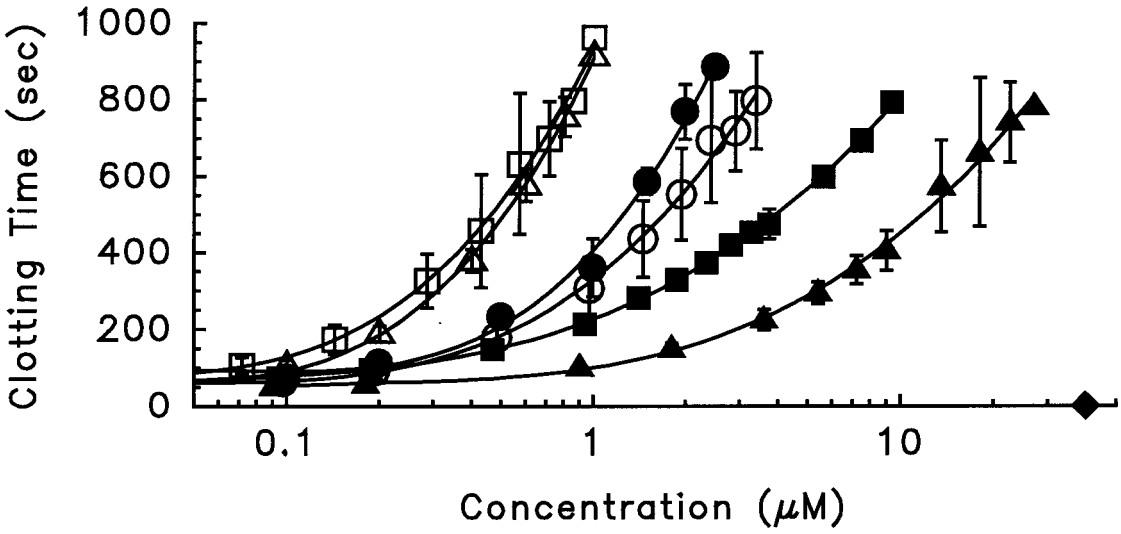


Fig. 28. Comparative anticoagulant effects of antithrombin agents, after supplementation to NHP, as studied in the ECT. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 16j-16k.

was added to the NHP. Fig. 28 depicts the inhibitory activities of thrombin inhibitors in this assay system. With the exception of heparin, the inhibitory activities of the other agents were concentration-dependent. Table 16 shows the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec. When comparing these CT_{100}s, it was found that the most potent inhibitor of the ECT was hirudin (CT_{100}: 0.065 μM), followed closely by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 0.095 μM). The effects of Ac-(D)Phe-Pro-boroArg-OH were followed by D-Phe-Pro-Arg-H (CT_{100}: 0.16 μM), Boc-D-Phe-Pro-Arg-H (CT_{100}: 0.89 μM) and lastly by argatroban (CT_{100}: 3.65 μM). In this assay, heparin was devoid of any effects at high concentrations (>0.91 μM). The individual clotting times for each thrombin inhibitor concentration point are given in Tables 16j and 16k.

5. Heptest

In the Heptest assay, factor Xa added to NHP activated the common pathway which lead to thrombin formation with subsequent fibrinogen conversion to fibrin clots. The inhibitory activities of antithrombin agents in this test were concentration-dependent, as seen in Fig. 29. When comparing the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec, as reported in Table 16, heparin was found to be the most potent inhibitor of the Heptest with a CT_{100} of 0.3 μM. The effects of heparin were followed closely by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 2.1 μM) and then by argatroban (CT_{100}: 3.7 μM), D-MePhe-Pro-Arg-H (CT_{100}: 11.2 μM), Boc-D-Phe-Pro-Arg-H (CT_{100}: 23.8 μM) and lastly by D-Phe-Pro-Arg-H (CT_{100}: 21.4 μM). The individual clotting times for each thrombin inhibitor concentration point are reported in Tables 16l - 16n.

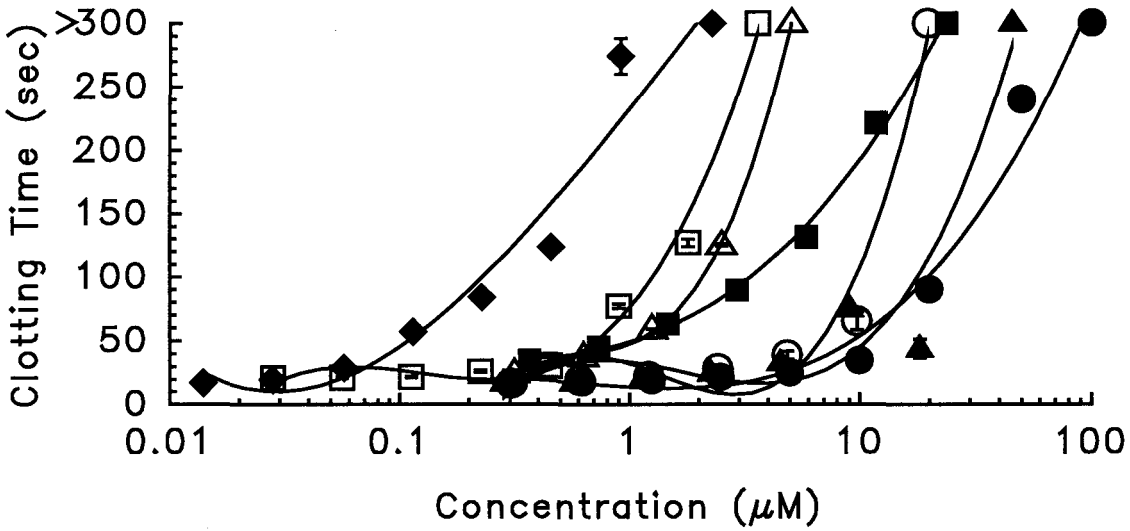


Fig. 29. Comparative anticoagulant effects of various agents, after supplementation to NHP, as studied in the Heptest. (●) D-Phe-Pro-Arg-H, (○) D-MePhe-Pro-Arg-H, (▲) Boc-D-Phe-Pro-Arg-H, (△) Ac-(D)Phe-Pro-boroArg-OH, (■) argatroban, (□) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Tables 16l-16n.

E. Anticoagulant Effects of Thrombin Inhibitors in Normal Human Whole Blood

The coagulation process *in vivo* takes place in whole blood, so that in addition to the coagulation factors present in plasma, the rest of the blood components, such as platelets and white cells may also play a role in the clotting process. To investigate the anticoagulant activities of thrombin inhibitors in normal human whole blood, the ACT and TEG were utilized to compare D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, argatroban, hirudin and heparin. Freshly drawn blood samples were supplemented *in vitro* with various concentrations of thrombin inhibitors prior to studying their effects in these assay systems.

1. Activated Clotting Time (ACT)

The results of the concentration-dependent anticoagulant activities of thrombin inhibitors in whole blood, as studied in the ACT, are depicted in Fig. 30. Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin produced similar concentration-dependent pattern of prolongation of the ACT with a prolongation of this assay to 300-370 sec at concentrations of 1 μM. D-MePhe-Pro-Arg-H and argatroban had weak anticoagulant activities even at the highest concentration, producing a prolongation of the ACT to 150-180 sec at 1 μM concentrations. The individual clotting times for each inhibitor concentration are reported in Table 16o.

2. Thrombelastographic Analysis (TEG)

The results of the concentration-dependent anticoagulant activities of thrombin inhibitors in whole blood, as studied in the TEG, are depicted in Fig. 31. As seen in this

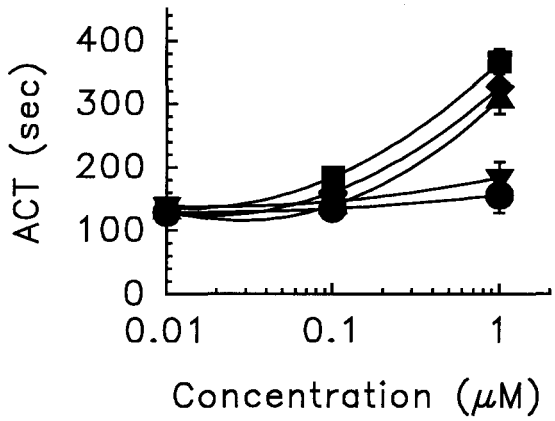


Fig. 30. Comparative anticoagulant effects of thrombin inhibitors, after supplementation to normal human whole blood, as detected in the ACT assay. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (▼) argatroban, (■) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 16o.

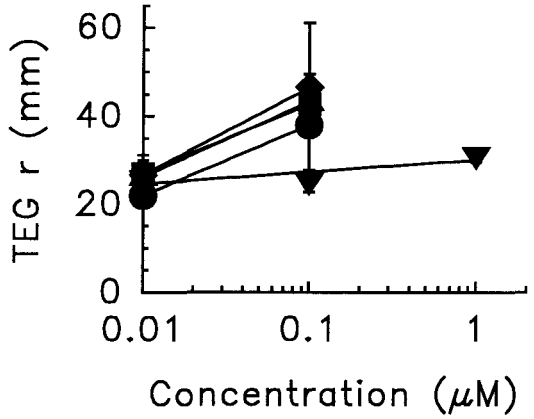


Fig. 31. Comparative anticoagulant effects of thrombin inhibitors, after supplementation to normal human whole blood, as studied in the TEG assay. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (▼) argatroban, (■) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 16p.

profile, D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin produced similar concentration-dependent pattern of prolongation of the r value of the TEG (38-47 mm) at equimolar concentrations (0.1 μM). Argatroban had weak anticoagulant activities even at the highest concentration, producing a prolongation of the TEG r value to 31 mm at 1 μM. The individual TEG r values for each inhibitor concentration are reported in Table 16p.

F. Anticoagulant Effects of Thrombin Inhibitors in NRP

Human plasma and rabbit plasma differ in their composition and the relative levels of clotting factors. Since it was shown in the biochemical studies that each thrombin inhibitor had a distinct pattern of inhibition of serine proteases and generation assays, it was reasonable to assume that the anticoagulant effects of these inhibitors would differ depending on the plasma origin. Therefore, to compare the *in vitro* anticoagulant effects of thrombin inhibitors, based on the inhibition of thrombin clotting activity in the following global clotting tests, D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin were supplemented to normal rabbit plasma. As with the NHP tests, each assay was based on the specific activation of the coagulation system, thus providing different insights into the inhibitory actions of these thrombin inhibitors. To compare the anticoagulant effects of thrombin inhibitors in these assay systems, the concentrations of each inhibitor that resulted in prolongation of the clotting time to 100 sec (CT_{100}) was calculated and compiled in Table 17.

Table 17 -- Comparative anticoagulant effects of thrombin inhibitors in NRP.

	Concentrations that prolong clotting time to 100 sec in [μM]			
	PT	APTT	TT	Heptest
D-MePhe-Pro-Arg-H	107 $\pm$ 4	4.8 $\pm$ 0.4	4.5 $\pm$ 0.4	3.3 $\pm$ 0.2
Ac-(D)Phe-Pro-boroArg-OH	>20.1	0.52 $\pm$ 0.08	1.0 $\pm$ 0.2	1.1 $\pm$ 0.1
Hirudin	3.6 $\pm$ 0.4	0.7 $\pm$ 0.1	0.18 $\pm$ 0.02	0.24 $\pm$ 0.03
Heparin	>9.3	0.38 $\pm$ 0.10	0.18 $\pm$ 0.02	0.27 $\pm$ 0.06

Each value represents the mean $\pm$ 1 SD of three separate determinations. In the PT assay, values produced by D-MePhe-Pro-Arg-H and hirudin were significantly different from each other. In the APTT assay, the value produced by D-MePhe-Pro-Arg-H was significantly different from those produced by the other thrombin inhibitors. In the 10 U Ca^{++}TT assay, values produced by all thrombin inhibitors were significantly different from each other, with the exception of the hirudin vs heparin comparison. In the Heptest assay, as in the TT assay, values produced by all thrombin inhibitors were significantly different from each other, with the exception of the hirudin vs heparin comparison. ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test).

1. Prothrombin Time (PT)

In the PT assay, thromboplastin added to NRP activated the coagulation cascade extrinsically (starting with factor VII), leading to thrombin formation which resulted in clot formation. The inhibitory activities of antithrombin agents in this assay are depicted in Fig. 32, panel A. Heparin and Ac-(D)Phe-Pro-boroArg-OH had minimal effects in this assay at high concentrations: heparin produced a pt of 17 sec at 9.4 μM, while Ac-(D)Phe-Pro-boroArg-OH produced a PT of 24 sec at 20 μM. When comparing the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec, given in Table 17, it was found that the most potent inhibitor of the PT was hirudin (CT_{100}: 3.6 μM) followed by D-MePhe-Pro-Arg-H (CT_{100}: 107 μM). Both hirudin and D-MePhe-Pro-Arg-H appeared to have an all-or-none effects on the PT. The individual clotting times for each thrombin inhibitor concentration point are reported in Table 17a.

2. Activated Partial Thromboplastin Time (APTT)

In the APTT, a contact activator mixture added to NRP activated the coagulation cascade intrinsically (starting with factor XII), leading to thrombin formation which resulted in clot formation. The inhibitory activities of thrombin inhibitors in this test were concentration-dependent, as seen in Fig. 32, panel B. Table 17 depicts the concentrations of each agent that resulted in prolongation of the clotting time to 100 sec. In comparing the CT_{100} value for each antithrombin agent, it was found that the most potent inhibitor of the APTT was heparin (CT_{100}: 0.4 μM) followed by closely by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 0.5 μM), hirudin (CT_{100}: 0.7 μM) and lastly by D-

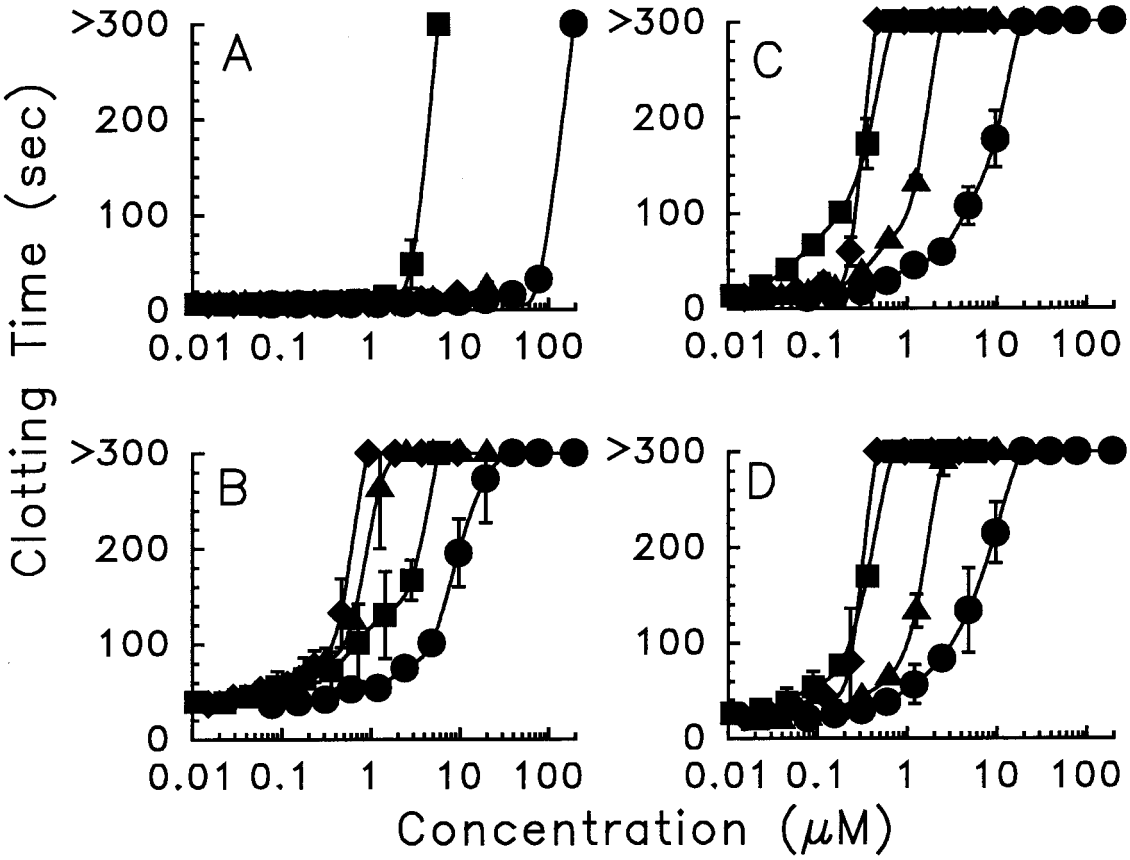


Fig. 32. Comparative anticoagulant effects of thrombin inhibitors, after supplementation to normal rabbit plasma, as studied in the global clotting tests. Panel A represents the effects of antithrombin agents in the PT assay. The individual values at each concentration point and their gravimetric equivalents are given in Table 17a. Panel B represents the effects of various agents in the APTT assay. The individual values at each concentration point and their gravimetric equivalents are given in Table 17b. Panel C represents the effects of agents in the TT assay. The individual values at each concentration point and their gravimetric equivalents are given in Table 17c. Panel D represents the effects of antithrombin agents in the Heptest. The individual values at each concentration point and their gravimetric equivalents are given in Table 17d. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean ± 1 SD of three individual determinations.

MePhe-Pro-Arg-H (CT_{100}: 4.8 μM). The individual clotting times for each thrombin inhibitor concentration point are reported in Table 17b.

3. Thrombin Time (TT)

In the TT, thrombin added to NRP directly converted the existing fibrinogen to fibrin clots. Furthermore, the added thrombin activated the coagulation cascade at multiple points (extrinsically via factors VII and V, intrinsically via factor XI and common pathway via factor VIII), leading to additional thrombin formation which resulted in clot formation. Fig. 32, panel C, represents the inhibitory activities of antithrombin agents. In this assay, all agents produced a concentration-dependent prolongation of the PT. When comparing the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec, reported in Table 17, it was found that the most potent inhibitor of the TT was hirudin (CT_{100}: 0.18 μM) followed closely by heparin (CT_{100}: 0.18 μM). However, prolongation of the TT to over 300 sec was achieved with a lower concentration of heparin (0.47 μM) than of hirudin (0.72 μM). The effects of heparin were followed by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 1 μM) and then by D-MePhe-Pro-Arg-H (CT_{100}: 4.5 μM). The individual clotting times for each thrombin inhibitor concentration point are reported in Table 17c.

4. Heptest

In the Heptest, factor Xa added to NRP activated the common pathway which lead to thrombin formation with subsequent fibrinogen conversion to fibrin clots. The inhibitory activities of antithrombin agents in this assay system were concentration-

dependent, as seen in Fig. 32, panel D. Table 17 reports the concentrations of each thrombin inhibitor that resulted in prolongation of the clotting time to 100 sec. When comparing the CT_{100} in this assay, it was found that the most potent inhibitors of the Heptest were heparin (CT_{100}: 0.27 μM) and hirudin (CT_{100}: 0.24 μM), which exhibited similar effects at the same concentrations. The effects of heparin and hirudin were followed by Ac-(D)Phe-Pro-boroArg-OH (CT_{100}: 1.1 μM) and then by D-MePhe-Pro-Arg-H (CT_{100}: 3.3 μM). The individual clotting times for each thrombin inhibitor concentration point are given in Table 17d.

G. Anticoagulant Effects of Thrombin Inhibitors in Normal Rabbit Whole Blood

Since the concentration of plasma factors differ depending on the species utilized, the thrombin inhibitors D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin were also supplemented to freshly drawn rabbit whole blood, to compare their anticoagulant effects to each other and to their respective effects in the normal human whole blood assays. The effects of these agents in the ACT and TEG assays were studied.

1. Activated Clotting Time (ACT)

The results of the anticoagulant activities of thrombin inhibitors in rabbit whole blood, as studied in the ACT, are depicted in Fig. 33. Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin produced similar prolongation of the ACT at the concentrations utilized, exhibiting a prolongation of the ACT of 180-230 sec from the baseline, at concentrations of 201, 144 and 187 nM respectively. D-MePhe-Pro-Arg-H had the

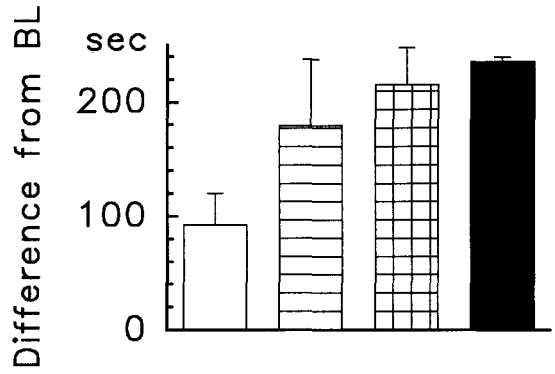


Fig. 33. Comparative anticoagulant effects of various agents, after supplementation to normal rabbit whole blood, as studied in the ACT assay. (□) D-MePhe-Pro-Arg-H at 389 nM, (≡) Ac-(D)Phe-Pro-boroArg-OH at 201 nM, (⫲) hirudin at 144 nM, (■) heparin at 235 nM. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 17e.

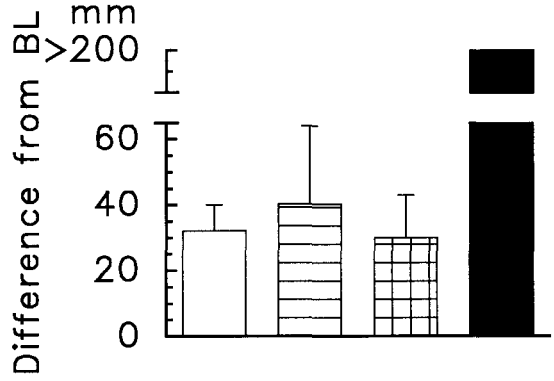


Fig. 34. Comparative anticoagulant effects of various agents, after supplementation to normal rabbit whole blood, as studied in the TEG assay. (□) D-MePhe-Pro-Arg-H at 389 nM, (≡) Ac-(D)Phe-Pro-boroArg-OH at 201 nM, (⫲) hirudin at 144 nM, (■) heparin at 235 nM. Each point represents a mean ± 1 SD of three individual determinations. The individual values at each concentration point and their gravimetric equivalents are given in Table 17f.

weakest anticoagulant activities, producing only a 92 sec prolongation of the ACT from the baseline at a concentration of 389 nM. The individual clotting times for each thrombin inhibitor are reported in Table 17e.

2. Thrombelastographic Analysis (TEG)

The results of the anticoagulant activities of thrombin inhibitors in whole rabbit blood, as studied in the TEG, are depicted in Fig. 34. D-MePhe-Pro-Arg-H produced similar prolongation of the TEG r value as Ac-(D)Phe-Pro-boroArg-OH and hirudin. This prolongation was 30-40 mm from the baseline value at 389, 201 and 144 μM respectively by the three thrombin inhibitors. Heparin produced the strongest prolongation of the r value of the TEG, to >200 mm at a concentration of 187 nM. The individual TEG r values for each thrombin inhibitor are reported in Table 17f.

H. Antithrombotic Effects of Thrombin Inhibitors as Studied in the Rabbit Stasis Thrombosis Model

The Wessler stasis thrombosis model (Wessler et al. 1959) is a rabbit model of venous thrombosis and has been widely used in the evaluation of antithrombotic activity of various substances. Although this model has provided useful information on the antithrombotic activities of various anticoagulant agents, the results obtained are relative to the type of thrombogenic challenge used to produce the thrombotic processes. Defined thrombogenic agents to activate the hemostatic system of rabbits have been previously utilized in a standardized manner where the site of activation is controlled (Fareed et al. 1985). In previous studies specific components of the hemostatic system were selectively activated with various agents such as Prothrombin Complex Concentrate (PCC) and

Russell's Viper Venom (RVV) (Fareed et al. 1985). However, additional activation of other enzymes and the presence of protein C in these activation mixtures may influence the results. The availability of TF in a recombinant form (Bach et al. 1986) allows for a specific investigation of thrombogenesis and its pathophysiological mechanisms. In this study, the objective was to characterize r-TF in terms of its thrombogenicity in a standardized model of stasis thrombosis and subsequently to compare the antithrombotic potential of thrombin inhibitors following I.V. or S.C. administration. In addition, the duration of the antithrombotic effects of thrombin inhibitors was also studied. Representative thrombin inhibitors compared in this model were D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin.

1. Standardization of the Rabbit Stasis Thrombosis Model

In these studies, r-TF was injected at various doses in the procedure described in Chapter III, Section I. 1. After I.V. injection of r-TF, at 1.351, 0.676, 0.338, 0.169 and 0.042 pmol/kg, followed by a 20 sec circulation time, the jugular vein segments of the rabbit were isolated and after 10 and 20 minutes of stasis, each of the jugular vein segments was dissected and the contained clots were graded on a previously established scale from 0 to +4 (Fareed et al. 1986). Blood samples were collected at baseline and after r-TF injection, for *ex vivo* whole blood and plasma analyses. Six rabbits were studied at each r-TF dose.

a. Clot Score Analyses

Injection of r-TF produced dose-dependent thrombotic effects, as depicted in Fig.

35. At doses of 1.35 pmol/kg the clot scores obtained were always maximal (+4) at both the 10 minute and the 20 minute endpoints. The estimated ED_{50} from this curve for r-TF was about 0.34 and 0.4 pmol/kg for the 10 and 20 min stasis respectively. At doses as low as 42.3 fmol/kg there was always a clot of at least +1 observed after a 20 minute stasis time. When saline was used in place of r-TF in this procedure, there were no clots observed (clot score=0). A dose of 0.68 pmol/kg consistently resulted in clot scores of at least +3. This was the dose selected for assessing the antithrombotic effects of thrombin inhibitors in this model in the following studies. The clot score value for each r-TF dose for both the 10 and 20 min stasis time are given in Table 18.

Table 18 -- Standardization of the rabbit model of jugular vein stasis thrombosis, with r-TF: dose-dependent thrombotic effects of r-TF after I.V. injection, 20 sec circulation, after 10 and 20 min stasis.

Dose ng/kg	Dose pmol/kg	Clot Score	
		10 min stasis	20 min stasis
50	1.351	4.0±0.0	4.0±0.0
25	0.676	3.2±0.2	3.6±0.3
12.5	0.338	1.7±0.6	2.0±0.0
6.25	0.169	1.2±0.4	1.8±0.4
1.56	0.042	1.0±0.0	1.5±0.0
0	0	0.0±0.0	0.0±0.0

Each value represents the mean ± SEM of 6 independent determinations.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

The *ex vivo* effect of r-TF on the protease inhibition profile of rabbit plasma as studied by the antithrombin and anti-Xa amidolytic methods did not reveal any detectable

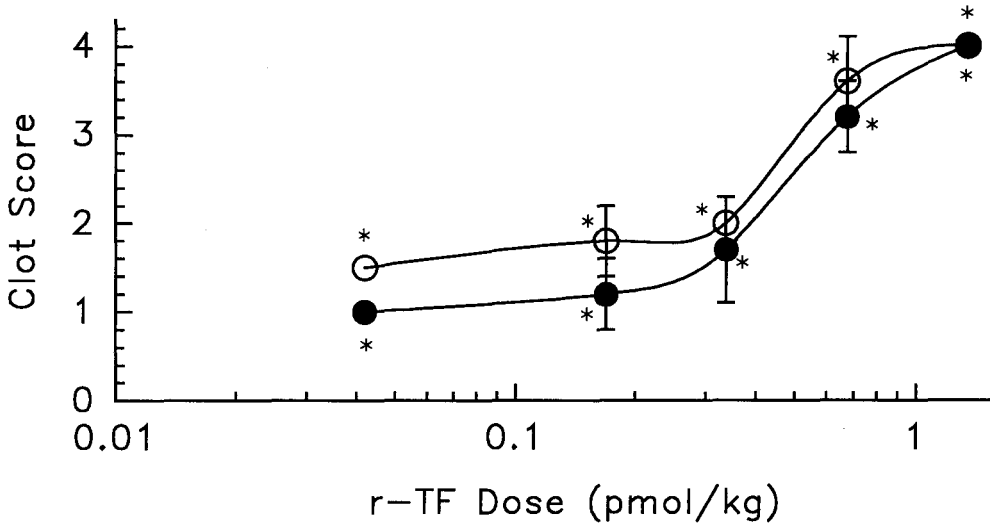


Fig. 35. Dose-dependent thrombotic effects of r-TF as studied in the rabbit model of jugular vein stasis thrombosis. r-TF was administered through I.V. injection and allowed to circulate for 20 sec prior to ligation of the jugular segments. (●) represent clot scores obtained after 10 minutes stasis, (○) represent clot scores obtained after 20 min stasis. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p<0.05$) when compared to control (saline produced 0.0 ± 0.0, both after 10 and 20 min stasis) as analyzed with the Kruskal-Wallis test. The individual clot score values at each rTF dose and their gravimetric equivalents are given in Table 18.

antithrombin or anti-Xa activity at any of the doses of r-TF administered. However, as shown in Fig. 36, comparison of the ACT values of samples obtained at baseline and 5 minutes after r-TF administration, revealed a prolongation of the ACT. This prolongation was dose-dependent, which was significant at r-TF doses of 0.68 pmol/kg or greater. However, at doses below 0.34 pmol/kg there was no prolongation in the ACT.

Fig. 37 depicts the comparison of the measured r distance on the TEG of samples obtained from the rabbits which had undergone the stasis model before and after the administration of r-TF at various doses. A clear dose-dependent prolongation of the TEG r duration occurred, which became significant when a 1.35 pmol/kg dose was administered. The other TEG parameters (k, rk and angle) also exhibited a parallel prolongation to the r value (data not shown).

When the plasma samples collected before and after r-TF injection were compared, r-TF had no effects on any of the global clotting tests, as seen in Fig. 38. r-TF did not exhibit any effect on the PT of rabbit plasma when administered *in vivo* at doses as high as 1.35 pmol/kg, as seen in Fig. 38, panel A. Panel B of Fig. 38 depicts the results from the APTT analysis of the samples obtained from the stasis thrombosis experiments. The *in vivo* administration of r-TF did not appear to have any effect on the APTT at doses up to 1.35 pmol/kg. Similar results to those from the PT and APTT (i.e. no difference between baseline and post r-TF injection) were obtained from the *ex vivo* Heptest analysis of the blood samples obtained during the stasis thrombosis experiments (Fig. 38, panel C). In addition, r-TF appeared to have no effect on the TT profile when

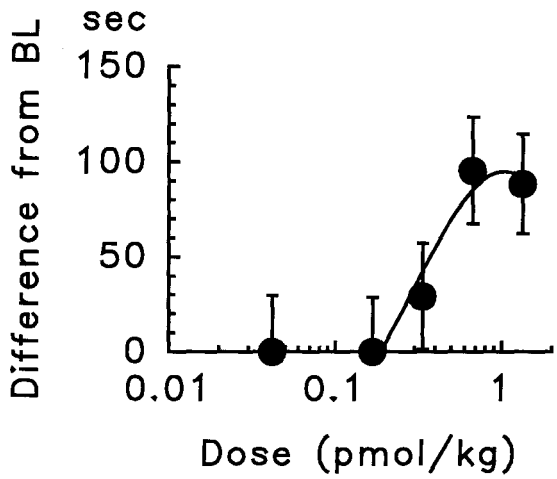


Fig. 36. Dose-dependent pro-coagulant *ex vivo* effects of r-TF as studied in the rabbit model of jugular vein stasis thrombosis, utilizing the ACT assay. Blood samples were collected prior to r-TF injection and 5 min following r-TF injection. Each point represents a mean of the difference between baseline and post-r-TF values for each rabbit ± S.E.M. (n=6 at each dose).

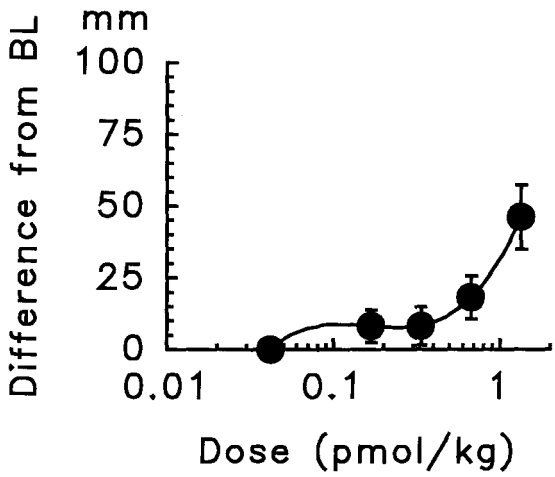


Fig. 37. Dose-dependent pro-coagulant *ex vivo* effects of r-TF as studied in the rabbit model of jugular vein stasis thrombosis, utilizing the TEG assay. Blood samples were collected prior to r-TF injection and 5 min following r-TF injection. Each point represents a mean of the difference between baseline and post-r-TF values for each rabbit ± S.E.M. (n=6 at each dose).

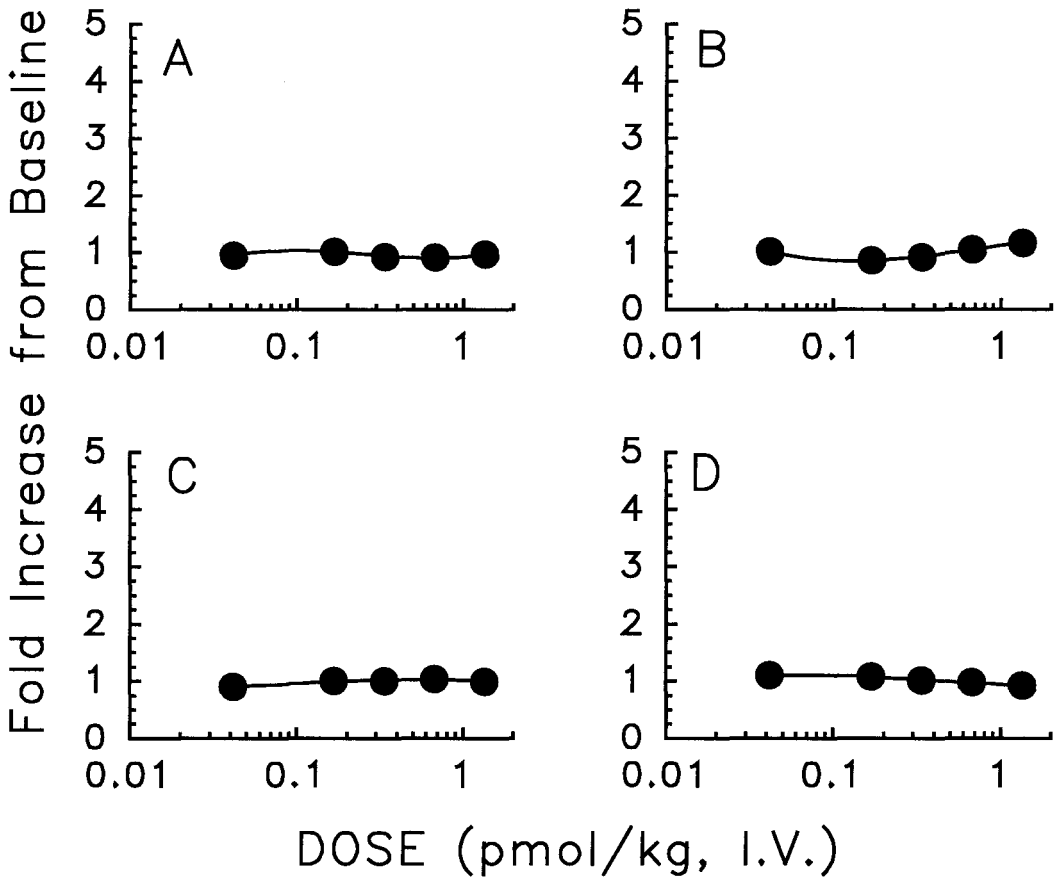


Fig. 38. Dose-dependent pro-coagulant *ex vivo* effects of r-TF as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to r-TF injection and 5 min following r-TF injection. Panel A depicts the results of the PT analysis. Panel B depicts the results from the APTT analysis. Panel C depicts the results of the Heptest analysis. Panel D depicts the results of the TT analysis. Each point represents a mean of the difference between baseline and post-r-TF values for each rabbit ± S.E.M. (n=6 at each dose).

administered at doses as high as 1.35 pmol/kg (Fig. 38, panel D).

2. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration at a Fixed Time Point

From the above standardization studies, an r-TF dose of 0.68 pmol/kg was chosen for the standard procedure as the thrombogenic source in this rabbit jugular vein stasis thrombosis model. The antithrombotic effects of D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin were compared in a dose-dependent manner, after I.V. injection and after a 5 min circulation time prior to injection of r-TF. Twenty sec after injection of r-TF, the jugular segments of the rabbit were isolated and after 10 and 20 minutes of stasis, each jugular segment was dissected and the contained clots were analyzed on a previously established scale from 0 to +4 (Fareed et al. 1986). Blood samples were collected at baseline and 5 min after thrombin inhibitor injection (prior to r-TF injection), for *ex vivo* whole blood and plasma analyses. Six rabbits were studied at each thrombin inhibitor dose.

a. Clot Score Analyses

Injection of all four thrombin inhibitors produced dose-dependent antithrombotic effects, after 10 and 20 min stasis time, in a dose range of 0.001 to 2 μmol/kg, as depicted in Fig. 39, panels A and B respectively. The strongest antithrombotic agent appeared to be heparin at doses in the nmol/kg range, both after 10 and 20 min stasis time, with ED_{50}s of 0.008 and 0.009 μmol/kg respectively. The effects of heparin were closely followed by those of hirudin, with ED_{50}s of 0.003 and 0.11 μmol/kg respectively. After 20 min stasis time, the antithrombotic effects of heparin and hirudin were

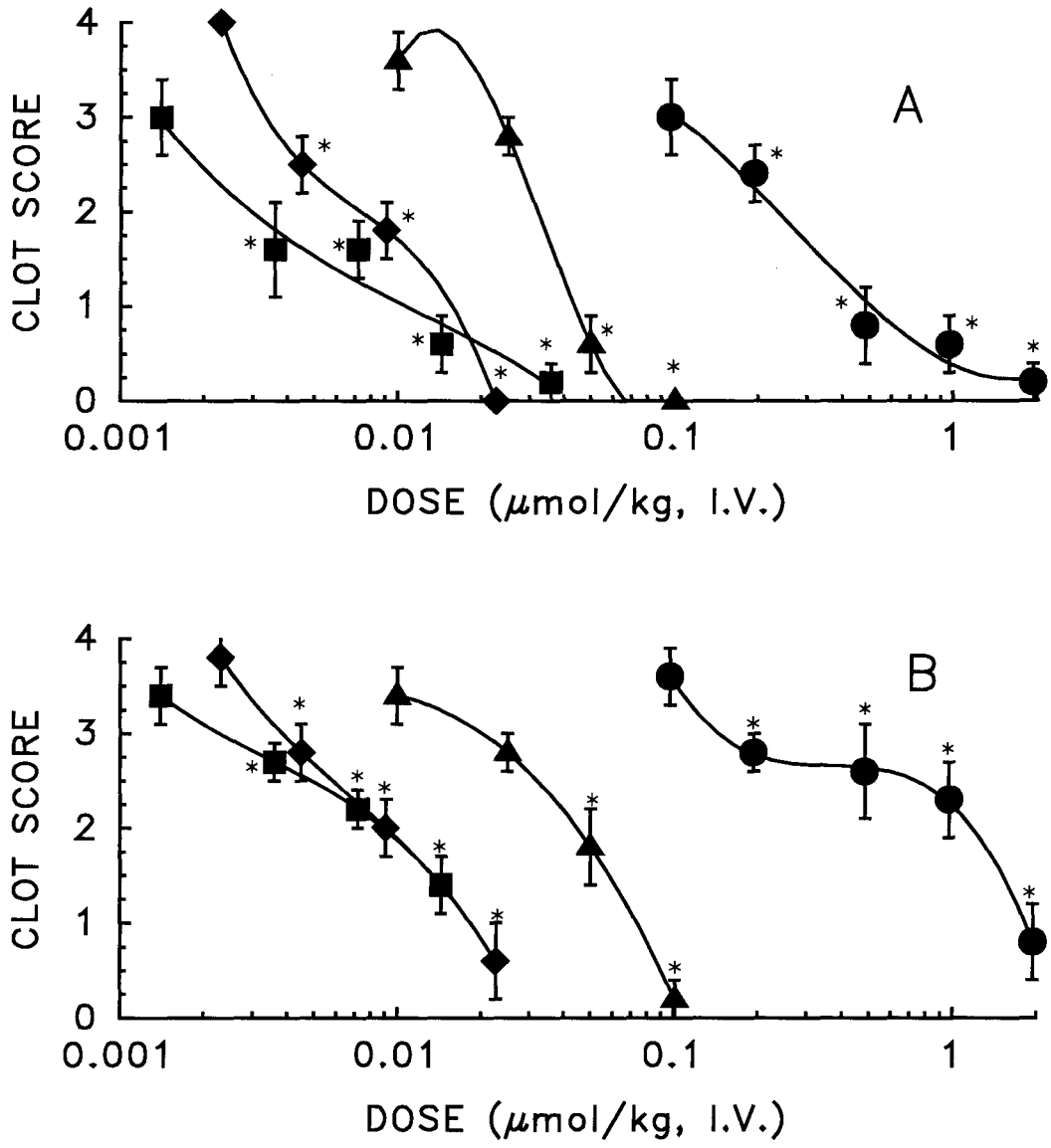


Fig. 39. Comparative dose-dependent antithrombotic effects of various agents as studied in the rabbit model of jugular vein stasis thrombosis. Each thrombin inhibitor was administered through I.V. injection and circulated for 5 minutes prior to initiation of the stasis procedure. The results depicted in panel A represent clot scores obtained after 10 minutes stasis. The results depicted in panel B represent clot scores obtained after 20 min stasis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-D-Phe-Pro-boroArg-OH, (■) hirudin, (♦) heparin. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (3.2 ± 0.2 after 10 min stasis in panel A, 3.6 ± 0.3 after 20 min stasis in panel B), as analyzed with the Kruskal-Wallis test. The individual clot score values at each antithrombin agent dose and their gravimetric equivalents at both the 10 and 20 min stasis end points are given in Tables 19a-19b.

superimposable at doses higher than 0.01 μmol/kg. The effects of heparin and hirudin were followed by those of Ac-(D)Phe-Pro-boroArg-OH, with ED_{50}s of 0.032 and 0.045 μmol/kg after 10 and 20 min stasis. D-MePhe-Pro-Arg-H was the weakest antithrombotic agent after both 10 and 20 min stasis time, with ED_{50}s of 0.242 and 1.60 μmol/kg respectively. The estimated ED_{50} from each thrombin inhibitor dose-response curve is reported in Table 19. The clot score value for each thrombin inhibitor dose after 10 and 20 min stasis time are found in Tables 19a - 19d.

Table 19 -- Comparative antithrombotic potencies of thrombin inhibitors after I.V. injection as determined in the rabbit model of jugular vein stasis thrombosis.

	Dose of each agent that results in a clot score of +2 in [μmol/kg].	
	10 min stasis time	20 min stasis time
D-MePhe-Pro-Arg-H	0.242	1.60
Ac-D-Phe-Pro-boroArg-OH	0.032	0.045
Hirudin	0.003	0.011
Heparin	0.008	0.009

Each value represents a single determination from the respective dose-response curve depicted in Fig. 39.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

Fig. 40 depicts the *ex vivo* effect of thrombin inhibitors on the protease inhibition profile of rabbit plasma as studied by the antithrombin and anti-Xa amidolytic methods revealing dose-dependent antithrombin effects but only minimal anti-Xa activities. Even at the highest doses administered, most of these agents produced only weak anti-Xa

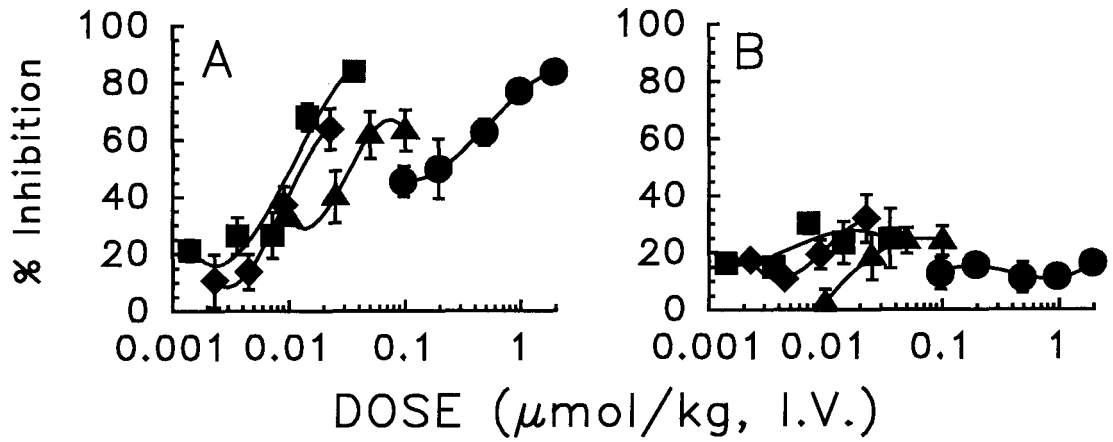


Fig. 40. Dose-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min post agent injection (prior to r-TF). Panel A depicts the results of the amidolytic antithrombin analysis. Panel B depicts the results from the amidolytic anti-Xa analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

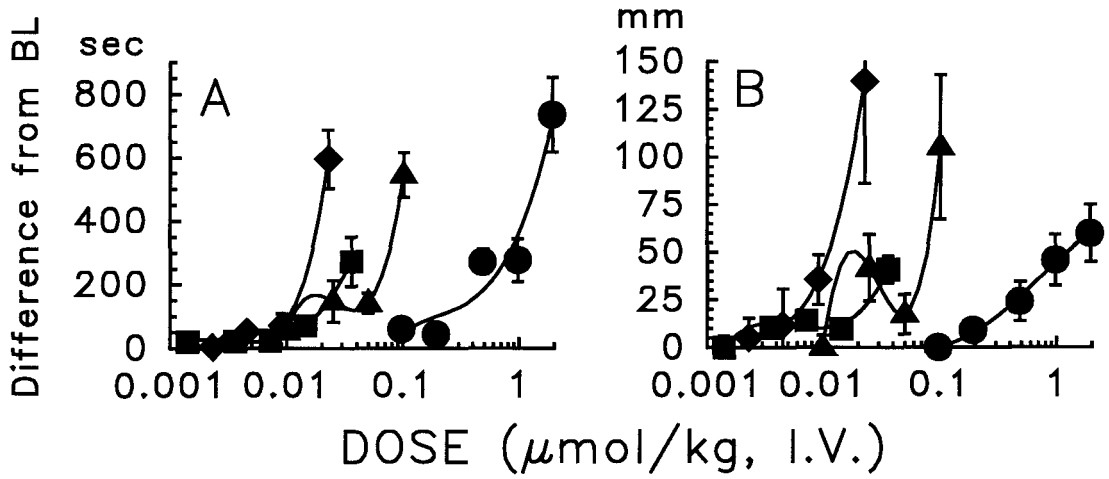


Fig. 41. Dose-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min post agent injection (prior to r-TF). Panel A depicts the results from the ACT analysis. Panel B depicts the results from the TEG r analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

actions. While heparin produced the strongest antithrombotic effects, as assessed by the clot scores, hirudin produced the most potent antithrombin effects. As in the clot score profiling, Ac-(D)Phe-Pro-boroArg-OH, relatively weaker than heparin and hirudin, it produced slightly stronger antithrombin effects in comparison to D-MePhe-Pro-Arg-H.

A comparison of the ACT values of samples obtained at baseline and 5 minutes after thrombin inhibitor injection, depicted in Fig. 41 panel A, showed a dose-dependent prolongation of the ACT, for all thrombin inhibitors, in a pattern parallel to the one emerging from the clot scores. The strongest prolongation of the ACT was produced by heparin producing a 600 sec ACT prolongation from the baseline value at a dose of 0.023 μmol/kg, followed by hirudin, Ac-(D)Phe-Pro-boroArg-OH and then D-MePhe-Pro-Arg-H. While Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H produced similar prolongation of the ACT at the highest dose used to that of heparin, hirudin resulted in only a 300 sec prolongation at the highest dose injected.

Fig. 41, panel B, depicts the comparison of the measured r distance on the TEG of samples obtained from the rabbits which underwent the stasis model before and after the administration of antithrombin agents at various doses. Dose-dependent prolongation of the TEG r duration occurred with all thrombin inhibitors, in a pattern similar to the ACT and to the clot score profile. At the highest doses injected, heparin produced the longest prolongation of the TEG r value, followed by Ac-(D)Phe-Pro-boroArg-OH, D-MePhe-Pro-Arg-H and lastly by hirudin.

When the plasma samples collected before and after thrombin inhibitor injection were compared, global clotting test-dependent results were obtained, as seen in Fig. 42.

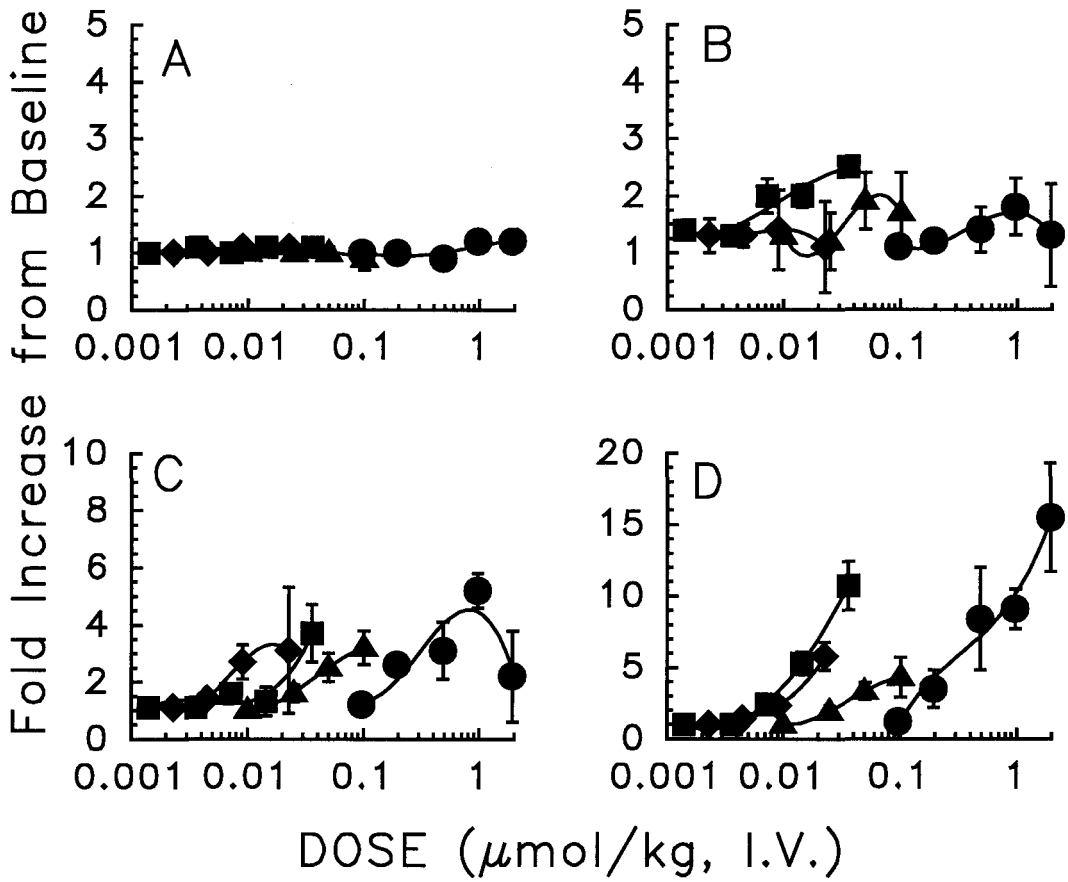


Fig. 42. Dose-dependent *ex vivo* anticoagulant effects of antithrombin agents after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min post agent injection (prior to r-TF). Panel A depicts the results of the PT analysis. Panel B depicts the results from the APTT analysis. Panel C depicts the results from the Heptest analysis. Panel D depicts the results from the TT analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

None of the thrombin inhibitors had any effects on the PT of rabbit plasma, even at the highest dose injected, as seen in Fig. 42, panel A. The panel B of Fig. 42 depicts the results from the APTT analysis and comparison of the samples obtained from these stasis thrombosis experiments. The *in vivo* administration of heparin and D-MePhe-Pro-Arg-H did not appear to have any effect on the APTT, even at the highest doses injected. Hirudin and Ac-(D)Phe-Pro-boroArg-OH produced mild dose-dependent prolongation of the APTT. When the plasma samples collected before and after the injection of thrombin inhibitors were compared in the Heptest assay (Fig. 42, panel C), it was found that all agents produced dose-dependent prolongation of the Heptest. In the Heptest, heparin appeared to produce more potent anticoagulant effects than hirudin, followed by Ac-(D)Phe-Pro-boroArg-OH and then by D-MePhe-Pro-Arg-H. When the same plasma samples were compared in the TT (Fig. 42, panel D), all thrombin inhibitors produced a prolongation of the TT. Hirudin produced the most potent prolongation of the TT, followed closely by heparin, then by Ac-(D)Phe-Pro-boroArg-OH and lastly by D-MePhe-Pro-Arg-H. While hirudin at the highest dose injected produced weaker antithrombotic activities than heparin and Ac-(D)Phe-Pro-boroArg-OH at their highest doses, hirudin produced the longer prolongation of the TT than the other two compounds. Furthermore, while D-MePhe-Pro-Arg-H at the highest dose injected produced similar antithrombotic effects to those of heparin and Ac-(D)Phe-Pro-boroArg-OH at the highest dose injected, the prolongation of the TT was longer with D-MePhe-Pro-Arg-H (about 15 fold increase from baseline) than with heparin and Ac-(D)Phe-Pro-boroArg-OH (about 5 fold increase from baseline).

3. Duration of Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration at a Fixed Dose

To determine the time-dependance of the antithrombotic effects of the thrombin inhibitors studied in this rabbit stasis thrombosis model after I.V. administration, the dose of each agent that produced sub-maximal antithrombotic effects was selected and studied at different circulation times, prior to injecting r-TF as described above. The dose of each thrombin inhibitor studied was: D-MePhe-Pro-Arg-H at 1.942 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, hirudin at 0.036 μmol/kg and heparin at 0.023 μmol/kg. The circulation time points selected for studying the antithrombotic effects of these thrombin inhibitors at these doses were 5, 30, 60 and 90 min (prior to r-TF injection). Six rabbits were tested at each time point for each thrombin inhibitor. Blood draws were performed at baseline and prior to r-TF injection (5 or 30 or 60 or 90 min post thrombin inhibitor injection) and utilized in the *ex vivo* whole blood and plasma analyses.

a. Clot Score Analyses

Intravenous administration of all four thrombin inhibitors produced time-dependent antithrombotic effects, as depicted in Fig. 43, after 10 and 20 min stasis time, panels A and B respectively. The thrombin inhibitor which lost its antithrombotic activities at the fastest rate was Ac-(D)Phe-Pro-boroArg-OH (t½ about 20 min post I.V. injection, after both the 10 and 20 min stasis time), followed by heparin (t½ around 40 min and 25 min post I.V. injection, after the 10 and 20 min stasis respectively) and then by D-MePhe-Pro-Arg-H (t½ around 60 min and 45 min post I.V. injection after the 10 and 20 min

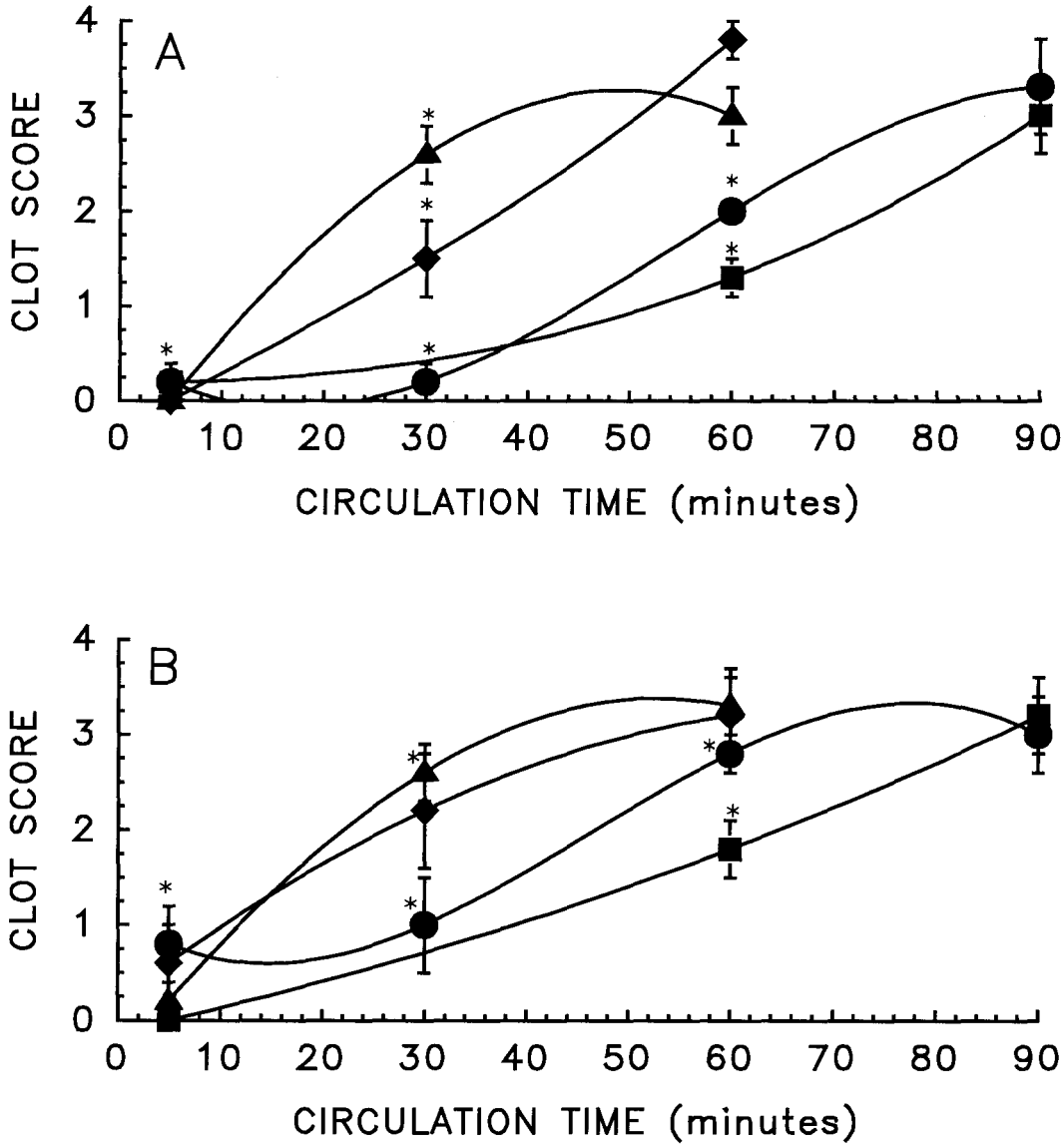


Fig. 43. Time dependance of antithrombotic effects of various agents in the rabbit model of jugular vein stasis thrombosis. Each agent was administered through I.V. injection and allowed to circulate for varying time periods prior to initiation of the stasis procedure. The results depicted in panel A represent clot scores obtained after 10 minutes stasis. The results depicted in panel B represent clot scores obtained after 20 min stasis. (●) D-MePhe-Pro-Arg-H at 1.942 μmol/kg, (▲) Ac-D-Phe-Pro-boroArg-OH at 0.101 μmol/kg, (■) hirudin at 0.036 μmol/kg, (◆) heparin at 0.023 μmol/kg. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p<0.05$) when compared to control (3.2 ± 0.2 after 10 min stasis in panel A, 3.6 ± 0.3 after 20 min stasis in panel B), as analyzed with the Kruskal-Wallis test. The individual clot score values for each thrombin inhibitor at each time point at both the 10 and 20 min stasis end points are given in Tables 19e-19f.

stasis respectively). Hirudin also exhibited a gradual diminution of its antithrombotic activities at the slowest rate (t½ around 75 min and 65 min post I.V. injection, after 10 and 20 min stasis respectively). The clot score values for each thrombin inhibitor at each circulation time are found in Tables 19e and 19f, following 10 and 20 min stasis respectively.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

The *ex vivo* effect of thrombin inhibitors on the protease inhibition profile of rabbit plasma as studied by the antithrombin and anti-Xa amidolytic methods revealed time-dependent antithrombin effects but minimal anti-Xa activities, as can be seen in Fig. 44, panels A and B respectively. The antithrombin effects of heparin were minimal as well. D-MePhe-Pro-Arg-H and hirudin produced an antithrombin pattern indicating peak antithrombin activity at 30 min post I.V. injection, in contrast to their time-dependent antithrombotic profile, in which their peak antithrombotic activities were observed 5 min after I.V. injection. The antithrombin activities of Ac-(D)Phe-Pro-boroArg-OH were steadily lost with time.

Comparison of the ACT values of samples obtained at baseline and 5, 30, 60 and 90 min after thrombin inhibitor injection, depicted in Fig. 45, panel A, revealed a time-dependent prolongation of the ACT, for all antithrombin agents, in a pattern parallel to the one emerging from the clot score time-dependent profile. The thrombin inhibitors that lost their ACT prolongation effects at the fastest rate were Ac-(D)Phe-Pro-boroArg-OH and heparin, followed by D-MePhe-Pro-Arg-H. Hirudin lost its ACT prolongation effects at the slowest rate.

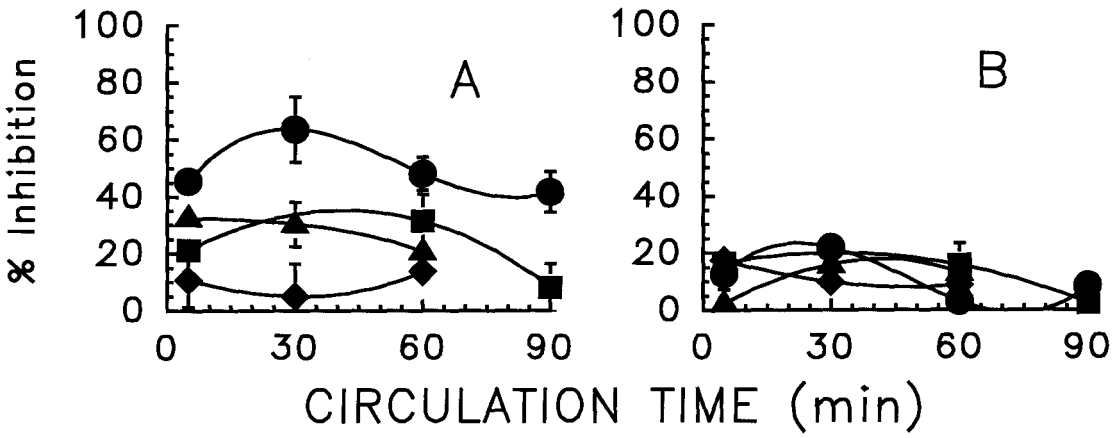


Fig. 44. Time-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection. Panel A depicts the results of the amidolytic antithrombin analysis. Panel B depicts the results from the amidolytic anti-Xa analysis. (●) D-MePhe-Pro-Arg-H at 1.942 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, (■) hirudin at 0.036 μmol/kg, (◆) heparin at 0.023 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

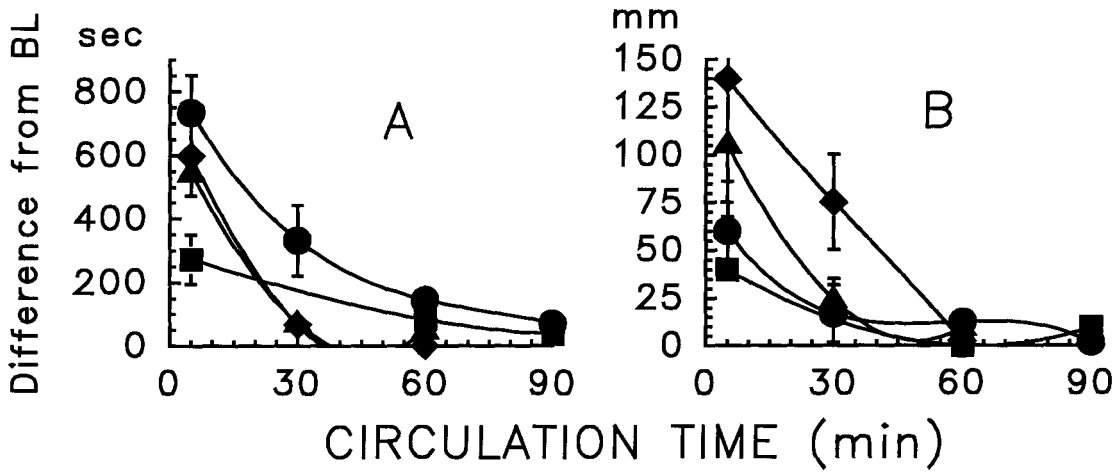


Fig. 45. Time-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection. Panel A depicts the results from the ACT analysis. Panel B depicts the results from the TEG r analysis. (●) D-MePhe-Pro-Arg-H at 1.942 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, (■) hirudin at 0.036 μmol/kg, (◆) heparin at 0.023 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

Fig. 45, panel B, depicts the comparison of the measured r distance on the TEG of samples obtained from the rabbits which underwent the stasis model before and after the administration of thrombin inhibitors at various blood sampling times. Time-dependent prolongation of the TEG r value was observed with all thrombin inhibitors, in a pattern parallel to the one emerging from the clot score time-dependent profile and the ACT profile. The thrombin inhibitor that lost its TEG prolongation effects at the fastest rate was Ac-(D)Phe-Pro-boroArg-OH, followed by heparin and then by D-MePhe-Pro-Arg-H. Hirudin lost its TEG prolongation effects at the slowest rate.

When the plasma samples collected before and 5, 30, 60 and 90 min after thrombin inhibitor injection were compared, global clotting test-dependent and time-dependent results were obtained, as seen in Fig. 46. None of the thrombin inhibitors had any effects on the PT of rabbit plasma as seen in Fig. 46, panel A. Panel B of Fig. 46 depicts the results from the APTT analysis and comparison of the samples obtained from these stasis thrombosis experiments. While the *in vivo* administration of heparin and D-MePhe-Pro-Arg-H did not appear to have any effect on the APTT, the anticoagulant effects produced by hirudin and Ac-(D)Phe-Pro-boroArg-OH were time-dependent. When the plasma samples collected before and after the injection of thrombin inhibitors were compared in the Heptest assay (Fig. 46, panel C), it was found that all thrombin inhibitors produced time-dependent prolongation of the Heptest. While the anticoagulant effects in the Heptest assay steadily decreased with time for Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin, D-MePhe-Pro-Arg-H appeared to produce peak anticoagulant activities abut 30 min post I.V. injection, similarly to its effects in the antithrombin

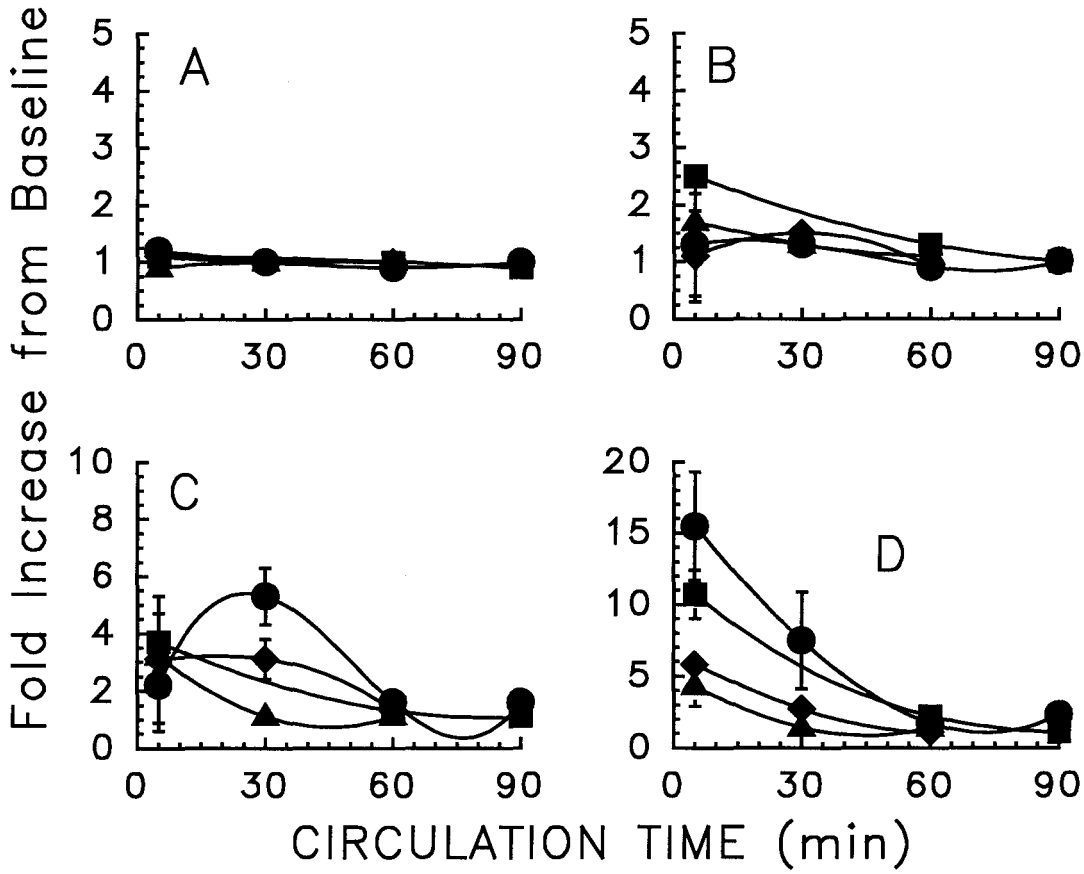


Fig. 46. Time-dependent *ex vivo* anticoagulant effects of antithrombin agents after I.V. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection (post-thrombin inhibitor injection for various circulating time periods). Panel A depicts the results of the PT analysis. Panel B depicts the results from the APTT analysis. Panel C depicts the results from the Heptest analysis. Panel D depicts the results from the TT analysis. (●) D-MePhe-Pro-Arg-H at 1.942 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, (■) hirudin at 0.036 μmol/kg, (◆) heparin at 0.023 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

assay. When the same plasma samples were compared in the TT (Fig. 46, panel D), all thrombin inhibitors produced a steadily declining time-dependent prolongation of the TT. The effects of D-MePhe-Pro-Arg-H were cleared at the fastest rate, in contrast to its time-dependent antithrombotic effects.

4. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration at a Fixed Time Point

As in the above studies, a r-TF dose of 0.68 pmol/kg was chosen for the standard procedure as the thrombogenic source in this rabbit jugular vein stasis thrombosis model. The antithrombotic effects of D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin were compared in a dose-dependent manner, after S.C. injection and after a 45 min circulation time prior to injection of r-TF. Twenty sec after injection of r-TF, the jugular segments of the rabbit were isolated and after 10 and 20 minutes of stasis, each jugular segment was dissected and the contained clots were analyzed on a previously established scale from 0 to +4 (Fareed et al. 1986). Blood samples were collected at baseline and 45 min after thrombin inhibitor injection (prior to r-TF injection), for *ex vivo* whole blood and plasma analyses. Six rabbits were studied at each thrombin inhibitor dose.

a. Clot Score Analyses

Injection of all four thrombin inhibitors produced dose-dependent antithrombotic effects, after 10 and 20 min stasis time, as depicted in Fig. 47, panels A and B respectively. The most potent antithrombotic agent appeared to be hirudin at doses in the nmol/kg range, both after 10 and 20 min stasis time, with ED_{50}s of 0.036 and 0.07

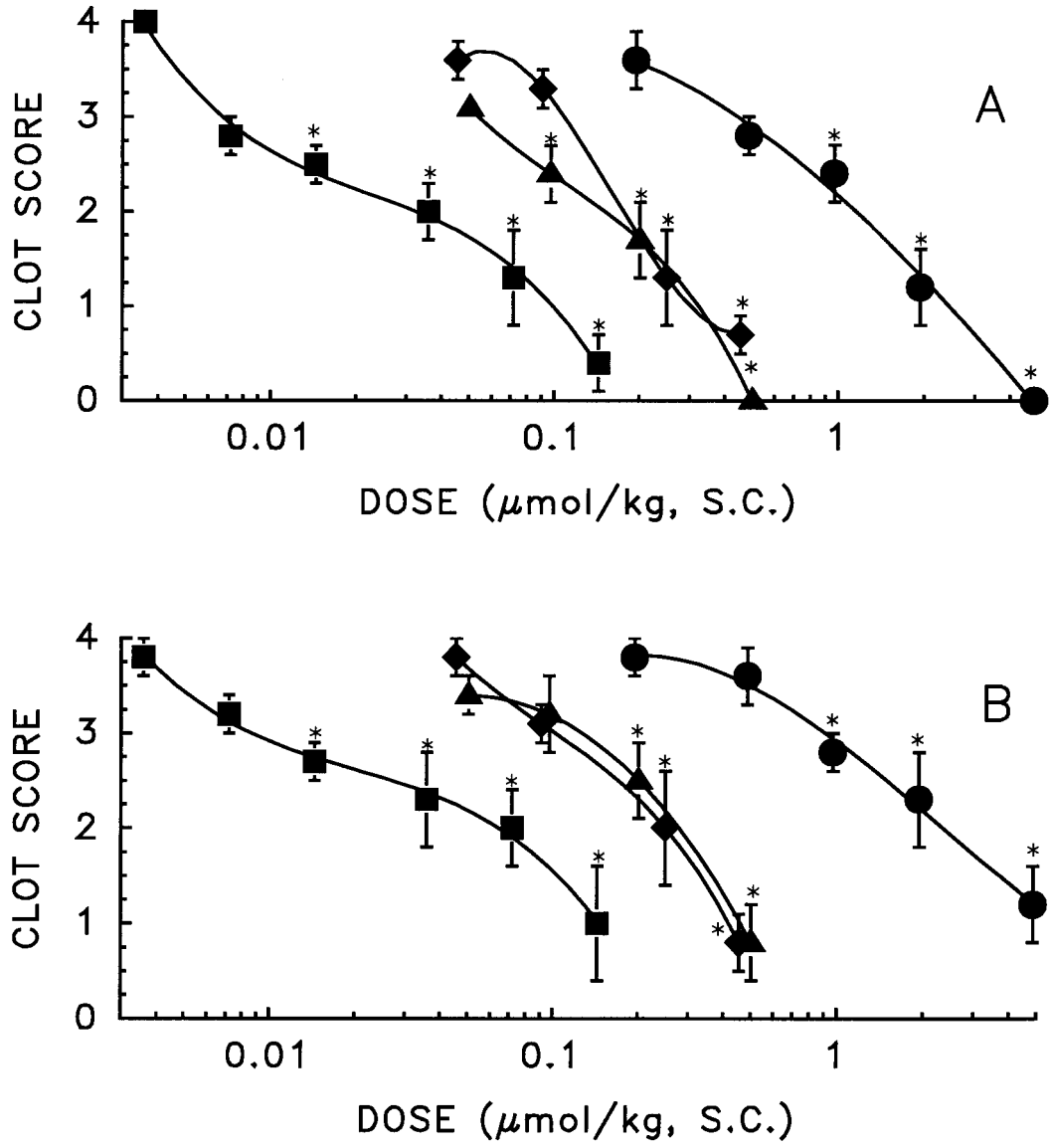


Fig. 47. Comparative dose-dependent antithrombotic effects of various agents as studied in the rabbit model of jugular vein stasis thrombosis. Each agent was administered through S.C. injection and allowed to circulate for 45 minutes prior to initiation of the stasis procedure. The results depicted in panel A represent clot scores obtained after 10 minutes stasis. The results depicted in panel B represent clot scores obtained after 20 min stasis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-D-Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (3.2 ± 0.2 after 10 min stasis in panel A, 3.6 ± 0.3 after 20 min stasis in panel B), as analyzed with the Kruskal-Wallis test. The individual clot score values at each thrombin inhibitor dose and their gravimetric equivalents at both the 10 and 20 min stasis end points are given in Tables 20a-20d.

μmol/kg respectively. The effects of hirudin were followed by those of Ac-(D)Phe-Pro-boroArg-OH (ED_{50}s of 0.152 and 0.29 μmol/kg after 10 and 20 min stasis respectively) and heparin (ED_{50}s od 0.185 and 0.25 μmol/kg respectively. After 20 min stasis time, the antithrombotic effects of Ac-(D)Phe-Pro-boroArg-OH and heparin were superimposable. Similar to the antithrombotic effects after I.V. administration, D-MePhe-Pro-Arg-H was the weakest antithrombotic agent after both 10 and 20 min stasis time, with ED_{50}s of 1.22 and 2.78 μmol/kg respectively. The estimated ED_{50} from each thrombin inhibitor dose-response curve is reported in Table 20. The clot score value for each thrombin inhibitor dose are found in Tables 20a - 20d, for the 10 and 20 min stasis time.

Table 20 -- Comparative antithrombotic potencies of thrombin inhibitors after S.C. injection, as determined in the rabbit model of jugular vein stasis thrombosis.

	Dose of each agent resulting in a clot score of +2 in [μmol/kg]	
	10 min stasis time	20 min stasis time
D-MePhe-Pro-Arg-H	1.22	2.78
Ac-D-Phe-Pro-boroArg-OH	0.152	0.29
Hirudin	0.036	0.07
Heparin	0.185	0.25

Each value represents a single determination from the respective dose-response curves depicted in Fig. 47.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

The *ex vivo* effect of thrombin inhibitors on the protease inhibition profile of

rabbit plasma as studied by the antithrombin and anti-Xa amidolytic methods revealed dose-dependent antithrombin effects but minimal anti-Xa activities even at the highest doses administered, as can be seen in Fig. 48, panels A and B respectively. Hirudin produced the strongest antithrombin effects, which corresponded to the antithrombotic effects. As in the clot score profiling, Ac-(D)Phe-Pro-boroArg-OH produced the second most potent antithrombin effects, followed by heparin and lastly by D-MePhe-Pro-Arg-H. Even though all thrombin inhibitors produced similar antithrombotic effects at the highest doses used, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H produced a markedly higher antithrombin effects than hirudin and heparin.

Comparison of the ACT values of samples obtained at baseline and 45 minutes after thrombin inhibitor S.C. injection, depicted in Fig. 49, panel A, revealed a dose-dependent prolongation of the ACT, for all thrombin inhibitors, in a pattern parallel to the one emerging from the clot scores. The strongest prolongation of the ACT was produced by hirudin, followed by Ac-(D)Phe-Pro-boroArg-OH and then by heparin and D-MePhe-Pro-Arg-H. Even though all agents produced similar antithrombotic effects at the highest doses used, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H produced a markedly longer ACT prolongation than hirudin and heparin, similar to the antithrombin effects.

Fig. 49, panel B, depicts the comparison of the measured r distance on the TEG of samples obtained from the rabbits which underwent the stasis model before and after the S.C. injection of thrombin inhibitors at various doses. Dose-dependent prolongation of the TEG r duration occurred with all thrombin inhibitors. Unlike the antithrombotic,

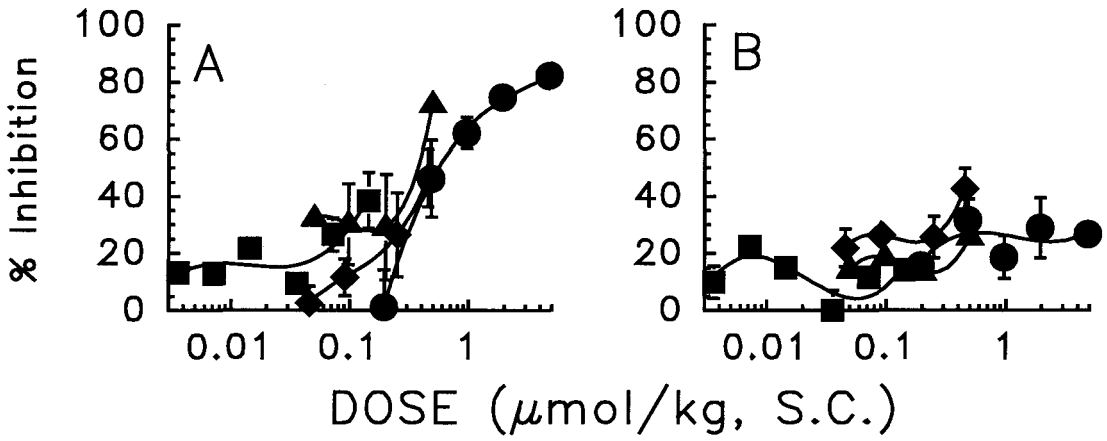


Fig. 48. Dose-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 45 min post agent injection. Panel A depicts the results of the amidolytic antithrombin analysis. Panel B depicts the results from the amidolytic anti-Xa analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

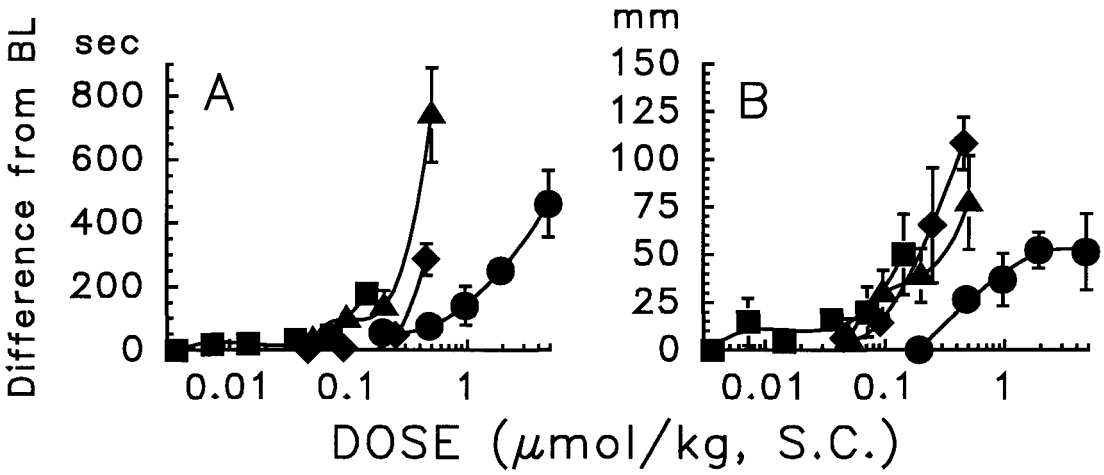


Fig. 49. Dose-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 45 min post agent injection. Panel A depicts the results from the ACT analysis. Panel B depicts the results from the TEG r analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

antithrombin and ACT effects, in the TEG r value comparisons it was found that the dose response effects of Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin overlapped. However, D-MePhe-Pro-Arg-H consistently resulted in the weakest effects on the TEG r value.

When the plasma samples collected before and after thrombin inhibitor S.C. injection were compared, global clotting test-dependent results were obtained, as seen in Fig. 50. None of the thrombin inhibitors had any effects on the PT of rabbit plasma, even at the highest dose injected, as seen in Fig. 50, panel A. Panel B of Fig. 50 depicts the results from the APTT analysis and comparison of the samples obtained from these stasis thrombosis experiments. All thrombin inhibitors produced dose-dependent anticoagulant effects after S.C. injection. Similar to the effects that these agents had on the TEG r value, the anticoagulant effects of Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin after S.C. administration overlapped. Even though all thrombin inhibitors produced similar antithrombotic effects at the highest doses used, the anticoagulant effects of heparin were markedly higher than those produced by the other agents. When the plasma samples collected before and 45 min after the S.C. injection of thrombin inhibitors were compared in the Heptest assay (Fig. 50, panel C), it was found that all agents produced dose-dependent prolongation of the Heptest. As seen in the antithrombotic profile, in the Heptest hirudin appeared to produce the strongest anticoagulant effects. These effects were followed by those produced by heparin and then by Ac-(D)Phe-Pro-boroArg-OH. Consistently with all other effects produced by D-MePhe-Pro-Arg-H after S.C. injection, this agent was the least potent anticoagulant in

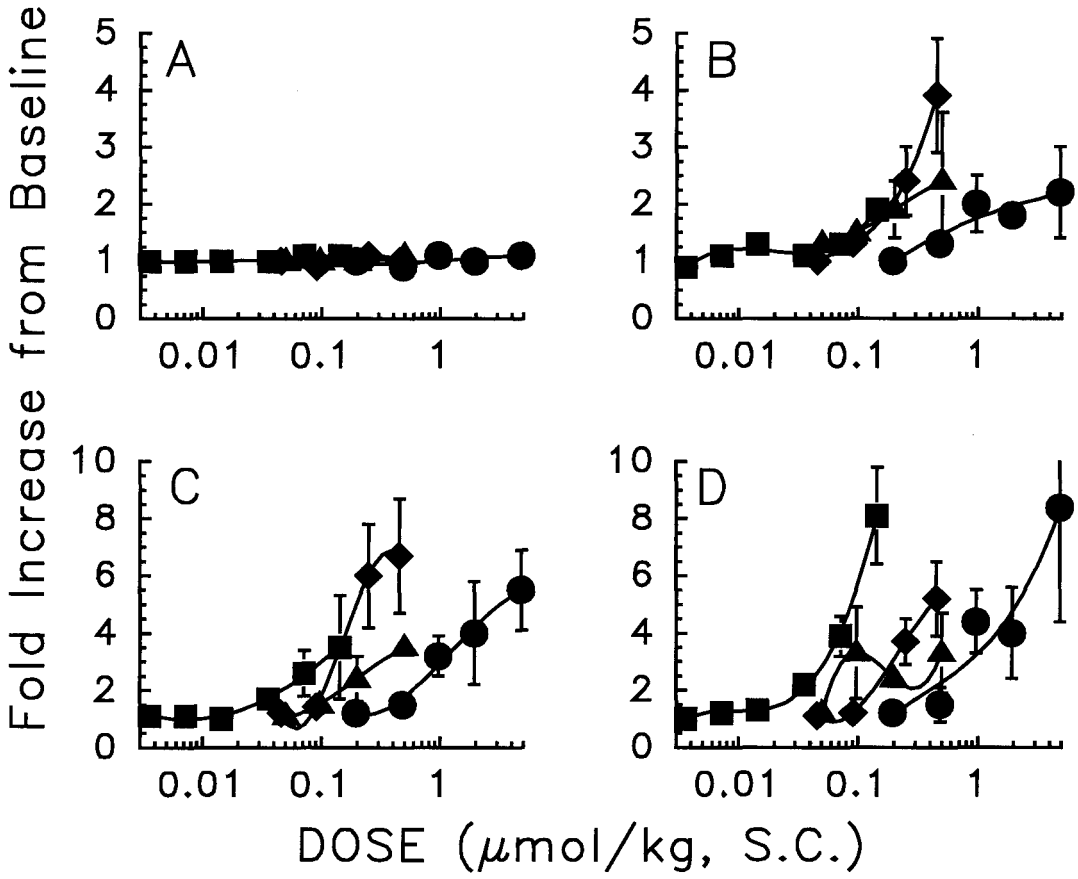


Fig. 50. Dose-dependent *ex vivo* anticoagulant effects of antithrombin agents after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 45 min post thrombin inhibitor injection (prior to r-TF). Panel A depicts the results of the PT analysis. Panel B depicts the results from the APTT analysis. Panel C depicts the results from the Heptest analysis. Panel D depicts the results from the TT analysis. (●) D-MePhe-Pro-Arg-H, (▲) Ac-(D)Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each dose, for each agent).

the Heptest assay as well. As observed with the anticoagulant effects in the APTT, even though all thrombin inhibitors produced similar antithrombotic effects at the highest doses used, the anticoagulant effects of heparin in the Heptest assay were markedly higher than those produced by Ac-(D)Phe-Pro-boroArg-OH and hirudin. Like heparin, D-MePhe-Pro-Arg-H also produced higher anticoagulant effects in the Heptest than Ac-(D)Phe-Pro-boroArg-OH and hirudin, at the highest doses injected. When the same plasma samples were compared in the TT (Fig. 50, panel D), all thrombin inhibitors produced a prolongation of the TT in manner parallel to their antithrombotic effects, so that hirudin produced the most potent prolongation of the TT, followed closely by heparin, then by Ac-(D)Phe-Pro-boroArg-OH and lastly by D-MePhe-Pro-Arg-H. While all thrombin inhibitors at the highest dose injected produced similar antithrombotic activities, hirudin and Ac-(D)Phe-Pro-boroArg-OH produced longer prolongation of the TT than the other two compounds.

5. Duration of Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration at a Fixed Dose

To determine the time-dependance of the antithrombotic effects of the thrombin inhibitors studied in this rabbit stasis thrombosis model after S.C. administration, the dose of each agent that produced sub-maximal antithrombotic effects in the dose-dependent S.C. studies was selected and studied at different circulation times, prior to injecting r-TF as described above. The dose of each antithrombin agent studied was: D-MePhe-Pro-Arg-H at 4.854 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.503 μmol/kg, hirudin at 0.144 μmol/kg and heparin at 0.455 μmol/kg. The circulation time points

selected for studying the antithrombotic effects of these thrombin inhibitors at these doses were 15, 45 and 90 min (prior to r-TF injection). Six rabbits were tested at each time point for each thrombin inhibitor. Blood samples were collected at baseline and prior to r-TF injection (15, 30, 45, 60 and 90 min post thrombin inhibitor injection) and utilized in the *ex vivo* whole blood and plasma analyses.

a. Clot Score Analyses

Subcutaneous administration of all four thrombin inhibitors produced a time-dependent antithrombotic effects, as depicted in Fig. 51, after 10 and 20 min stasis time, panels A and B respectively. In the 10 min stasis clot score results (Fig. 51, panel A), it was observed that while Ac-(D)Phe-Pro-boroArg-OH lost its antithrombotic activities progressively with time after 15 min post S.C. injection, the effects of D-MePhe-Pro-Arg-H and hirudin started to diminish after 45 min post S.C. injection. In contrast to the antithrombotic effects produced by the other thrombin inhibitors, the effects of heparin steadily increased with time, with the maximal antithrombotic effects achieved 90 min post S.C. injection.

The duration of the antithrombotic effects of thrombin inhibitors after S.C. injection, as assessed by the clot scores obtained after 20 min stasis time (Fig. 51, panel B), differed only slightly from those obtained after 10 min stasis time. The antithrombotic effects of Ac-(D)Phe-Pro-boroArg-OH appeared to have steady antithrombotic effects over the 90 min period studied, rather than being progressively lost with time after 15 min post S.C. injection, as observed in the 10 min stasis panel. D-MePhe-Pro-Arg-H appeared to time-dependently lose its antithrombotic activities starting

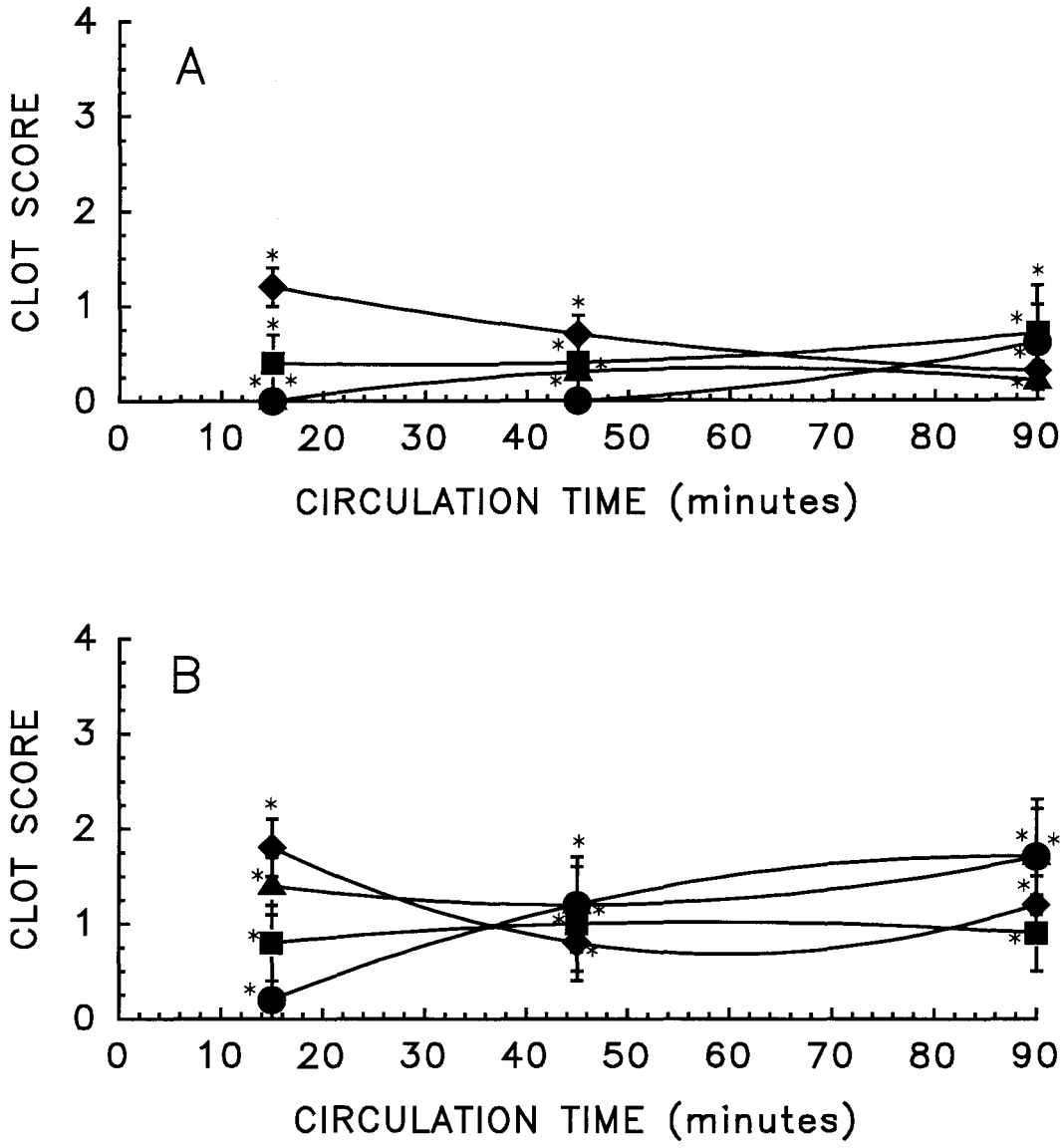


Fig. 51. Time dependance of antithrombotic effects of various agents in the rabbit model of jugular vein stasis thrombosis. Each agent was administered through S.C. injection and allowed to circulate for varying time periods prior to initiation of the stasis procedure. The results depicted in panel A represent clot scores obtained after 10 minutes stasis. The results depicted in panel B represent clot scores obtained after 20 min stasis. (●) D-MePhe-Pro-Arg-H at 4.854 μmol/kg, (▲) Ac-D-Phe-Pro-boroArg-OH at 0.503 μmol/kg, (■) hirudin at 0.144 μmol/kg, (◆) heparin at 0.455 μmol/kg. Each point represents a mean $\pm$ S.E.M. (n=6). * denotes statistical significance ($p<0.05$) when compared to control (3.2 $\pm$ 0.2 after 10 min stasis in panel A, 3.6 $\pm$ 0.3 after 20 min stasis in panel B), as analyzed with the Kruskal-Wallis test. The individual clot score values for each thrombin inhibitor at each time point at both the 10 and 20 min stasis end points are given in Tables 20e-20f.

at 15 min post S.C. injection, as opposed to 45 min post S.C. injection, as observed in the 10 min stasis panel. Hirudin, like Ac-(D)Phe-Pro-boroArg-OH, appeared to have steady antithrombotic effects over the 90 min period studied, rather than being progressively lost with time after 45 min post S.C. injection, as observed in the 10 min stasis panel. Heparin time-dependently increased its antithrombotic effects, reaching a maximum around 60 min post S.C. injection, as opposed to 90 min post S.C. injection, as observed in the 10 min stasis panel. The clot score values for each thrombin inhibitor at each circulation time are found in Tables 20e and 20f, following 10 and 20 min stasis respectively.

b. *Ex Vivo* Analyses of Plasma and Whole Blood Samples

The *ex vivo* effect of thrombin inhibitors on the protease inhibition profile of rabbit plasma as studied by the antithrombin amidolytic method revealed time-dependent antithrombin effects, as can be seen in Fig. 52, panel A. While the antithrombin effects of D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH were steady over the 90 min period monitored, the effects of hirudin and heparin steadily increased with time, reaching a maximum at 60 min post S.C. injection. Heparin exhibited time-dependent anti-Xa effects, as measured in the amidolytic assay, depicted in Fig. 52, panel B. These effects reached a maximum about 60 min post S.C. injection of heparin. The other three agents had weak effects on the inhibition factor Xa, as measured in this assay.

Comparison of the ACT values on samples obtained at baseline and 5, 30, 60 and 90 min after thrombin inhibitor injection, depicted in Fig. 53, panel A, revealed a time-dependent prolongation of the ACT, for all thrombin inhibitors. Parallel to the

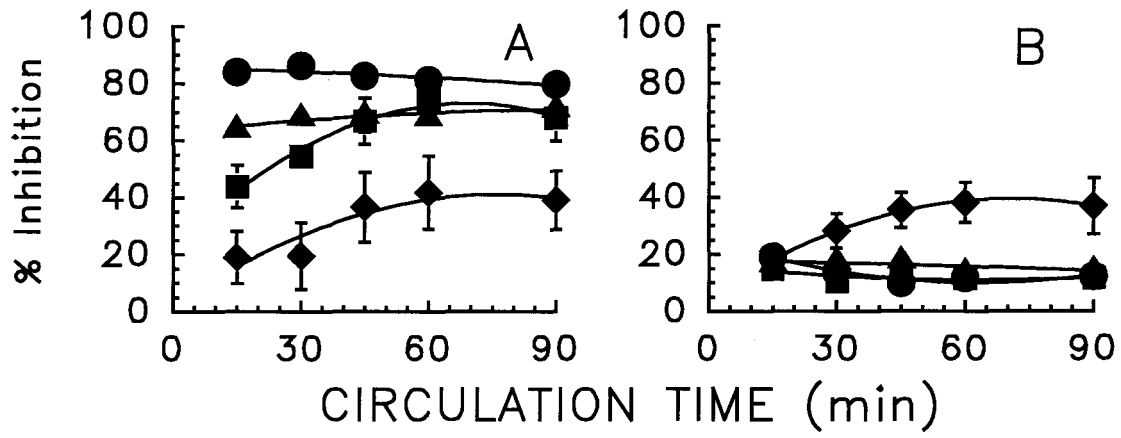


Fig. 52. Time-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection. Panel A depicts the results of the amidolytic antithrombin analysis. Panel B depicts the results from the amidolytic anti-Xa analysis. (●) D-MePhe-Pro-Arg-H at 4.854 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.503 μmol/kg, (■) hirudin at 0.144 μmol/kg, (◆) heparin at 0.455 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

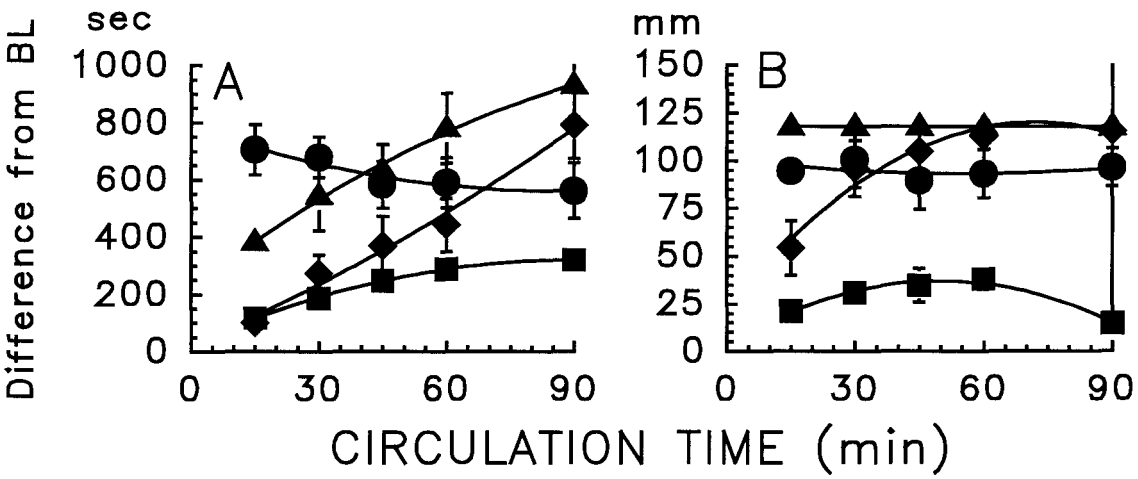


Fig. 53. Time-dependent *ex vivo* anticoagulant effects of thrombin inhibitors after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection. Panel A depicts the results from the ACT analysis. Panel B depicts the results from the TEG r analysis. (●) D-MePhe-Pro-Arg-H at 4.854 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.503 μmol/kg, (■) hirudin at 0.144 μmol/kg, (◆) heparin at 0.455 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

antithrombotic effects, D-MePhe-Pro-Arg-H gradually lost its anticoagulant effects in the ACT. In contrast to their antithrombotic effects, Ac-(D)Phe-Pro-boroArg-OH and hirudin time-dependently increased their anticoagulant activities in the ACT during the 90 min following S.C. injection. Heparin, parallel to its antithrombotic effects after S.C. injection, also time-dependently increased its anticoagulant actions in the ACT.

Fig. 53, panel B depicts the comparison of the measured r distance on the TEG of samples obtained from the rabbits which underwent the stasis model before and after the S.C. injection of thrombin inhibitors at various blood sampling times. Time-dependent prolongation of the TEG r value was observed with hirudin and heparin. The effects of both agents were maximal at 60 min post S.C. injection, parallel to their antithrombotic effects. D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH produced maximal TEG r value prolongation from the first blood sampling time at 15 min, and these effects were steady throughout the 90 min post S.C. injection period, even though their respective antithrombotic effects were slowly diminishing.

When the plasma samples collected at the baseline and 5, 30, 60 and 90 min after thrombin inhibitor injection were compared, global clotting test-dependent and time-dependent results were obtained, as seen in Fig. 54. With the exception of D-MePhe-Pro-Arg-H, none of the thrombin inhibitors had any effects on the PT of rabbit plasma as seen in Fig. 54, panel A. The effects of D-MePhe-Pro-Arg-H on the PT were noticeable only 45 min post S.C. injection. Panel B of Fig. 54 depicts the results from the APTT analysis and comparison of the samples obtained from these stasis thrombosis experiments. D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH produced maximal

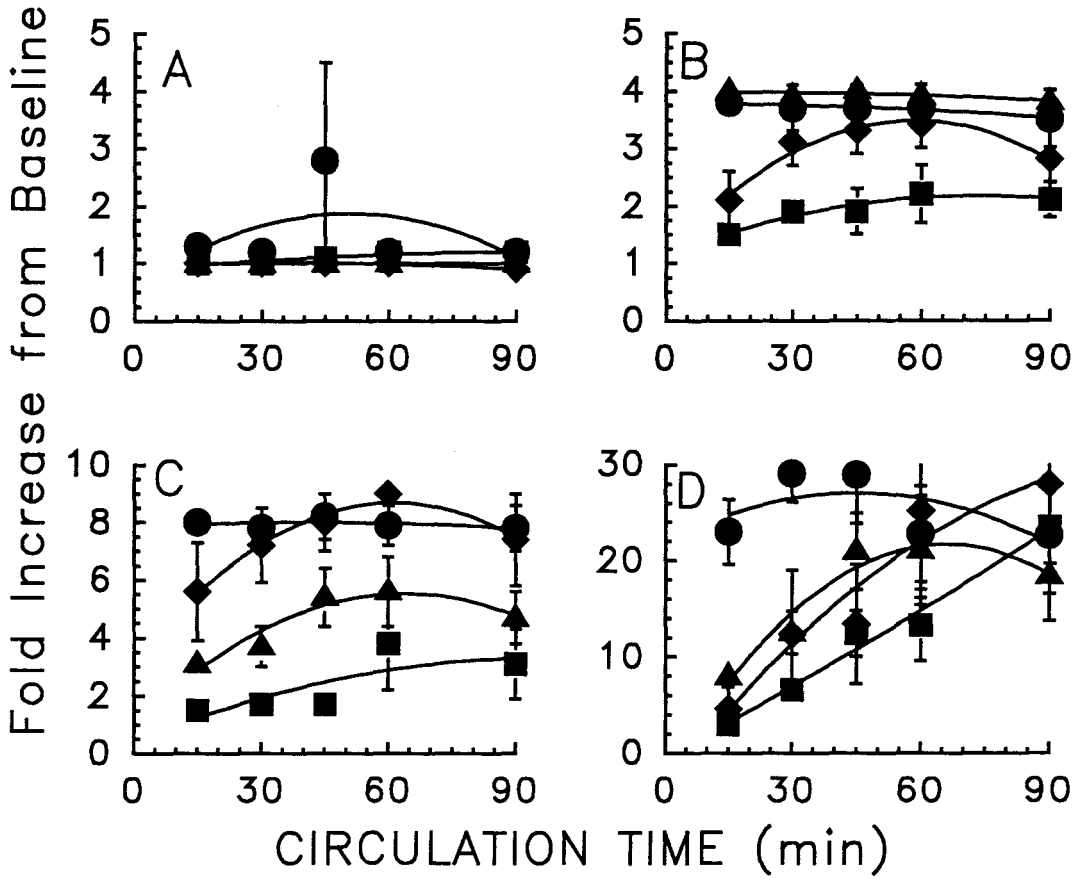


Fig. 54. Time-dependent *ex vivo* anticoagulant effects of antithrombin agents after S.C. injection as studied in the rabbit model of jugular vein stasis thrombosis. Blood samples were collected prior to and 5 min prior to r-TF injection (post-agent S.C. injection for various circulating time periods). Panel A depicts the results of the PT analysis. Panel B depicts the results from the APTT analysis. Panel C depicts the results from the Heptest analysis. Panel D depicts the results from the TT analysis. (●) D-MePhe-Pro-Arg-H at 4.854 μmol/kg, (▲) Ac-(D)Phe-Pro-boroArg-OH at 0.503 μmol/kg, (■) hirudin at 0.144 μmol/kg, (◆) heparin at 0.455 μmol/kg. Each point represents a mean of the difference between baseline and post-agent values for each rabbit ± S.E.M. (n=6 at each time point, for each agent).

prolongation of the APTT throughout the 90 min post S.C. injection period, even though the corresponding antithrombotic effects slowly decreased with time. The anticoagulant effects of hirudin on the APTT increased with time and plateaued around 60 min after S.C. injection, similarly to its antithrombotic effects. The anticoagulant effects of heparin on the APTT closely paralleled the antithrombotic effects after S.C. injection, 20 min stasis time. Time-dependent increase in the antithrombotic and anticoagulant activity of heparin was observed until 60 min post S.C. injection, after which time point, both activities declined. When the plasma samples collected prior to and after the injection of thrombin inhibitors were compared in the Heptest assay (Fig. 54, panel C), it was found Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin produced time-dependent prolongation of the Heptest. D-MePhe-Pro-Arg-H produced maximal prolongation of the Heptest throughout the 90 min period post S.C. injection. The anticoagulant effects of the other three inhibitors in the Heptest, steadily increased with time, reaching a peak at 60 min post S.C. injection, after which the anticoagulant effects started to decrease. When the same plasma samples were compared in the TT (Fig. 54, panel D), hirudin and heparin produced a steadily increasing time-dependent prolongation of the TT. The anticoagulant effects of D-MePhe-Pro-Arg-H reached a maximum at 30 min post S.C. injection, which started to decrease 45 min post S.C. injection. Ac-(D)Phe-Pro-boroArg-OH also produced time-dependent prolongation of the TT after S.C. injection, similar to its anticoagulant effects in the APTT and Heptest, reaching a maximum at 60 min post S.C. injection and then declining with time.

I. Antithrombotic Effects of Thrombin Inhibitors as Studied in the Rat Model of Laser-Induced Thrombosis

The rabbit jugular vein stasis thrombosis model used in the previous experiments is venous model of thrombosis that results in fibrin-rich clots. Since veins and arteries have different physiologic make ups (hemodynamics, smooth muscle, size) and exhibit distinct pathophysiologic responses, it is probable that thrombin inhibitors have different antithrombotic effects depending on the type of blood vessel studied and the thrombotic injury. Therefore, in the following studies, a rat model of laser-induced thrombosis was utilized to compare the antithrombotic effects of D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin. This model of arterial thrombosis is based on the development of a platelet-rich thrombus in response to focused laser-mediated thermal injuries to the vascular wall. The endpoint in this model was the number of standardized laser exposures required to produce either a clot completely occluding the arteriole injured or a semi-occluding clot with a length equal to 1.5 x the vessel diameter. Thrombin inhibitors were injected at various concentrations through the rat tail vein 5 min prior to induction of the laser injury.

The results of these studies are depicted in Fig. 55. In control rats (injected with saline) 3 laser shots were needed to reach an endpoint. All thrombin inhibitors produced dose-dependent antithrombotic effects in this model. In comparing the dose of each agent required to extend the endpoint to 6 laser shots, as given in Table 21, heparin was the most potent antithrombotic agent (0.08 μmol/kg), followed by Ac-(D)Phe-Pro-boroArg-OH (0.154 μmol/kg) and then by hirudin (0.28 μmol/kg). Consistent with the rabbit jugular vein stasis thrombosis model, D-MePhe-Pro-Arg-H had the weakest effects in the

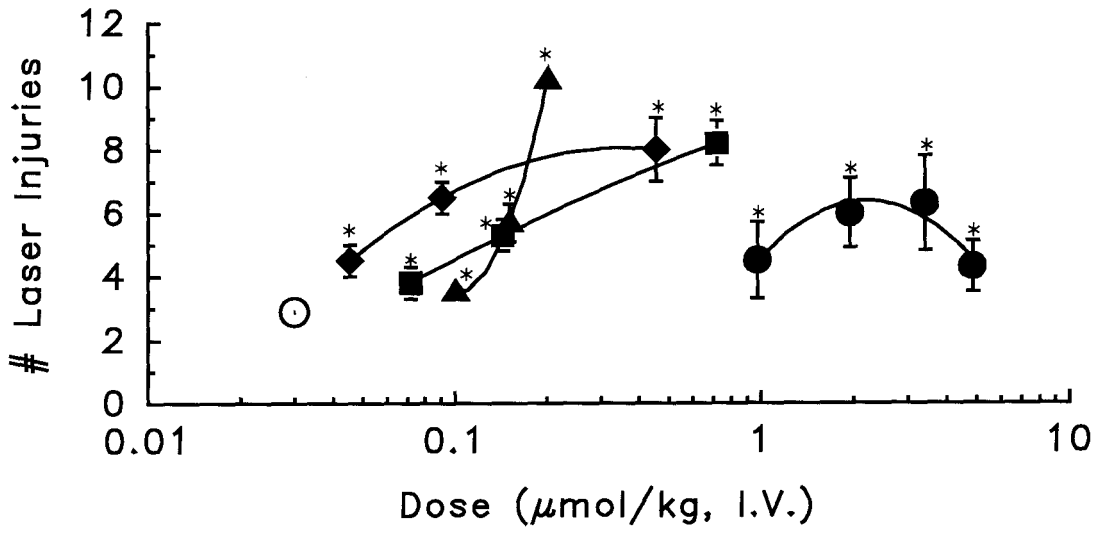


Fig. 55. Comparative dose-dependent antithrombotic effects of various agents as studied in the rat laser-induced thrombosis model. Each thrombin inhibitor was injected through the rat tail vein circulated for 5 minutes prior to initiation of the laser injury procedure. (●) D-MePhe-Pro-Arg-H, (▲) Ac-D-Phe-Pro-boroArg-OH, (■) hirudin, (◆) heparin. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (2.9 ± 0.1) as analyzed with ANOVA followed by the Student-Newman-Keuls test. The individual values for each thrombin inhibitor dose and their gravimetric equivalents are given in Tables 21a-21d.

rat laser-induced thrombosis model as well (2 μmol/kg). In this model, the ceiling for heparin and hirudin appeared to be around 8 laser exposures. However, in the case of Ac-(D)Phe-Pro-boroArg-OH, this ceiling was extended to about 10 laser shots. Furthermore, D-MePhe-Pro-Arg-H produced dose-dependent antithrombotic effects up to a dose of 3.4 μmol/kg, while with higher doses the antithrombotic effects were weaker. In the rats that received D-MePhe-Pro-Arg-H at doses higher than 3.4 μmol/kg, it was visually observed that the blood flow was reduced within 3-10 min after injection of the thrombin inhibitor. Similar observations were made in the rats that received Ac-(D)Phe-Pro-boroArg-OH at doses higher than 0.2 μmol/kg. These results were not reported, since the blood flow completely stopped prior to induction of laser injury. The number of laser exposures required to achieve the endpoint for each thrombin inhibitor at each dose are reported in Tables 21a - 21d.

Table 21 -- Comparative antithrombotic potencies of thrombin inhibitors, as determined in the rat model of laser-induced thrombosis in isolated mesenterial arterioles.

	Dose of each agent that results in increase of laser shots to 6 in [μmol/kg].
D-MePhe-Pro-Arg-H	2.0
Ac-D-Phe-Pro-boroArg-OH	0.15
Hirudin	0.28
Heparin	0.08

Each value represents a single determination from the respective dose-response curves depicted in Fig. 53. Saline, as the control, required 2.9 ± 0.1 laser shots for occlusive thrombus formation.

J. Hemorrhagic Effects of Thrombin Inhibitors as Studied in the Rabbit Ear Bleeding Model

Thrombin inhibitors can be potent antithrombotic agents, as seen in the previous results on the thrombosis models. While species-dependent, their antithrombotic actions are mediated mainly through their inhibitory actions against thrombin. However, the same process is involved in normal hemostasis, where the primary hemostatic plug is formed by activated platelets (which thrombin can activate) and reinforced by fibrin deposition (formed by the catalytic action of thrombin). Inhibition of these processes may lead to hemorrhage. To compare the hemorrhagic potential of thrombin inhibitors, D-MePhe-Pro-Arg-H, Ac-(D)Phe-Pro-boroArg-OH, hirudin and heparin were studied in a rabbit model of ear bleeding (Cade et al. 1984). The thrombin inhibitors were administered to rabbits at various doses and following varying circulation times, 5 standardized cuts were made into their ears. The blood released from the punctured sites for the ensuing 10 min was collected and measured. To compare the hemorrhagic potential of these agents, an index was devised, whereby the dose of the antithrombin agent that produced bleeding of $2 \cdot 10^9$ RBC/L was estimated as the BD_2. Six rabbits were tested at each thrombin inhibitor dose and circulation time studied.

1. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After I.V. Administration at Fixed Time Points

The dose-dependent hemorrhagic effects of thrombin inhibitors after I.V. injection, as assessed in the rabbit ear bleeding model are depicted in Fig. 56. In panel A, the hemorrhagic procedure was initiated 5 min after I.V. injection of the thrombin inhibitor, while in panel B, the hemorrhagic procedure was initiated following 20 min

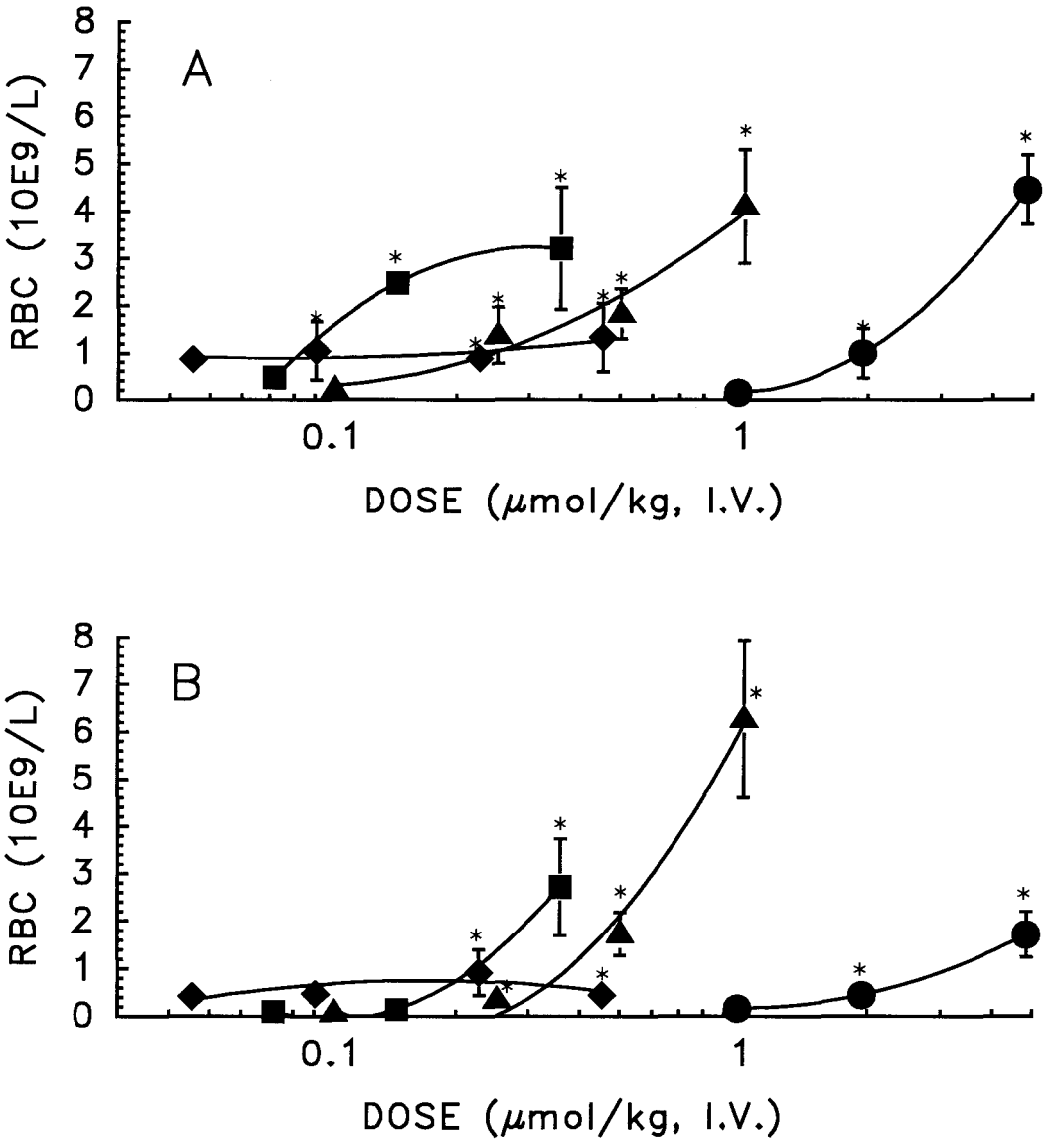


Fig. 56. Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model. In panel A, each agent was administered through I.V. injection and allowed to circulate for 5 minutes prior to initiation of the incision procedure. In panel B, each agent was administered through I.V. injection and circulated for 20 minutes prior to initiation of the incision procedure. (●) D-MePhe-Pro-Arg-H, (▲) Ac-D-Phe-Pro-boroArg-OH, (■) hirudin, (♦) heparin. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (0.100 ± 0.060) as analyzed with ANOVA followed by the Student-Newman-Keuls test. The individual values for each thrombin inhibitor dose and their gravimetric equivalents for both the 5 an 10 min post I.V. end points are given in Tables 22a-22d.

I.V. injection of the thrombin inhibitor. Except for heparin, the other thrombin inhibitors resulted in dose-dependent hemorrhagic effects. The agent with the strongest hemorrhagic effects was hirudin, 5 min post and 10 min post I.V. injection, with BD_2 of 0.11 and 0.30 μmol/kg respectively. The effects of hirudin were followed by Ac-(D)Phe-Pro-boroArg-OH (BD_2s of 0.55 and 0.54 μmol/kg after 5 and 20 min injection) and then by D-MePhe-Pro-Arg-H (BD_2s of 2.82 and >4.85 μmol/kg, after 5 and 20 min post injection). Heparin exhibited minimal hemorrhagic effects, as seen in both the 5 min and 20 min post I.V. injection panels (BD_2s of >0.45 μmol/kg in both cases). While the hemorrhagic effects of D-MePhe-Pro-Arg-H and hirudin were decreased 20 min post I.V. injection when compared to 5 min post I.V. injection, the effects of Ac-(D)Phe-Pro-boroArg-OH appeared to increase with time. The dose of each thrombin inhibitor that resulted in bleeding of $2 \cdot 10^9$ RBC/L, 5 min and 20 min post I.V. injection, is reported in Table 22. The amount of RBCs released at each thrombin inhibitor dose is reported in Tables 22a - 22d for the 5 min and 10 min post I.V. injection.

Table 22 -- Comparative hemorrhagic potencies of thrombin inhibitors after I.V. injection, as determined in the rabbit model of ear bleeding.

	Dose of each agent that resulted in bleeding of $2 \cdot 10^9$ RBC/L in [μmol/kg].	
	5 min post I.V injection	20 min post I.V. injection
D-MePhe-Pro-Arg-H	2.82	>4.85
Ac-D-Phe-Pro-boroArg-OH	0.55	0.54
Hirudin	0.11	0.30
Heparin	>0.45	>0.45

Each value represents a single determination from the respective dose-response curves depicted in Fig. 56. Saline produced bleeding of 0.100 ± 0.070 10^9 RBC/L.

2. Duration of Hemorrhagic Effects of Thrombin Inhibitors After I.V. Administration at a Fixed Dose

To determine the duration of the hemorrhagic effects of thrombin inhibitors, the dose of each agent that produced sub-maximal hemorrhagic effects in the above studies was selected and the hemorrhagic procedure was initiated at different times post I.V. injection of these agents. The individual doses for these studies were 4.85 μmol/kg for D-MePhe-Pro-Arg-H, 0.50 μmol/kg for Ac-(D)Phe-Pro-boroArg-OH, 0.36 μmol/kg for hirudin and 0.46 μmol/kg for heparin. The hemorrhagic process was initiated 5, 20, 30, 45, 60 and 90 min post I.V. injection of each thrombin inhibitor. Six rabbits were studied at each circulation time point.

The data of the comparative hemorrhagic effects are depicted in Fig. 57. All thrombin inhibitors exhibited time-dependent hemorrhagic profiles which generally diminished within 45 min post I.V. injection. D-MePhe-Pro-Arg-H lost its hemorrhagic effects at the fastest rate ($t_{1/2}$ around 15 min), while Ac-(D)Phe-Pro-boroArg-OH ($t_{1/2}$ around 35 min) and hirudin ($t_{1/2}$ around 25 min) showed a decrease in their hemorrhagic effects only after 20 min post I.V. injection. The individual data on the hemorrhagic effects of each antithrombin agent at each time point are found in Table 22e.

3. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After S.C. Administration at Fixed Time Points

The dose-dependent hemorrhagic effects of thrombin inhibitors after S.C. injection, as assessed in the rabbit ear bleeding model are depicted in Fig. 58. In panel A, the hemorrhagic procedure was initiated 45 min after S.C. injection of the thrombin inhibitor, while in panel B, the hemorrhagic procedure was initiated following 60 min

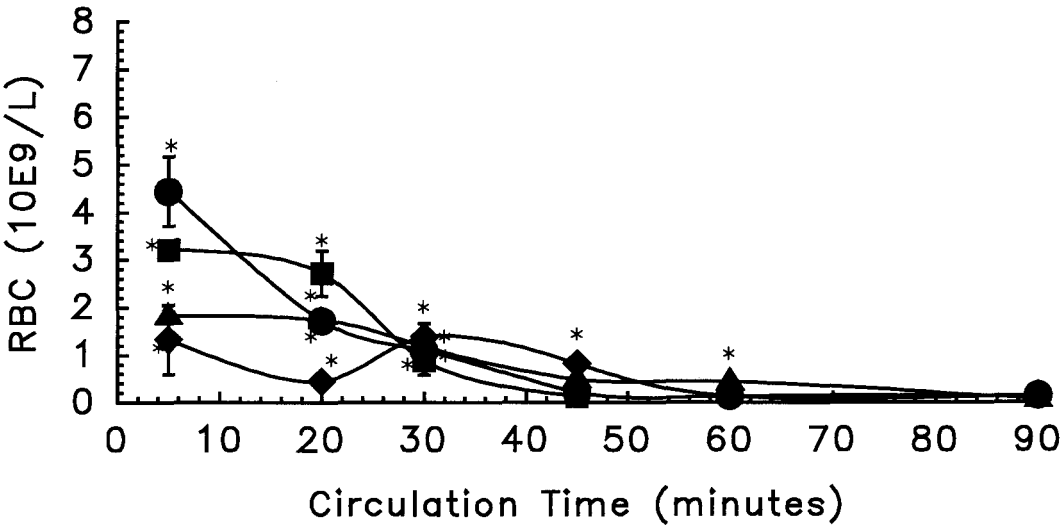


Fig. 57. Time dependance of hemorrhagic effects of antithrombin agents in the rabbit ear bleeding model. Each agent was administered through I.V. injection and allowed to circulate for varying time periods prior to initiation of the incision procedure. (●) D-MePhe-Pro-Arg-H at 4.85 μmol/kg, (▲) Ac-D-Phe-Pro-boroArg-OH at 0.50 μmol/kg, (■) hirudin at 0.36 μmol/kg, (◆) heparin at 0.46 μmol/kg. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (0.100 ± 0.060) as analyzed with ANOVA followed by Student-Newman-Keuls test. The individual values for each thrombin inhibitor at each time point are given in Table 22e.

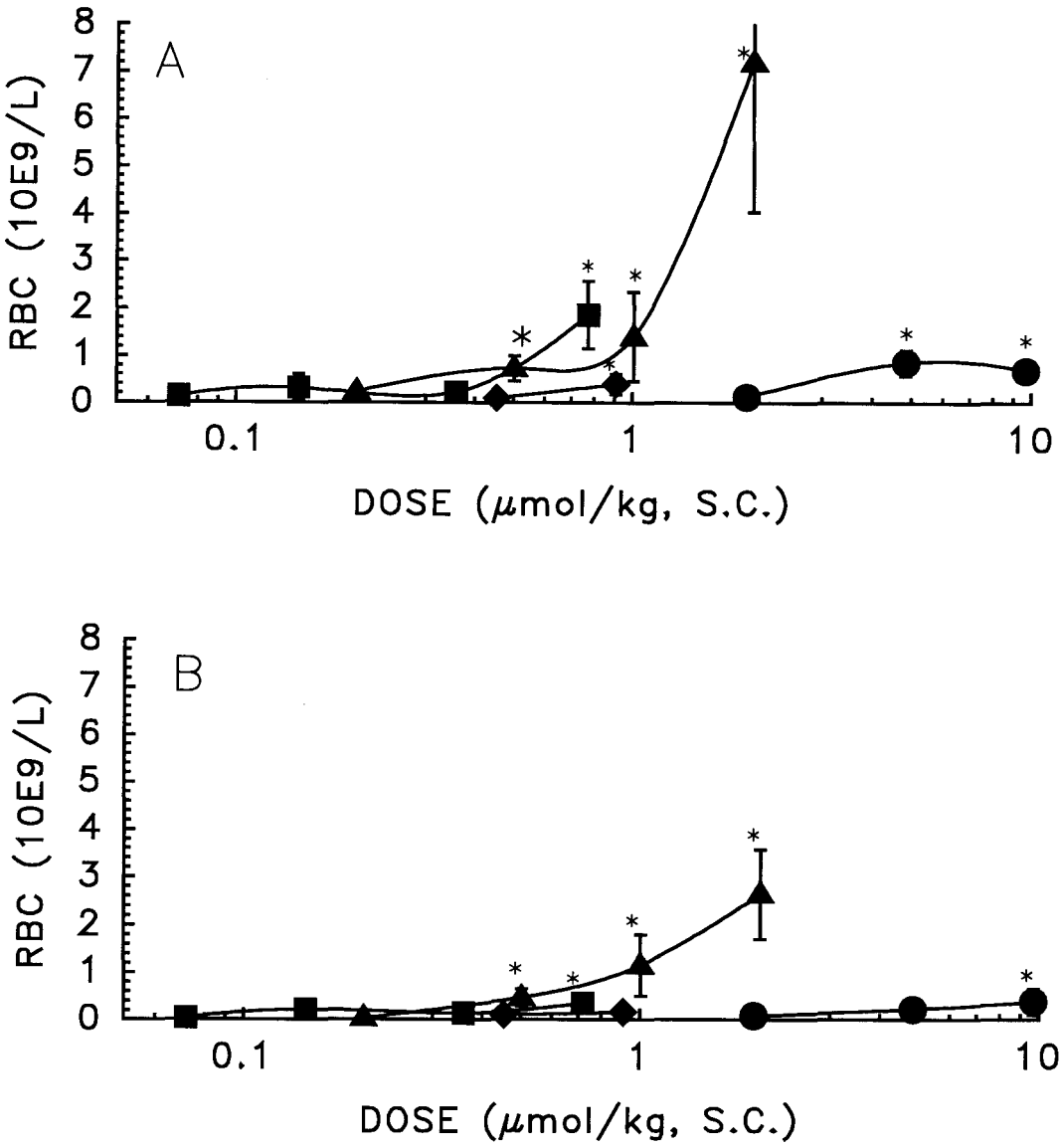


Fig. 58. Comparative dose-dependent hemorrhagic effects of various agents as studied in the rabbit ear bleeding model. In panel A, each agent was administered through S.C. injection and allowed to circulated for 45 minutes prior to initiation of the incision procedure. In panel B, each agent was administered through S.C. injection and circulated for 60 minutes prior to initiation of the incision procedure. (●) D-MePhe-Pro-Arg-H, (▲) Ac-D-Phe-Pro-boroArg-OH, (■) hirudin, (♦) heparin. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (0.100 ± 0.060) as analyzed with ANOVA followed by the Student-Newman-Keuls test. The individual values for each thrombin inhibitor dose and their gravimetric equivalents for both the 5 an 10 min post I.V. end points are given in Tables 23a-23d.

S.C. injection of the antithrombin agent. Similar to the hemorrhagic effects following I.V. administration, except for heparin, the other thrombin inhibitors resulted in dose-dependent hemorrhagic effects. As observed after I.V. injection, the agent with the strongest hemorrhagic effects 45 min post S.C. administration, was hirudin (BD_2 0.80 μmol/kg), followed by Ac-(D)Phe-Pro-boroArg-OH (BD_2 1.13 μmol/kg) and then by D-MePhe-Pro-Arg-H (BD_2 >9.7 μmol/kg). Heparin had minimal hemorrhagic effects, as seen in both the 45 min and 60 min post S.C. injection panels, with BD_2 of >0.9 μmol/kg in both cases. Except for Ac-(D)Phe-Pro-boroArg-OH, none of the other inhibitors appeared to have any hemorrhagic effects 60 min post S.C. injection, as seen in panel B of Fig. 58. However, the effects of Ac-(D)Phe-Pro-boroArg-OH were lower at 60 min post S.C. injection, when compared to the effects produced 45 min post S.C. injection. The dose of each thrombin inhibitor that resulted in bleeding of $2 \cdot 10^9$ RBC/L, 45 min and 60 min post S.C. injection, is reported in Table 23. The amount of RBCs released at each thrombin inhibitor dose is reported in Tables 23a - 23d, for the 45 min and 60 min post S.C. injection.

4. Duration of Hemorrhagic Effects of Thrombin Inhibitors After S.C. Administration at a Fixed Dose

To determine the duration of the hemorrhagic effects of thrombin inhibitors after S.C. injection, the dose of each agent that produced sub-maximal hemorrhagic effects in the above studies was selected and the hemorrhagic procedure was initiated at different times post S.C. injection of these agents. The dose of each thrombin inhibitor selected was: D-MePhe-Pro-Arg-H at 9.71 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 2.01

μmol/kg, hirudin at 0.77 μmol/kg and heparin at 0.91 μmol/kg. The hemorrhagic procedure was initiated 15, 30, 45, 60 and 90 min post S.C. injection of each thrombin inhibitor. Six rabbits were studied at each circulation time point.

Table 23 -- Comparative hemorrhagic potencies of thrombin inhibitors after S.C. injection, as determined in the rabbit model of ear bleeding.

	Dose of each agent that resulted in bleeding of $2 \cdot 10^9$ RBC/L in [μmol/kg].	
	45 min circulation	60 min circulation
D-MePhe-Pro-Arg-H	>9.71	>9.7
Ac-D-Phe-Pro-boroArg-OH	1.13	1.57
Hirudin	0.80	>0.7
Heparin	>0.91	>0.9

Each value represents a single determination from the respective dose-response curves depicted in Fig. 58. Saline produced bleeding of 0.100 ± 0.070 10^9 RBC/L.

The results of these studies are depicted in Fig. 59. Except for heparin, the other thrombin inhibitors had time-dependent hemorrhagic effects. For Ac-(D)Phe-Pro-boroArg-OH and hirudin, their hemorrhagic effects became maximal at 45 min post S.C. injection. While both thrombin inhibitors progressively lost their hemorrhagic activities after 45 min, the effects of hirudin were abolished at 60 min post S.C. injection. D-MePhe-Pro-Arg-H lost its hemorrhagic effects progressively with time, similar to its time-dependent hemorrhagic effects after I.V. injection. Heparin, as in all other hemorrhagic studies, had minimal effects at all time points. The hemorrhagic effects of each thrombin inhibitor at each time point are found in Table 23e.

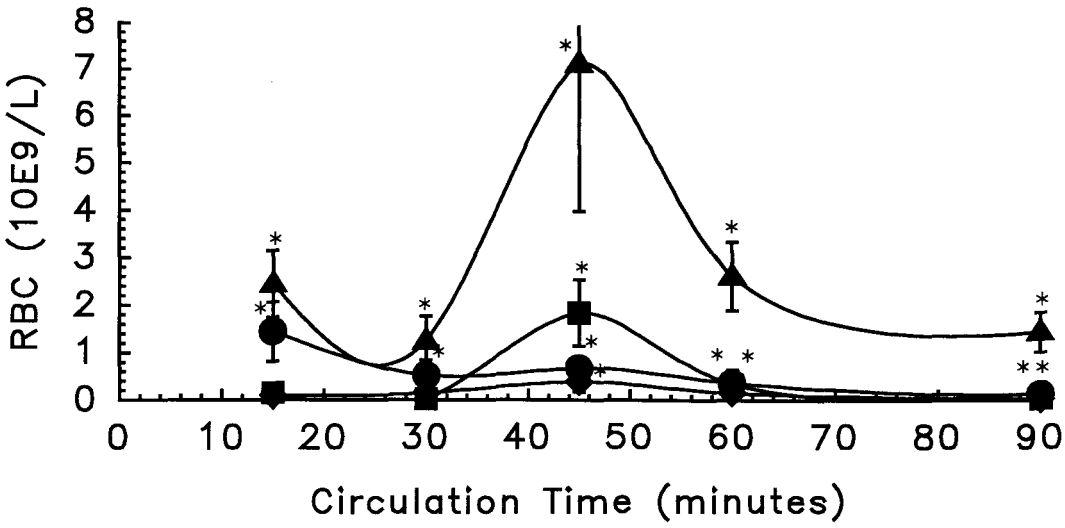


Fig. 59. Time dependance of hemorrhagic effects of thrombin inhibitors in the rabbit ear bleeding model. Each agent was administered through S.C. injection and allowed to circulate for varying time periods prior to initiation of the incision procedure. (●) D-MePhe-Pro-Arg-H at 9.71 μmol/kg, (▲) Ac-D-Phe-Pro-boroArg-OH at 2.01 μmol/kg, (■) hirudin at 0.77 μmol/kg, (♦) heparin at 0.91 μmol/kg. Each point represents a mean ± S.E.M. (n=6). * denotes statistical significance ($p < 0.05$) when compared to control (0.100 ± 0.060) as analyzed with ANOVA followed by the Student-Newman-Keuls test. The individual values for each thrombin inhibitor at each time point are given in Table 23e.

K. Effects of Thrombin Inhibitors on Blood Pressure After I.V. Administration in Rabbits

Thrombin inhibitors may mediate vascular effects independent of their antithrombin activities. To asses whether the thrombin inhibitors used in the rabbit model of ear blood loss had any effects on the blood pressure of rabbits, the blood pressure during the stasis thrombosis procedure was monitored for the highest dose of each thrombin used after I.V. injection (D-MePhe-Pro-Arg-H at 1.942 μmol/kg, Ac-(D)Phe-Pro-boroArg-OH at 0.101 μmol/kg, hirudin at μmol/kg and heparin at 0.023 μmol/kg).

The blood pressure monitoring of the rabbits during the stasis thrombosis procedure, in the studies where the dose-dependent effects of r-TF were also studied. These studies revealed a slight transient drop in the blood pressure lasting 5 $\pm$ 1 minutes after the first baseline blood draw and the subsequent infusion of saline (Fig. 60, panel A). Similar results were obtained when r-TF was used in place of saline, as seen in Fig. 60, panel B. The results obtained with each of the thrombin inhibitors (injected 5 min prior to the r-TF injection) were similar to those obtained with r-TF alone. Representative graphs from a rabbit treated with hirudin and Ac-(D)Phe-Pro-boroArg-OH are seen in Fig. 60, panels C and D respectively. Panel E shows the effect of D-MePhe-Pro-Arg-H on the blood pressure response. Consistent with the results obtained with the other agents, this peptide derivative did not produce any significant effects on the blood pressure.

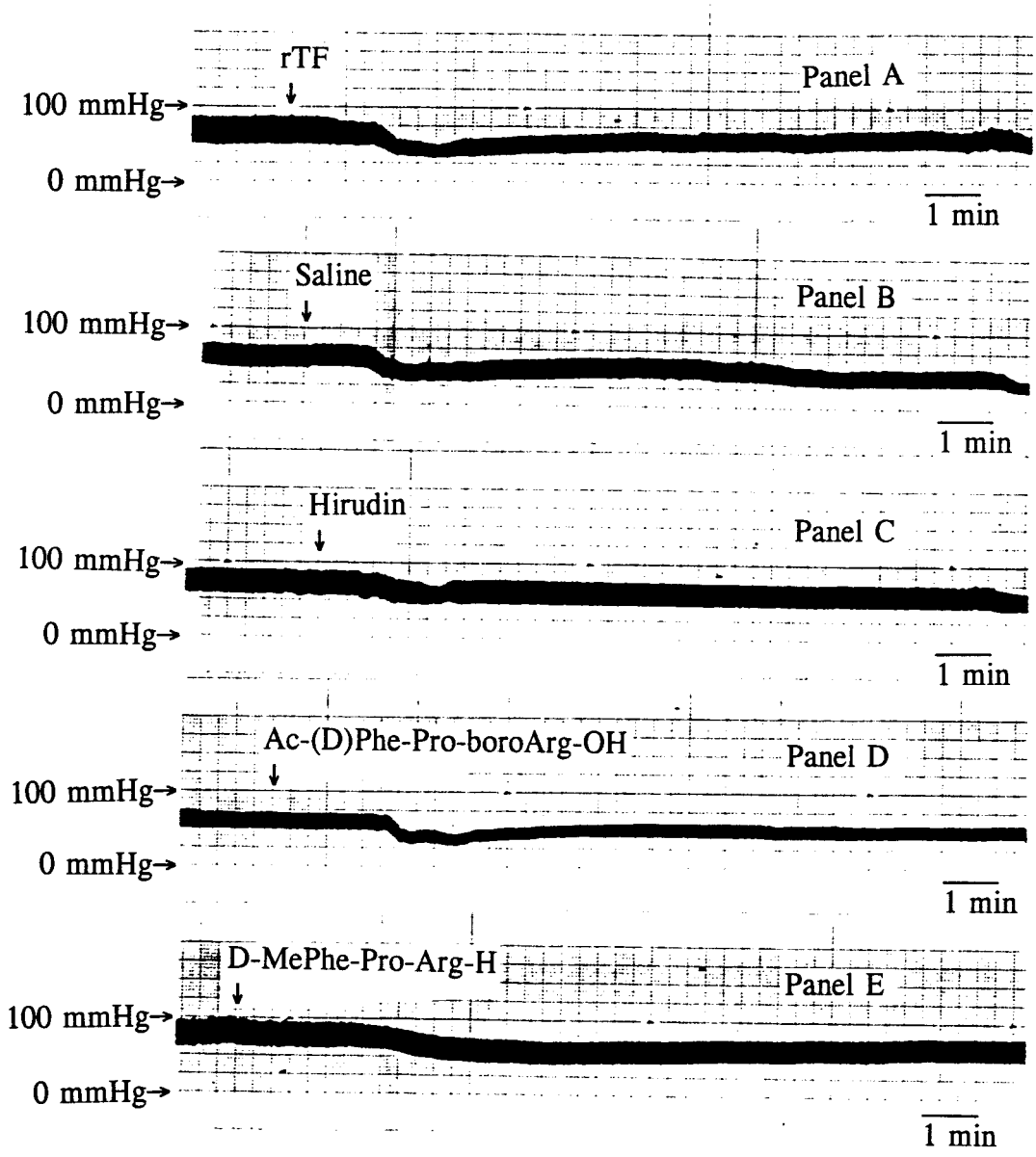


Fig. 60. Intra-arterial blood pressure monitoring of rabbits during *in vivo* investigation with rTF and antithrombotic agents. Five min following the I.V. injection of each agent, 9 ml of blood was collected via the carotid artery. Panel A depicts the effects of rTF at 1.35 pmol/kg, I.V. Panel B depicts the effects of saline. Panel C depicts the effects of hirudin at 0.04 μmol/kg, I.V. Panel D depicts the effects of Ac-(D)Phe-Pro-boroArg-OH at 0.1 μmol/kg, I.V. Panel E depicts the effects of D-MePhe-Pro-Arg-H at 1.9 μmol/kg, I.V.

CHAPTER V

DISCUSSION

This dissertation represents an integrated account of the pharmacological properties of several thrombin inhibitors which are not different only in their structural characteristics, but also exhibit varying degrees of specificity towards thrombin. The experimental approaches are designed to test the hypothesis that the inhibition of thrombin may not be the sole determinant of their endogenous antithrombotic and hemorrhagic actions. It is likely that several other endogenous factors are responsible for their pharmacologic effects. These effects include both the plasmatic and cellular sites. Furthermore, metabolic transformations of the individual thrombin inhibitors could also contribute to their endogenous pharmacodynamics.

In this dissertation, heparin has been used as a reference anticoagulant. However, unlike all of the antithrombin agents used, this drug produces its actions through potentiation of antithrombin and HC-II. Furthermore, this agent exhibits poly-pharmacologic behavior and targets several plasmatic and vascular sites (Lindahl et al. 1991, Jeske et al. 1995). On the other hand, the synthetic and recombinant thrombin inhibitors included in this study represent chemically pure homogeneous agents. A comparison of the biochemical and pharmacological characteristics of heparin and thrombin inhibitors is given in Table 24.

Table 24 -- Comparative properties of heparin and thrombin inhibitors.

	Heparin	Thrombin Inhibitors
Thrombin inhibition	Indirect	Direct
Clot-bound thrombin	No interaction	Inactivation
Inhibition of other serine proteases	Indirect, Xa and to a lesser extent XIIa, XIa, Xa and IXa.	Variable
Fibrinolytic interactions	Activation	Variable
Serpin interactions	Requires antithrombin, potentiates HC-II, releases TFPI	None
Platelet interactions	Inhibition	Inhibition only of thrombin-induced aggregation
Endothelial interactions	↑ vascular permeability, release of TFPI, binds endothelial lining	Unknown
Hemodynamic modulation	Activation	Unknown
Immunogenicity	HIT	Barely immunogenic
Bioavailability	20-30% S.C., <5% oral	80-100% S.C., 5-30% oral
Liver toxicity	↑ liver enzymes	None
Antagonists	Protamine, PF4, several serpins	None

Beside the antithrombin mediated inhibition of thrombin, heparin produces its overall antithrombotic effects through various mechanisms, while thrombin inhibitors are believed to act only through the inhibition of thrombin. Thus, while heparin is capable of inhibiting other coagulation factors via antithrombin and HC-II, antithrombin agents antagonize the final stages of coagulation. In addition, heparin interacts ionically with the vascular lining to mediate a series of antithrombotic and pro-fibrinolytic effects, such as release of tPA, TFPI and antithrombotic glycosaminoglycans from the endothelial lining (Verstraete and Zoldelhyi 1995). Heparin is also capable of inhibiting platelet functions, while antithrombin agents only inhibit thrombin-induced aggregation. On the other hand, most of the antithrombin agents are smaller in size and therefore are capable of inhibiting clot-bound thrombin, which may be important in clot stabilization and its subsequent lysis (Gast et al. 1994, Berry et al. 1994). The main reason for clinical interest in antithrombin agents as a potential replacement for heparin is due to the fact that heparin has certain limitations, which include wide subject-to-subject variability, poor response and allergic manifestations. One of the most serious adverse effects with heparin is heparin induced thrombocytopenia (HIT), a deleterious immune reaction where the patient's platelets aggregate in response to heparin to form white clots. Both heparin and the higher molecular weight thrombin inhibitors (hirudin) exhibit immunogenicity (Eichler and Greinacher 1996). The clinical implications of the anti-hirudin antibodies are not clear at this time. Another reason for evaluating alternative antithrombotic agents to heparin is the limited S.C. bioavailability of heparin. Heparin exhibits S.C. bio-availability of 20-30% whereas the thrombin inhibitors have 80-100% S.C.

bioavailability. The oral bioavailability of the thrombin inhibitors ranges from 5-30% whereas heparin has a rather limits absorption (<5%) by this route. Another major a problem that needs to be addressed for all direct thrombin inhibitors is their pharmacologic neutralization. While heparin is readily neutralizable by protamine, currently, there is no available pharmacologic antagonist for any of the direct thrombin inhibitors.

Thrombin inhibitors as a class of new anticoagulants represents compounds with significant structural variations. Recombinant hirudin represents a protein analog of natural hirudin with absolute specificity for thrombin. The peptide arginals represent low molecular weight peptide derivatives with strong antithrombin potency. However, they also exhibit varying degrees of inhibitory actions towards other serine proteases. The boronic acid tripeptide Ac-(D)Phe-Pro-boroArg-OH represents a transition state analogue with a high degree of reactivity and broad serine protease inhibitory spectrum. The peptidomimetic agent argatroban, represents a relatively weaker inhibitor of thrombin, with a high degree of specificity.

While the antiprotease spectrum of these agents has been extensively investigated, very little is known regarding their interactions with the cellular components of blood and vasculature. It is generally perceived that the antithrombin actions of these agents correlate directly with their *in vivo* pharmacologic effects. Theoretically, this hypothesis appears logical, however, experimental validation of this widely accepted concept is not available until now. The data reported in this dissertation represent the first comprehensive account where the different antithrombin agents are compared in defined

biochemical systems mimicking thrombogenesis and valid pharmacological models are utilized to investigate the relative antithrombotic and bleeding actions of these agents. This research provides an integrated account of the biochemical and pharmacologic actions of these agents, that may be useful in predicting the relative therapeutic index of these new anticoagulants.

Despite the extensive trials on the use of the new thrombin inhibitors in appropriately chosen clinical indications, supportive studies on their biochemical and pharmacologic influences of these agents have been rather limited. Due to the absence of this important fundamental information, both the safety and efficacy problems have been obvious during the newer clinical trials. The comparative studies reported in this dissertation address the following key questions:

1. Is the sole inhibition of thrombin sufficient to control thrombogenesis in various thrombotic states?
2. Are thrombin inhibitors similar to heparin in their pharmacologic actions?
3. Are there any differences among the currently available thrombin inhibitors?
4. Do thrombin inhibitors manifest non-thrombin mediated pharmacologic actions?

This dissertation therefore attempts to address some of the previously unresolved issues in carefully designed biochemical and pharmacologic studies. A direct comparison of thrombin inhibitors as a new class of drugs with conventional heparin provides an important database for the relative antithrombotic and hemorrhagic profiles of these drugs, which will be helpful in their clinical development.

A. Molecular and Structural Characterization of Heparin and Antithrombin agents

The peptide aldehyde D-Phe-Pro-Arg-H has been reported to be unstable in solution (Bajusz et al. 1990). The peptides D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H were developed to minimize cyclization and racemization. To determine the extent of cyclization and racemization in all of these agents, the HPLC studies were undertaken. All peptides resulted in elution profiles composed of more than one peaks. The identity of the structure which was represented by each one of the background-adjusted peaks was deciphered by isolating the material represented by each peak and then analyzing it with NMR techniques, which have been previously published (Bajusz et al. 1990). The HPLC studies revealed that almost 90% of the D-Phe-Pro-Arg-H, 95% of the D-MePhe-Pro-Arg-H and 100% of the Boc-D-Phe-Pro-Arg-H remained in the active form, in three equilibrium structures, after reconstitution in distilled H_2O.

The HPLC analysis of the boronic acid peptide derivative Ac-(D)Phe-Pro-boroArg-OH revealed that this compound eluted in several peaks. However, the identity of the structures represented by each one of these peaks is not known at this time. Furthermore, in contrast to the peptide arginals, the elution profile of this agent was rather complex. Because of the highly reactive nature of this agent, molecular interactions and other structural transformations may be responsible for this complex behavior. The boronic acid residue is highly reactive and the unusual HPLC elution profile may also be attributable to the interaction of this reactive peptide to the column.

The heparin preparation employed in these studies is a polycomponent mixture of glycosaminoglycans of different molecular weights. To determine the homogeneity

of this preparation of porcine origin, the GPC studies were carried out. The heparin preparation was examined by using two different detectors, the UV and the RI. The peaks resolved in the UV detector are generally due to the amount of sulfation and the presence of double bonds in the heparin. The peaks detected with the RI are due to the mass of the material studied. The results obtained from both detectors were in close agreement. The molecular weight average of this particular heparin preparation was determined to be approximately 10,700 and about half of the material was determined to be in the 8,000-12,000 range. While no analytical studies were carried out on the recombinant hirudin, this preparation was certified to be homogeneous and 95% pure by the manufacturer. It is apparent that the agents used in this investigation represent wide differences in their physicochemical and structural characteristics. While hirudin is a recombinant protein and the synthetic thrombin inhibitors represent structurally distinct peptidomimetics, heparin is a complex mixture of sulfated glycosaminoglycans. Therefore, molar conversions of the effects of heparin in these reported studies were based on the apparent molecular weight of heparin. In contrast, the molecular weights of all other agents were based on their formula molecular weights.

B. Relative Antithrombin Potencies of Thrombin Inhibitors

Thrombin inhibitors are being extensively investigated for a variety of clinical applications. As the number of thrombin inhibitors is increasing, there is a growing need for the development of a method for the potency standardization of these agents. Currently, outside the structural characterization using HPLC methods and the biochemical characterization of these agents measuring inhibitory constants for thrombin

(Bajusz et al. 1978, Bagdy et al. 1994, Kettner et al. 1990, Kikumoto et al. 1984, Braun et al. 1988), no other valid methods are available for the direct comparison of the antithrombin potency of these agents. Furthermore, a study where antithrombin potency for a specific antithrombin agent is directly compared with its pharmacologic actions is not available.

In the thrombin titration studies, where the amidolytic effect of thrombin on a chromogenic substrate was used to determine the inhibitory activities of antithrombin agents, an attempt was made to evaluate and compare various thrombin inhibitors in terms of ATU potencies. The order of potency of the thrombin inhibitors was found to be hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-OH > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban. With the exception of argatroban, the order of potencies of the other agents matched the order of their respective Ki values. Even though all of these thrombin inhibitors interact with thrombin on a 1:1 stoichiometric ratio, these agents form complexes with thrombin in kinetically distinct manners. More specifically, the tripeptide aldehydes (D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H) are slowly reversible thrombin inhibitors with Ki values of around 80 nM (Bajusz et al. 1978, Bagdy et al. 1994), Ac-(D)Phe-Pro-boroArg-OH is a transition state analogue with a Ki of 41 pM, which means a tight-binding and slowly reversible competitive thrombin inhibitor (Kettner et al. 1990), argatroban is a fast reversible antithrombin agent with a Ki of 39 nM (Kikumoto et al. 1984), while hirudin is a tight-binding inhibitor with a Ki of 21 fM (Stürzebecher and Walzman 1991). To overcome these kinetic differences, prior to the addition of the substrate, each of the thrombin

inhibitors was pre-incubated with thrombin, to achieve steady-state kinetics. Despite this pre-incubation period, the activity of argatroban in this amidolytic assay in relationship to the other thrombin inhibitors was not correlated with the order of their Ki values.

The amidolytic activity of thrombin is not predictive of the clotting activity of thrombin. The amidolytic activity of thrombin may be unaffected even if the clotting activity of this enzyme is diminished (Sonder and Fenton 1986). Furthermore, the affinity (K_m) and the specificity constant (k_{cat}/K_m) of thrombin for the chromogenic substrate used in this method ($51 \cdot 10^6$ $M^{-1}s^{-1}$) are different from those of the natural substrates for thrombin (Sonder and Fenton 1986), such as factor V ($3.26 \cdot 10^6$ $M^{-1}s^{-1}$, Monkovic and Tracy 1990), factor XI ($0.0026 \cdot 10^6$ $M^{-1}s^{-1}$, Gailiani and Broze 1991), factor XIII ($0.12 \cdot 10^6$ $M^{-1}s^{-1}$, Janus et al. 1983), protein C (Esmon et al. 1983) and fibrinogen ($3.8 \cdot 10^6$ $M^{-1}s^{-1}$ and $1.1 \cdot 10^6$ $M^{-1}s^{-1}$, for fibrinopeptides A and B, De Cristofaro and Di Cera 1992, Picozzi et al. 1994). Therefore, a competition between the chromogenic substrate and each of the thrombin inhibitors cannot be readily related to and be predictive of pathophysiologic events.

To determine the relative antithrombin effects of these inhibitory agents utilizing the functional property of thrombin to cleave fibrinogen into fibrin, the fibrinogen-based clotting systems were developed. In these assays, the order of potency of the antithrombin agents was found to be hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > argatroban > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H. As with their respective Ki values, hirudin and Ac-(D)Phe-Pro-boroArg-OH were the strongest thrombin inhibitors in this test, while Boc-D-Phe-Pro-Arg-H was the weakest. However,

the order of potencies of D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and argatroban were of a different sequence in this assay than the order of their Ki values. Furthermore, argatroban exhibited more potent activities in relationship to the rest of the antithrombin agents in this clot-based system, unlike its weak effects in the amidolytic systems.

As published previously (Iyer and Fareed, 1995), the amidolytic thrombin titration assay is a rapid and reproducible method that can be used for the *in vitro* potency evaluation and quality assurance purposes for a specific thrombin inhibitor. However, the value of this method in screening all new thrombin inhibitors for their anticoagulant and antithrombotic potential may be limited and can be applied only to the agents that are known to be tight, slowly reversible thrombin inhibitors. In this method, only the IC_{50} values are comparable and doubling of this value may not give the correct number for 100% thrombin inactivation (Dr. J. Fenton II and Ms. Brezniak, New York State Department of Health, personal communication). The example of argatroban illustrates this point: argatroban is found to be 100 fold weaker than hirudin (on a molar basis) in this assay. However, in the fibrinogen-based method, where the end-point is clot formation and is also a rapid and reliable screening method for antithrombin activities of agents, argatroban exhibited comparable antithrombin potencies to those of D-MePhe-Pro-Arg-H and D-Phe-Pro-Arg-H. Furthermore, argatroban has been used clinically for a variety of indications in Japan (Kario et al. 1995) and is in clinical trials in both Europe and the U.S., with promising efficacy and beneficial outcomes. Thus, the *in vitro* potency evaluation of these agents may or may not be relevant to the *in vivo* actions. This chromogenic method may be useful in the comparison of various batches

of the same thrombin inhibitor, or even in the comparison of different thrombin inhibitors with the exact mechanism of thrombin inhibition. The fibrinogen-based method may be more relevant in comparing thrombin inhibitors that differ in their mechanisms of actions. As demonstrated recently by Bagdy et al. 1994, it is important to utilize a battery of several different methods to assess relative potencies of thrombin inhibitors, since it appears that different methods may be more suitable for evaluating one agent in comparison with other thrombin inhibitors.

C. Antithrombin and Anti-Xa Activities of Thrombin Inhibitors in Plasmatic Systems

While defined assay systems, such as the amidolytic tests discussed earlier, can provide useful information on the relative antithrombin potency of various agents, these assays are based on buffered conditions where thrombin is the only enzyme present. To examine the matrix effect that plasma has on the antithrombin activities of thrombin inhibitors, the human plasma-based amidolytic antithrombin assay was utilized. The order of potency of the antithrombin agents was found to be hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban. In this system, heparin was also studied and was found to be an even more potent antithrombin agent than hirudin. The effects of heparin in this system are mediated through the antithrombin contained within the plasma and therefore the antithrombin effects exhibited by this drug are a reflection of the potentiation of antithrombin and not of direct effects of heparin on thrombin. Since the previously discussed thrombin titration systems are devoid of antithrombin, heparin had no

antithrombin effect and comparisons with the other thrombin inhibitors were not possible.

When rabbit plasma was used instead of human plasma in the same assay, heparin was again found to be the strongest thrombin inhibitor. However, the order of potencies of hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H were reversed, although very close in absolute value. This difference in the order of potency in the human and rabbit plasma systems is probably a reflection of the differences in the plasma matrices and the concentrations of various factors. Rabbit plasma contains higher concentrations than human plasma of most coagulation factors, with the exception of prekallikrein, factor X, prothrombin and fibrinogen (Walenga 1987). In addition, rabbit plasma contains about the same amount of antithrombin as human plasma (Walenga 1987). Since rabbit plasma contains 18 fold higher concentrations of factor V than human plasma, which serves as a cofactor for factor Xa, and since addition of thrombin to plasma activates the coagulation cascade via multiple feedback mechanisms, it may be that direct inhibition of factor Xa in the rabbit plasma-based assay by such agents as Ac-(D)Phe-Pro-boroArg-OH may be more important in the generation and subsequent inhibition of generated thrombin than in the human plasma-based system. On the other hand, in the human plasma-based test, the direct inhibition of the added thrombin may mask any effects that thrombin inhibitors may have on the generation of thrombin from the endogenous factors by inhibiting other coagulation factors. Thus, the inhibitory constants for thrombin, as represented by the reported Ki values, may be more relevant in predicting the antithrombin potency of these agents.

An automated method for the determination of hirudin in plasma has been recently

published (Hafner et al. 1995). This method is also based on the amidolytic activity of thrombin on a chromogenic substrate. However, in this method bovine thrombin is used instead of human enzyme as is in the studies described in this dissertation. This may also influence the comparative effects of various thrombin inhibitors on the amidolytic activity of this thrombin. Furthermore, the chromogenic substrate is added to the plasma before thrombin, therefore decreasing the possibility that low reaction velocity thrombin inhibitors, such as heparin/antithrombin complex, will have an effect on this assay. The comparative effects of hirudin in the chromogenic assay and the APTT results reported by Hafner et al. 1996 are in agreement with the results reported in this dissertation, where no correlation was found between the antithrombin amidolytic activities of various thrombin inhibitors and their effects in the APTT assay.

To examine the specificity of the antithrombin agents compared in these studies for thrombin, the amidolytic anti-Xa assays were also utilized. These assays were plasma-based systems and it was found that with the exception of heparin and Ac-(D)Phe-Pro-boroArg-OH, the other thrombin inhibitors were devoid of anti-Xa activity. As expected, heparin exhibited measurable anti-Xa activity in this assay which is mediated by plasmatic antithrombin. Antithrombin also inhibits most of the factors in the coagulation cascade, especially thrombin and factor Xa.

The effects of Ac-(D)Phe-Pro-boroArg-OH validate the observation that this compound is not a specific thrombin inhibitor, even though its potency for factor Xa inhibition is lower than that of heparin. When the same inhibitors were studied in the rabbit plasma-based assay, similar results were obtained: except for heparin and Ac-

(D)Phe-Pro-boroArg-OH, none of the other agents had any anti-Xa effects. Even though heparin was again found to be a more potent factor Xa inhibitor than Ac-(D)Phe-Pro-boroArg-OH, its anti-Xa potency in the human plasma in comparison to the rabbit plasma was 10 fold stronger, while Ac-(D)Phe-Pro-boroArg-OH's potency was only 3 fold stronger in the human plasma than in the rabbit plasma. This difference in the ratios is most likely reflective of the limiting factor in heparin's activity, namely the antithrombin concentration in each plasma system in relation to the factor Xa/factor Va levels.

Another plasma-based assay that provides data on the antithrombin and anti-Xa effects of various agents is the Heptest, which is a clot-based method, unlike the previously discussed plasma-based assays, which are amidolytic methods. In this assay, activated factor Xa is added to the plasma, which results in direct activation of the common pathway and generation of thrombin. The order of potency of thrombin inhibitors in the human plasma-based Heptest assay was heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > argatroban > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H. With the exception of heparin, and the change in the rank order of argatroban and D-MePhe-Pro-Arg-H, this potency order closely followed that obtained in the fibrinogen-based antithrombin assay. In addition, the order of potency of the antithrombin agents in the Heptest closely followed the order of their respective Ki values. When rabbit plasma was used instead of human plasma in the Heptest assay, hirudin was found to be more potent than heparin. This difference is probably due to the difference in antithrombin concentration in relation to factor Xa/factor Va concentration in the two plasmas, which limits the heparin antithrombin and anti-Xa activity.

In all of the systems discussed earlier, argatroban was found to be a weak inhibitor in the amidolytic assays, while in the clotting assays its activity appeared to be more potent in relation to the tripeptide aldehydes, corresponding to their respective Ki values. Since argatroban is the most reversible of all thrombin inhibitors examined, this indicates that an agent's antithrombin potency as measured by a Ki value rather than its amidolytic potential may be more predictive of its anticoagulant effects.

D. Antiprotease Spectrum of Various Thrombin Inhibitors

The objective of adjunctive therapy of anticoagulants with fibrinolytics is to simultaneously control thrombogenesis and facilitate thrombolysis. However, the specific agents which are to be combined should be carefully selected. Many of the new antithrombin agents target the catalytic site of thrombin, which is similar in many blood-borne enzymes, including most of the enzymes involved in coagulation and fibrinolysis. Thus, it is possible that some of these catalytic site directed thrombin inhibitors may inhibit other enzymes, involved in both coagulation and fibrinolysis, regardless of their relative affinity for thrombin. In addition, highly specific thrombin inhibitors without additional antiprotease activities, such as hirudin, may also impair physiological fibrinolysis by inactivating thrombomodulin-bound thrombin. Since thrombomodulin-bound thrombin has an anticoagulant action and no procoagulant activities, by activating protein C, which in turn inhibits the coagulation cascade by inactivating cofactors Va and VIIIa and promotes the fibrinolytic system by inactivating one of its inhibitors (PAI-1), inhibition of this bound thrombin would compromise the endogenous fibrinolytic system.

These experimental studies, where the inhibitory effects of various thrombin

agents on the amidolytic activities of purified enzymes were studied and designed to assess the inhibitory range of several thrombin inhibitors on pro-fibrinolytic enzymes. In the defined enzyme systems, it was evident that Ac-D-Phe-Pro-boroArg-OH was the thrombin inhibitor with the widest and strongest range of antiprotease actions. Boc-D-Phe-Pro-Arg-H also had a wide range of antiprotease activities, although these were achieved at higher concentrations (0.24 - 15 μM). D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H did not affect kallikrein and urokinase but did inhibit the other enzyme systems at concentrations comparable to those of Ac-D-Phe-Pro-boroArg-OH or Boc-D-Phe-Pro-Arg-H. In contrast to these tripeptide derived thrombin inhibitors, argatroban, a peptidomimetic of comparable mass, hirudin and heparin/antithrombin had minimal activities on the enzymes systems examined. Heparin was used in conjunction with antithrombin, since heparin mediates its inhibitory activities through potentiation of antithrombin. Aprotinin, an anti-fibrinolytic agent, followed the inhibitory profile of Ac-(D)Phe-Pro-boroArg-OH at equivalent concentrations. This agent however is devoid of antithrombin activity at this concentration range. It should be noted that the inhibition of these purified enzymes by thrombin inhibitors is competitive utilizing a substrate which is different than the natural ones for each enzyme, e.g. the natural substrates of APC are factors Va and VIIIa and their Ki values are different from that of the chromogenic substrate used in the amidolytic assay. Therefore, the *in vivo* and clinical relevance of these data is questionable and will depend among other factors on the local concentrations of the antithrombin agent, the endogenous and exogenous enzymes and the natural substrates. A valid approach to demonstrate the inhibitory effects of thrombin

inhibitors on APC is to utilize an animal model in which the regulatory physiologic effects of this anticoagulant protein can be assessed (≤ 100 μM). Nevertheless, these results suggest that agents such as the tripeptide derivatives may exhibit direct interactions with these enzymes and they may show varying degrees of compatibility with fibrinolytic agents since they can directly interact and compromise these actions. Agents such as argatroban, hirudin and heparin that lack direct inhibitory activities against the fibrinolytic enzymes, are expected to behave differently when administered with thrombolytic agents.

The relationship of these *in vitro* non-thrombin effects of the antithrombin agents to the *in vivo* process of fibrinolysis was examined in a rabbit model of jugular vein thrombolysis (Callas et al. 1995). In contrast to most animal models of thrombolysis used by other investigators where the clots formed are platelet-rich and are designed to detect thrombolytic facilitation, the clots formed and lysed in this model are fibrin-rich clots and the model is designed to detect thrombolytic compromise. The studies on the effects of thrombin inhibitors on this thrombolytic model confirm the results from the *in vitro* studies. The antithrombin agent with the broadest and strongest spectrum of activity against the fibrinolytic enzymes, Ac-D-Phe-Pro-boroArg-OH, also compromised thrombolysis the most. D-MePhe-Pro-Arg-H and aprotinin also had marked anti-fibrinolytic activities in this animal model. In contrast, argatroban, hirudin and heparin had minimal effects, correlating with their narrow spectrum of activity. Interestingly, the last three agents did have some activity, which may be due to inhibition of the thrombomodulin-bound thrombin. Although the doses used in this rabbit model of

jugular clot lysis were higher than those required for antithrombotic effects of these thrombin inhibitors, these levels may be relevant to those achieved clinically in localized delivery in procedures such as coronary thrombolysis. Preliminary results of a clinical trial where patients with acute myocardial infarction were administered adjunctive therapy of streptokinase and hirulog-I indicate that lower doses of hirulog-I are more beneficial than higher concentrations (Théroux 1995). This unexpected effect may be attributed to inhibition of thrombomodulin-bound thrombin at higher doses of hirulog-I, so that inhibition of the activation of protein C and subsequent compromise of the physiological anticoagulant and fibrinolytic system is strong enough to manifest a clinical impact.

Several investigators have published experimental studies on the interactions of various thrombin inhibitors with thrombolytic processes. A summary of these studies is given in the last section of the literature review. The difference between the previous studies and those reported in this dissertation is that these assays were developed so that only inhibitory effects on pro-fibrinolytic enzymes were detected, while any potential facilitating activities were not accountable.

Currently there are several thrombin inhibitors in clinical development as anticoagulants for the management of vascular and cardiovascular disorders. Hirudin, hirulog-I, efegatran are being developed for the management of abrupt closure during coronary angioplasty. The on-site concentrated delivery of these agents has also been advocated. In such clinical applications a direct fibrinolytic compromise by the inhibition of tPA and plasmin may seriously impair thrombolytic processes. An indirect fibrinolytic deficit due to the inhibition of thrombin's regulatory effect on activated protein C

formation can also lead to impaired fibrinolysis. Thus, prophylactic antithrombotic use of newly developed antithrombin drugs may also lead to an impairment of physiologic fibrinolysis which can manifest itself into various forms of thrombotic syndromes. Therefore, it is important to determine the relative fibrinolytic compromising actions of these agents to achieve optimal clinical results.

E. Inhibitory Actions of Thrombin Inhibitors in Protease Generating Systems

While in the previously discussed assays, the direct inhibitory activities of various agents on serine proteases was examined, the effects of the same agents on more complex assays, where activation of one factor leads to the generation of another was studied in a series of appropriately designed assays. Three different assays were used to study different aspects of coagulation and fibrinolysis:

1. Extrinsic generation systems, where the coagulation cascade was activated with thromboplastin and the source of clotting factors ranged from fibrinogen-deficient plasma to prothrombin complexes.
2. Intrinsic generation systems, where coagulation was activated by a contact activator and the source of clotting factors was fibrinogen-deficient plasma.
3. Plasminogenolysis systems, where streptokinase or tPA mediated plasmin generation from either human plasma derived or purified plasminogen.

In the first two types of assays, the detection of generation of both factor Xa and thrombin was possible. Thus, data was obtained on the importance of inhibition of thrombin versus factor Xa and the impact of these inhibitions on the further amplification of each system.

1. Extrinsic Activation Systems

In the fibrinogen deficient plasma-based assay where thrombin and factor Xa are generated after extrinsic activation, the order of potency of the various thrombin inhibitors studied varied depending on whether thrombin or factor Xa were detected. Thus, with regards to thrombin, the order of potency was hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban. Hirudin however, plateaued at about 80% inhibition of thrombin generation, while the other antithrombin agents were able to completely block this process, with the exception of argatroban. When considering the factor Xa generated in the same system, the order of potency of the various agents changed to heparin > Ac-(D)Phe-Pro-boroArg-OH > Boc-D-Phe-Pro-Arg-H, D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H. Hirudin and argatroban had minimal inhibitory activities in the generation of factor Xa, never exceeding about 20% inhibition. Considering the hirudin results and knowing that hirudin inhibits only thrombin, it seems that the feedback activation of factor Xa by the generated thrombin accounts for about 20% of the generated factor Xa in this system, or the amount that hirudin is able to block through thrombin inhibition. Alternatively, hirudin maximally inhibits 80% of the generated thrombin, which indicates that the remaining 20% of thrombin is responsible for 80% of factor Xa generation, after extrinsic activation. These explanations on the differential activities of hirudin do not contradict the results obtained with heparin and Ac-(D)Phe-Pro-boroArg-OH, which are capable of completely inhibiting the generation of both thrombin and factor Xa, probably due in part to their ability to directly inhibit factor Xa. However, when considering the

activities of the tripeptide aldehydes, it is difficult to understand why these thrombin inhibitors, unlike hirudin, are capable of completely blocking both thrombin and factor Xa generation. The differences in the kinetics of thrombin inhibition may be accountable for these differences from hirudin, so that if hirudin binds thrombin relatively slower as it is generated, enough thrombin "escapes" to perpetuate the feedback amplifications and further generation of thrombin. In addition, while the tripeptide aldehydes do not inhibit factor Xa, they may have inhibitory activities on other coagulation factors in addition to thrombin, since as seen in the pro-fibrinolytic enzyme assay studies, the tripeptide aldehydes are capable of inhibiting other serine proteases to varying degrees. These explanations are in accordance with the actions of argatroban, the other monospecific thrombin inhibitor which, like hirudin, is not capable of completely inhibiting the generation of either thrombin or factor Xa. The comparative effects of heparin and hirudin utilizing this system have been previously published (Kaiser et al. 1992) and are in agreement with the results described in this dissertation.

In the fibrinogen deficient plasma assays, the intrinsic pathway is also activated at some point via the feedback actions of thrombin. However, in the KONYNE® and FEIBA® based tests, only factors involved in the extrinsic and common pathways of coagulation are present. In these systems, heparin exhibited markedly weaker inhibitory activities in relation to the other thrombin inhibitors, in contrast to its profile in the fibrinogen-deficient plasma-based assays. This difference in the actions of heparin are probably due to the absence of antithrombin from the KONYNE® and FEIBA® preparations. Trace amounts of antithrombin or HC-II must exist however, since heparin

has some effects. The comparative effects of hirudin and heparin and Ac-(D)Phe-Pro-boroArg-OH in the KONYNE® system have been recently published (Kaiser et al. 1994). In this publication, addition of antithrombin is shown to potentiate the inhibitory activities of heparin. However, addition of factor V does not influence the inhibitory activities of any of the three studied agents, indicating that the factor V concentration in the KONYNE® preparation and consequently in this system, is not a rate limiting a factor.

The difference between the KONYNE® and FEIBA® preparations is that FEIBA® contains factor VIIa in addition to factors II, VII, IX and X. In the KONYNE® based assays, Ac-(D)Phe-Pro-boroArg-OH was the only inhibitor that was capable of completely inhibiting the generation thrombin and factor Xa, while the other antithrombin agents exhibited activities similar to those of hirudin in the fibrinogen deficient plasma-based systems. The difference in activity between Ac-(D)Phe-Pro-boroArg-OH and the other agents is probably due to the ability of Ac-(D)Phe-Pro-boroArg-OH to directly inhibit both thrombin and factor Xa. Furthermore, since the tripeptide aldehydes also lose their ability to inhibit the generation of both thrombin and factor Xa in this assay, in contrast to their activities in the fibrinogen deficient plasma systems, the previous hypothesis that these aldehydes may exert their inhibitory effects via inhibition of other coagulation factors can be narrowed to the intrinsic coagulation factors.

In the FEIBA® based assay, while Ac-(D)Phe-Pro-boroArg-OH remains a potent inhibitor of both the thrombin and factor Xa generation, hirudin is also a potent inhibitor of this generation test. In this assay, hirudin is capable of almost completely blocking the generation of both thrombin and factor Xa, in contrast to the previous systems.

These results suggest that the relative speed or the activation status of coagulation at which thrombin is generated is important in the activity of thrombin inhibitors. The results obtained from the KONYNE® and FEIBA® assays are difficult to interpret in light of the fact that thrombin and factor Xa are generated even in the absence of cofactors VIII and V, according to the specification sheets of the products. This indicates that either both products or thromboplastin which is added to extrinsically activate the coagulation system retain trace amounts of these cofactors.

2. Intrinsic Activation Systems

To examine the effects of antithrombin agents on the inhibition of the intrinsic generation of thrombin and factor Xa, the fibrinogen deficient plasma was activated with a contact activator and the generated thrombin and factor Xa were detected with chromogenic substrates. With the exception of heparin, all antithrombin agents inhibited the intrinsic generation of thrombin in a manner parallel to their inhibitory profiles in the extrinsic generation of thrombin and similar explanations for their activities can be offered. However, in contrast to its extrinsic thrombin activation profile, the activities of heparin plateaued around 80% inhibition of intrinsic thrombin generation. Similarly, argatroban and hirudin also produced comparable results to heparin in both systems. The difference in the activities of heparin is probably due to the differences in the inhibition of various clotting factors by antithrombin, through which heparin exerts its actions. When examining the effects of antithrombin agents on the generation of factor Xa in the same system, all agents including heparin, hirudin and argatroban, were capable of completely inhibiting the generation of factor Xa. Thus, it appears that none of the

factor Xa generated is a result of feedback activation through thrombin. These results are in agreement with the comparative effects of hirudin and heparin in these systems reported in a previous publication (Kaiser et al. 1992).

3. Plasminogenolysis Systems

Since various antithrombin agents were found to inhibit various fibrinolytic enzymes, to assess their effects on the process of activation of plasminogen, the *in vitro* plasminogenolysis experiments were carried out. Activation of plasminogen to plasmin was also inhibited to various degrees by the different agents, with the activities of hirudin and heparin being always minimal. The inhibitory profile of the thrombin inhibitors remained the same regardless of activators, streptokinase and tPA, or source of plasminogen, purified or human plasma. Therefore, the inhibitory effects of the antithrombin agents on these processes are probably due to direct inhibition of plasmin, while direct inhibition of the plasminogen activators may also contribute to the overall inhibitory effects of each agent in this system. These results are in agreement with the published effects of thrombin inhibitors on fibrinolytic enzymes (Barabas et al. 1993), where it was shown that the tripeptide aldehydes D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H, produced concentration-dependent antifibrinolytic effects in the fibrin plate assay and the TEG. However, according to these published studies, while these thrombin inhibitors produce clear inhibitory effects on TEG measured fibrinolysis, they also decrease the elasticity of the clots formed, which is suggested to be a pro-fibrinolytic effect. The results of these studies reinforce the importance of the specificity of antithrombin agents for thrombin.

F. Global Anticoagulant Effects of Thrombin Inhibitors: PT, APTT, ACT and TEG

The information provided by the antiprotease and protease generation assays is valuable in understanding the mechanism of action of each agent in more global settings, such as in plasma and whole blood clotting processes. The PT and APTT are the plasmatic clot-based assays that are the equivalents of the extrinsic and intrinsic fibrinogen deficient plasma amidolytic-based assays respectively.

In the PT assay, heparin has no anticoagulant effects in contrast to its effects in the extrinsic generation system. The differences in the plasma dilutions and the concentrations of the activator (thromboplastin) in the two systems may be responsible for this difference in heparin's action. With the exception of heparin and hirudin, the order of potency of the anticoagulant effects of the other antithrombin agents was the same as in the extrinsic thrombin generation system and similar to their Ki value rank order: hirudin > Ac-(D)Phe-Pro-boroArg-OH > argatroban > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H. The observations on the effects of argatroban reinforce the concept that the effects of an agent on the clotting activity of thrombin correlate better with its Ki value than its effects on the amidolytic activity of thrombin.

In the APTT assay, the order of potency of the antithrombin agents was found to be heparin > Ac-(D)Phe-Pro-boroArg-OH > hirudin > argatroban > Boc-D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H. This rank order does not correspond to the one obtained from the intrinsic thrombin generation system, or to any other obtained from the rest of the assays. This is the only test where Ac-(D)Phe-Pro-boroArg-OH was

found to be a more potent anticoagulant than hirudin. However, heparin was the most effective anticoagulant overall in this assay, as it was in the Heptest and the intrinsic and extrinsic generation of factor Xa in the fibrinogen deficient plasma-based assays. This is probably reflective of the importance of generation of factor Xa in the coagulation pathway after intrinsic activation, which is the rate limiting step in the generation of thrombin.

Species differences between human and rabbit plasma affect the anticoagulant activities of thrombin inhibitors, as seen when studying the effects of heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H in the rabbit plasma-based PT assay. In this assay, while hirudin was a stronger anticoagulant than D-MePhe-Pro-Arg-H and heparin exhibited no anticoagulant effects, Ac-(D)Phe-Pro-boroArg-OH also exhibited no anticoagulant effects, in contrast to its activities in the human plasma-based PT. However, when examining the anticoagulant effects of the same inhibitors in the rabbit plasma-based APTT, the rank order of anticoagulant potencies of these agents was the same as in the human plasma-based APTT.

The ACT and TEG assays provide tools for studying the anticoagulant effects of thrombin inhibitors in whole blood, so that clot formation is the composite result of the intrinsic activation of the coagulation cascade and its interactions with other blood components. When heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH, D-MePhe-Pro-Arg-H and argatroban were compared in these assays, both of these tests provided comparable results. In both systems, hirudin, heparin and Ac-(D)Phe-Pro-boroArg-OH appeared to be stronger anticoagulants than D-MePhe-Pro-Arg-H and argatroban. However, the TEG

assay appeared to be more sensitive to the anticoagulant effects of these agents, in that their differences in the extent of anticoagulation were more pronounced. Similar results were obtained when heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H were compared in rabbit whole blood, where again the TEG assay appeared to be more sensitive to the anticoagulant effects of these agents than the ACT assay.

G. Specific Measurements of Antithrombin Actions: TT and ECT

The plasma-based amidolytic antithrombin assay gives useful information on the relative antithrombin activities of various agents. However, as seen from the previous studies, the results of the amidolytic assays are not always correlated with the Ki values of thrombin inhibitors and the anticoagulant effects of these compounds depends on the assay end-point, amidolysis versus clotting. The clot-based equivalent test of this antithrombin assay is the TT, where a specific amount of thrombin is added to plasma to stimulate clot production. The relative activities of various agents in this clotting assay revealed that they all exhibited concentration-dependent anticoagulation with a rank order of potency of hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban. This rank order is quite different from that obtained in the amidolytic plasma-based antithrombin assay, where heparin was found to be the strongest antithrombin agent. Interestingly, the TT derived order of potency of thrombin inhibitors was exactly the same as that obtained from the thrombin titration assays where the ATU potency for each agent was determined. Similarly, the TT derived rank potency order paralleled that obtained from the extrinsic generation of thrombin in the fibrinogen deficient plasma-based system.

Another interesting observation in the TT assay was that this was the only clot-based assay in which argatroban was the weakest anticoagulant, similar to its inhibitory amidolytic actions in the previous studies.

The ECT assay is a newly developed assay that utilizes the snake venom derived enzyme ecarin to activate prothrombin in the plasma to procoagulant thrombin intermediates, rather than adding exogenous thrombin as in the TT assay. This assay was developed as a plasma clotting test that is specific for direct thrombin inhibitors. Since heparin inhibits thrombin only through potentiation of the activities of antithrombin and HC-II and since the thrombin resulting from ecarin activation is not fully formed and the antithrombin recognition sites are not exposed, heparin has no effects in this assay. Hirudin and Ac-(D)Phe-Pro-boroArg-OH had the strongest anticoagulant effects in this assay, as seen in all other tests. However, the effects of the other antithrombin agents had the following rank order of potency: D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > argatroban > Boc-D-Phe-Pro-Arg-H, which did not correlate with the results of any of the other assays apart from Boc-D-Phe-Pro-Arg-H being the weakest agent in some assays.

Even though the ECT may not be an optimal assay to compare the anticoagulant effects of antithrombin agents in relationship to their Ki values for thrombin, this test may be of clinical usefulness in the monitoring of thrombin inhibitor levels in patients receiving antithrombin drugs in conjunction with other anticoagulant agents, eg. in a patient receiving hirudin while on oral anticoagulants or heparin. In a recent report (Callas et al. 1996), it was shown that when plasma from patients on oral anticoagulants

or heparin was supplemented with hirudin or other thrombin inhibitors, the ECT assay was not affected, as compared with their effects after supplementation to normal human plasma. Furthermore, two recent publications (Rübsamen et al. 1996, Radziwon et al. 1996) report on the effectiveness of the ECT test in patient bedside monitoring of PEG-hirudin. Both studies show that the ECT is not influenced by other anticoagulant treatments such as aspirin, low molecular weight heparins and unfractionated heparin. In addition, the ECT results correlated with the absolute PEG-hirudin concentration in the plasma of the patients systemically administered with PEG-hirudin. However, the results obtained with the APTT and the ACT assays did not produce a similar correlation. The wide range of sensitivity of this assay is an added advantage (Radziwon et al. 1996).

Most of the patients treated with antithrombin agents are simultaneously administered with heparin or oral anticoagulant drugs. The conventional clotting tests such as the PT, APTT and TT are not reliable measures of the specific effects of antithrombin agents in these circumstances. The results reported in this dissertation suggest that ECT can be used for the absolute monitoring of the antithrombin agents in the presence of other anticoagulants. Therefore, this test is of major value in the specific monitoring of antithrombin drugs during PTCA, thrombolysis and stenting.

H. Comparative Antithrombotic Actions of Various Thrombin Inhibitors

The *in vitro* antithrombin and anticoagulant actions of various agents did not correlate with their *in vivo* antithrombotic effects. To examine the relative antithrombotic effects of selected thrombin inhibitors, four of the agents studied in the *in vitro* assays

were compared in the modified rabbit model of jugular vein stasis thrombosis. rTF was used as a thrombogenic challenge to determine the relative antithrombotic effects of various thrombin inhibitors. Unlike the previously reported studies, this thrombogenic trigger provided a more defined activation approach. This is also more relevant to the interventional cardiovascular procedures where TF is generated. The agents selected were heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H. Heparin was studied because it has been the traditional antithrombotic agent used clinically, in addition to being the target for replacement by some of the newer antithrombin agents. Since hirudin is currently in many clinical trials for various indications and is the strongest and specific thrombin inhibitor, it was also used in these comparative studies. Based on the broad anti-serine protease profile of Ac-(D)Phe-Pro-boroArg-OH, this agent was suitable for examining the relative contribution of thrombin inhibition in relation to inhibition of other clotting factors and serine proteases in antithrombotic efficacy. D-MePhe-Pro-Arg-H was compared with the previous antithrombin agents since its inhibitory spectrum is narrower than Ac-(D)Phe-Pro-boroArg-OH but not as specific as hirudin. During the course of these experiments, several *ex vivo* whole blood and plasma analyses were made, in an attempt to determine which test would be optimal and correlate best with the observed antithrombotic effects of each agent.

1. Standardization of rTF as a Thrombogenic Trigger in the Rabbit Stasis Thrombosis Model

The Wessler stasis thrombosis model (Wessler et al. 1959) is a rabbit model of venous thrombosis and has been widely used in the evaluation of antithrombotic activity

of various substances. Although this model has provided useful information on the antithrombotic activities of various anticoagulant agents, the results obtained are relative to the type of thrombogenic trigger used to produce the thrombotic processes (Fareed et al. 1985). Defined thrombogenic agents to activate the hemostatic system of rabbits have been previously utilized in a standardized manner where the site of activation is controlled (Fareed et al. 1985). In previous studies, specific components of the hemostatic system were selectively activated with various agents such as Prothrombin Complex Concentrate (PCC) and Russell's Viper Venom (RVV) (Fareed et al. 1985). The availability of TF in a recombinant form (Bach et al. 1986) allows for an in-depth investigation of the activation of hemostasis and its pathophysiological mechanisms.

TF is a 37,000 MW protein that is required for the activation of factor VII (Nemerson 1988). This TF/VIIa complex is then capable of converting factor X to Xa directly or via activation of factor IX to IXa (Österud and Rapaport 1977). Activated factor Xa can then form a complex with factor V and this complex catalyzes the conversion of prothrombin to active thrombin, which then in turn converts fibrinogen to fibrin. The concentration of factor V in plasma is lower than that of factor X (Nesheim et al. 1981, Miletich et al. 1981) and without TF available, as under normal conditions, only the VIIIa/IXa complex can catalyze the activation of factor X (Moshier 1990). Thus, as in the PT assay, the supplementation of large exogenous TF, after complexation with factor VII, causes a direct activation of factor X that is then able to saturate all of the available factor V. The generated factor Xa can then further catalyze the activation of factor VII, thus resulting in an amplified response of the extrinsic pathway of

coagulation, leading to generation of massive amounts of thrombin with the end result of formation of thrombi. The effectiveness of TF in initiating this amplified cascade is evident in the clotting results of the modified stasis thrombosis model as demonstrated in these studies, where rTF was capable of producing clots in this model at doses as low as 0.04 pmol/kg.

From the *ex vivo* analysis of the blood samples collected before and after administration of rTF in this rabbit model, a decrease in the clotting times of the global clotting tests would have been expected after the rTF administration. However, in the *ex vivo* studies performed in the rTF treated animals, no shortening of any of the plasma clotting assays (PT, APTT, TT and Heptest) was observed. This may be due to assay limitations, since these tests have been designed to detect anticoagulant effects in plasma samples and they may not be sensitive to procoagulant states. Another possible explanation is that rTF may bind to the cellular components of blood and vasculature, so that in preparing and testing the plasma fraction all rTF is removed. Furthermore, it is also conceivable that the administered rTF may have been neutralized by TFPI at the endothelial or plasmatic sites. The possibility that rTF may not exert any procoagulant effects in plasma systems has been excluded, since in a previous publication of these studies (Callas et al. 1995), the *in vitro* effects of rTF on plasma were studied in the PT assay in place of thromboplastin and it was observed that r-TF produced concentration-dependent shortening of the PT test.

The *ex vivo* ACT and TEG methods of monitoring the degree of whole blood coagulation in these experiments revealed unexpected results at the higher doses of rTF

(0.68 and 1.35 pmol/kg), where a significant prolongation of these tests was observed, in contrast to what was indicated from the clot scores obtained from the same animals. While the *ex vivo* analyses of plasma samples obtained after rTF administration in rabbits revealed no procoagulant effects in comparison to the baseline plasma samples, none of the plasma samples were found to be in an anticoagulant state either. This was in contrast to the *ex vivo* whole blood TEG and ACT results. Thus, prolongation of the whole blood clotting parameters is not due to hypofibrinogenemia or coagulation factor depletion effect due to excessive amounts of circulating TF since decrease in fibrinogen would result in prolongation of the plasma clotting assay results as well.

There are several possible explanations for this apparent *ex vivo* anticoagulant activity of rTF at the higher doses. The biological actions of TF, outside from its role in the coagulation cascade have been poorly characterized and it may well be that TF interacts with other cells, maybe endothelial cells or platelets, and through signal transduction mechanisms TF may promote fibrin formation and deposition so that even though in the end there is clot formation, it is not due to extrinsic pathway activation of blood coagulation alone and activation of coagulation may not be the only rTF mediated effect.

Another explanation for this ACT and TEG prolongation may be related to tissue factor pathway inhibitor (TFPI) which is present in plasma in different molecular forms which are primarily determined by its association with circulating lipoproteins. TFPI is a Kunitz-type protease inhibitor (Wun et al. 1988, Girard et al. 1989). One domain of this protease binds to the factor VII/TF complex while the other binds to factor Xa, thus

blocking both the extrinsic pathway leading to thrombin formation and the amplification of the same pathway by factor Xa (Broze 1989, Broze et al. 1988). TFPI is present in the plasma at ng/ml concentrations (Gramzinski et al. 1989) and thus it may inactivate all the factor VII/TF complexes more so when TF is available at higher concentrations, thus leaving only the factor IXa/factor VIII complex available for initiation of coagulation procedures. The vascular endothelium secretes TFPI in culture (Broze and Miletich 1987, Warr et al. 1989) but the mechanisms that lead to release or increase in plasma TFPI are not well defined and neither is their mode of action. Also, there is evidence that intravenous heparin may cause the release of TFPI bound to the luminal surface of the vascular endothelium (Sandset et al. 1988). It may be that rTF at higher doses displaces TFPI bound to the endothelium. Normal plasma levels of TFPI are unable to protect rabbits against DIC and glomerular thrombosis after 4 hours infusion of rabbit brain TF (Warr et al. 1990). Rapaport reported that the ability of TFPI to protect against lower concentrations of TF in the rabbits is under study (Rapaport 1991). However, no defined studies on the thrombogenic effects of rTF have been reported, where any simultaneous measurements of TFPI have been obtained.

In addition to cofactors, Ca^{++} and the coagulation factors, the following steps in the coagulation cascade require a phospholipid surface: activation of factors IX and X by factor VIIa in the presence of TF, factor X activation by factor IXa in the presence of factor VIIIa, and thrombin formation (from prothrombin) by factor Xa in the presence of factor Va. In each reaction both the factor acting as the activator (protease) as well as the inactive zymogen form of the factor to be activated bind to the phospholipid

matrix. The cofactors have specific domains for binding to proteases, zymogens, Ca^{++} and phospholipids (Moshier 1990). This phospholipid matrix is normally supplied by the platelets. However, in these studies the rTF used is already packaged in phospholipid vesicles. It is thus possible that the large doses of rTF provide a phospholipid surface which can sufficiently bind cofactors and the other components of the previously listed steps in a manner that they are less likely to produce procoagulant effects which are measurable by TEG and ACT methods. On the other hand, in the tests performed on plasma samples, a procoagulant effect as measured by the shortening of the clotting time may not be detectable due to assay limitations. In order to fully assess the effects of rTF, measurements of markers of protease activation such as the F_{1+2} fragment, fibrinopeptide A and thrombin-antithrombin complex may have been useful. However, these tests are only available for the human markers and it was not possible to perform these tests in the rabbit stasis thrombosis model.

Thus, the *in vivo* hypercoagulable state (formation of thrombi) as observed with the modified stasis model, cannot be correlated with either the *ex vivo* whole blood clotting tests (ACT and TEG) or the *ex vivo* plasma clotting tests (PT, APTT, Heptest, TT). Furthermore, while the two whole blood clotting tests correlate well with each other, they do not correlate with the plasma clotting tests.

As reported in the previous publication of this study (Callas et al. 1995), when the surface of clots generated in this animal model were examined under electron microscopy, the clots generated were found to be dense fibrin-rich clots with few other cellular components entrapped. When these clots were compared to the clots obtained

from the FEIBA® treated rabbits, the morphologic characteristics of these clots differed, with the rTF clots appearing denser and of finer fibrin structure. Therefore, activation of the coagulation pathway at different levels influences the quality of the generated clot. This may be of clinical relevance and may aid in understanding qualitative differences in various thrombotic states.

The current model used for the study of the thrombogenic effects of rTF represents a defined pathophysiologic model in which the cumulative clot formation is dependent on both the rTF and vascular stasis. Although in the whole blood system the suggested evidence on the release of TFPI or a TFPI-like substance is observed, the created thrombogenic event in the isolated jugular segment is strong enough to overpower the anticoagulant effects observed in the whole blood clotting assays.

2. Comparative Antithrombotic Actions of Thrombin Inhibitors

From the studies of the dose-dependent thrombogenic effects of rTF in the rabbit model of jugular vein stasis thrombosis, a dose of 0.68 pmol/kg was selected for the subsequent studies in which the antithrombotic effects of various agents were compared. This dose was selected because it consistently resulted in clot scores of at least +3, both after 10 and 20 min stasis. The following thrombin inhibitors were compared in this model: heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H.

a. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration

All agents produced dose-dependent antithrombotic effects after I.V. injection. The clots obtained after 10 min stasis revealed that the order of antithrombotic potency

of these agents was hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H. However, the shape of the dose-response curve for each agent was different and while heparin and Ac-(D)Phe-Pro-boroArg-OH produced sigmoid responses, hirudin and D-MePhe-Pro-Arg-H resulted in more linear responses indicating a decreased efficacy in comparison to heparin and Ac-(D)Phe-Pro-boroArg-OH. When examining the results from the 20 min stasis, again all antithrombin agents produced dose-dependent antithrombotic effects. The order of potency of these agents was different from the 10 min results in that the dose-response curves obtained with hirudin and heparin were almost superimposable, indicating that heparin retains its antithrombotic effects relatively longer than hirudin. The dose-response relationships by all thrombin inhibitors were linear, indicating decreased efficacy in comparison to their 10 min stasis effects. This decreased efficacy may be due to the clots becoming more stable with time via the cross-linking activities of factor XIIIa.

The comparative antithrombotic effects of heparin, hirudin, hirulog and argatroban in a rat model of jugular vein thrombosis have been recently published (Hayes et al. 1996). In this report, consistent with the earlier described results, on a molar basis hirudin was found to be a stronger antithrombotic agent than heparin after I.V. injection.

The *ex vivo* analyses of blood samples obtained before and after the administration of each of the antithrombins, revealed that the relative effects of each agent were assay-dependent. In the amidolytic antithrombin assay, all agents produced dose-dependent thrombin inhibition and their order of potency in this assay correlated with their relative antithrombotic responses. However, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-

Arg-H produced 30 and 50% thrombin inhibition at the lowest doses injected, even though their corresponding antithrombotic effects were minimal. In the anti-Xa assay heparin and Ac-(D)Phe-Pro-boroArg-OH produced dose-dependent effects, but these never exceeded 40 and 30% inhibition respectively. These anti-Xa effects produced by heparin and Ac-(D)Phe-Pro-boroArg-OH are not surprising since both agents produced concentration-dependent anti-Xa effects in the *in vitro* studies, which were also weaker than their amidolytic antithrombin effects. Their relative *ex vivo* anti-Xa activities also corresponded with their relative *in vitro* anti-Xa effects. Minimal anti-Xa effects were also produced by hirudin and D-MePhe-Pro-Arg-H and these were probably indirect and due to inhibition of thrombin and subsequent suppression of feedback amplifications.

The *ex vivo* whole blood analyses of the samples obtained prior and post thrombin inhibitor administration revealed that all agents resulted in dose-dependent prolongation of both assays, which correlated with each other although they did not correlate with the relative antithrombotic effects of these agents. With the exception of hirudin which was weaker than heparin, the other agents retained the rank order of potency in the whole blood clotting assays. While both the ACT and the TEG were sensitive to the anticoagulant effects of heparin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H, neither assay was sensitive to the effects of hirudin: at the highest doses that each agent was administered, hirudin produced the weakest *ex vivo* ACT and TEG prolongation, even though the antithrombotic effects produced by each agent were similar. The anticoagulant responses of heparin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H at the higher concentrations were similar only in the ACT, correlating to the similarity

of their antithrombotic effects at these doses. Furthermore, the inter-animal variations were less pronounced in the ACT than in the TEG, as measured by the S.E.M.

The *ex vivo* anticoagulant effects of antithrombin agents as measured in the plasma clotting assays revealed that these effects were assay-dependent. None of the thrombin inhibitors produced any effects in the PT, while the dose-dependent effects produced in the APTT were minimal. On the other hand, the Heptest and the TT revealed clear dose-dependent anticoagulant effects of all thrombin inhibitors after I.V. injection. The rank order of potency of these effects in the Heptest was heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H, which was similar to the whole blood assay results but different from the antithrombotic effects. The TT appeared to be the most sensitive test in detecting the anticoagulant effects of all thrombin inhibitors, as determined by the fold increase from baseline values. At the highest doses used, none of the thrombin inhibitors resulted in more than a 5-fold increase in the Heptest, while in the TT, hirudin and D-MePhe-Pro-Arg-H exceeded 10-fold increase from baseline values. Furthermore, the rank order of potency of these agents in the TT followed that observed in the antithrombotic effects: hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H. However, the extent of the TT anticoagulant effects achieved with the highest dose of each thrombin inhibitor was different and did not correspond to the extent of their antithrombotic actions.

b. Dose-Dependent Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration

The relative antithrombotic actions of antithrombin agents are dependent on the

route of administration, as seen in the rabbit stasis thrombosis studies where all agents were injected subcutaneously. While all agents produced dose-dependent antithrombotic effects and the rank order of antithrombotic actions was similar to those obtained after I.V. administration of these agents in the same model, the dose-response curve of heparin was close to that of Ac-(D)Phe-Pro-boroArg-OH in the S.C. studies after 10 min stasis and was the same after 20 min stasis. This was different from the response observed in the I.V. studies, where the effects of heparin were closer to those of hirudin. A possible explanation for this relative decrease in the activities of heparin is that this agent, being a multi-component drug of varying molecular weights, does not cross the subcutaneous barrier entirely, so that the higher molecular weight components do not reach the circulation. Based on the molecular weight of hirudin (6963 DA), this compound is expected to have weaker antithrombotic effects after S.C. administration, as opposed to I.V. administration, relative to Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H. Taking into consideration the ratio of the ED_{50} values for each agent in the I.V. and S.C. studies, while there was a 5-fold decrease in the absolute value for Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H, for hirudin this decrease was 12-fold and for heparin it was 23-fold, which supports the view regarding the S.C. barrier influence on the transport of heparin and hirudin. Another difference in the two studies (I.V. versus S.C. administration) was that the dose-response relationship for all thrombin inhibitors was linear after both 10 and 20 min stasis. As seen in the I.V. studies, after 20 min stasis, all agents decreased in efficacy (the slope of the dose-response curves decreased) in comparison to their 10 min stasis effects.

The *ex vivo* amidolytic antithrombin effects of the same agents after S.C. administration were dose-dependent. However, the extent of antithrombin activity did not correlate with the extent of antithrombotic effects, as seen when comparing these agents at the highest doses administered. With the exception of heparin which produced weak dose-dependent anti-Xa effects, none of the other three agents had any apparent dose-dependent effects on the amidolytic anti-Xa assay. The effects of heparin can be explained by the ability of antithrombin, through which heparin mediates its anticoagulant effects, to inhibit factor Xa in addition to thrombin. The whole blood clotting analyses of blood samples obtained before and after thrombin inhibitor S.C. injection revealed that all agents produced dose-dependent anticoagulant effects. The potency order of the anticoagulant effects of these agents was similar to their antithrombotic order of potency. The TEG assay appeared to be more sensitive to the anticoagulant effects of these agents than the ACT, where hirudin and heparin produced about 25% and 35% of the anticoagulant effects of Ac-(D)Phe-Pro-boroArg-OH at the highest doses where their antithrombotic effects where equivalent.

The anticoagulant effects of antithrombin agents after S.C. administration, as detected in the plasma-based clotting assays revealed that, as with the I.V. studies, the effects were test-dependent. As seen in the I.V. studies, none of these agents produced any effects in the PT assay. The effects on the APTT, Heptest and TT were dose-dependent. Hirudin exhibited the strongest effects, correlating with the antithrombotic effects produced by this agent. However, the TT was the most sensitive assay to the effects of hirudin at the highest injected dose in comparison with the other agents.

c. Time-Dependance of Antithrombotic Effects of Thrombin Inhibitors After I.V. Administration

The studies on the time-dependance of the antithrombotic effects of heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H after I.V. administration showed that the effects of all agents decreased with time, both after 10 and 20 min stasis. Ac-(D)Phe-Pro-boroArg-OH and heparin lost their antithrombotic activities at a faster rate than hirudin and D-MePhe-Pro-Arg-H, as determined by the slopes of their time-dependent responses. The *ex vivo* antithrombin analyses of the plasma samples obtained before and after the injection of each antithrombin agent, showed that with exception of heparin which had minimal antithrombin activities, the antithrombin effects of the other agents where time-dependent. While the effects of Ac-(D)Phe-Pro-boroArg-OH decreased steadily with time, the effects of D-MePhe-Pro-Arg-H where maximal at 30 min and those of hirudin where maximal around 45 min. The antithrombotic effects of D-MePhe-Pro-Arg-H were steadily maximal for the first 30 min after administration (correlating with the antithrombin effects) but the antithrombotic effects of hirudin steadily declined after I.V. injection. This dichotomy in the antithrombotic/ antithrombin effects of hirudin may be due to compartmentalization of hirudin and subsequent slow release of this agent so that the *ex vivo* antithrombin effects are sustained, even though the antithrombotic effects are diminished. As seen in the previous studies, none of the agents had any substantial effects on the anti-Xa assay. In contrast to the plasma amidolytic antithrombin and anti-Xa assays, the whole blood clotting tests, ACT and TEG, showed that all agents produced time-dependent anticoagulant effects that paralleled their antithrombotic effects, with heparin and Ac-(D)Phe-Pro-boroArg-OH exhibiting the

fastest rates of decrease of their anticoagulant activities, similar to their antithrombotic activities.

As observed in the previous studies, none of the antithrombin agents had any effects on the PT assay after I.V. administration during the observation period. In contrast, the other plasma-based clotting assays revealed a time-dependent decrease of the anticoagulant activities of all thrombin inhibitors, with one notable exception: D-MePhe-Pro-Arg-H produced a time-dependent increase in the Heptest assay until 30 min post I.V. injection, after which these effects started to rapidly decrease. This particular effect of D-MePhe-Pro-Arg-H is similar to that seen in the antithrombin amidolytic assay and is consistent with the antithrombotic effects of this agent, which are maximally sustained for the first 30 min post I.V. administration. Overall, as seen in the previous studies, the TT was the most sensitive assay to the anticoagulant effects produced by thrombin inhibitors.

d. Time-Dependent Antithrombotic Effects of Thrombin Inhibitors After S.C. Administration

The studies on the time dependance of the antithrombotic effects of various agents after S.C. administration revealed the differences in the ability of each agent to cross through the S.C. barrier. When examining the comparative antithrombotic effects of heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H in the rabbit stasis thrombosis model after 10 min stasis, it was observed that the agents that exhibited the maximal antithrombotic effects at the shortest interval after S.C. injection were hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H, in that order. The two

tripeptide derivatives have similar low molecular weights (496.8 and 514.6 DA respectively), which probably allows them to cross rapidly through to the circulation. While the effects of Ac-(D)Phe-Pro-boroArg-OH steadily and slowly declined with time after 15 min, the effects of D-MePhe-Pro-Arg-H remained maximal up to 45 min after S.C. administration before declining, indicating perhaps a difference in the clearance rates of the two agents. Similar to the effects of D-MePhe-Pro-Arg-H, hirudin also exhibited steady maximal antithrombotic effects for 45 min after S.C. injection, before these effects started to subside. The molecular weight difference between the two tripeptides and hirudin is 14-fold, but this does not compromise the ability of hirudin to cross the S.C. barrier. In contrast to the time-dependent effects of these agents, heparin exhibited a steady increase in its antithrombotic effects after S.C. administration up to the last time point examined at 90 min post injection. This is to be expected considering the multi-component nature of this drug and expecting the smaller antithrombotic components to cross the S.C. barrier earlier than the heavier counterparts.

The time-dependent effects of these agents are also dependent on the duration of stasis, so that at the 20 min stasis end point, the time-dependent antithrombotic profile of each agent changed. While the effects of D-MePhe-Pro-Arg-H were maximal 15 min post S.C. administration, these effects steadily declined, rather than remaining constant for 45 min as observed in the 10 min stasis. On the other hand, the effects of Ac-(D)Phe-Pro-boroArg-OH and hirudin remained constant over the 90 min period following post S.C. injection, in contrast to their 10 min stasis effects which slowly declined after 15 min. The effects of hirudin after 20 min stasis were steady over the 90 min period

studied as well and this was again in contrast to the effects of this agent after 10 min stasis, where a decline was observed after 45 min post S.C. administration. However, the sustained effects of both Ac-(D)Phe-Pro-boroArg-OH and hirudin after 20 min stasis were decreased when compared to the maximal effects at the 10 min stasis end point, indicating that these agents had decreased antithrombotic effects against cross-linked fibrin. Heparin exhibited a gradual and continual increase in its antithrombotic effects over the 90 min observation period, following S.C. injection and 10 min stasis. This was also in contrast to its effects after 20 min stasis, where heparin increased its antithrombotic effects until 45 min post S.C. administration, after which these antithrombotic activities gradually decreased. This may also be indicative of the limited antithrombotic potential of heparin on cross-linked fibrin.

The time-dependent *ex vivo* amidolytic antithrombin effects of heparin after S.C. injection, revealed a correlation with its antithrombotic effects, as did the amidolytic anti-Xa test and the whole blood clotting tests (ACT and TEG). As seen previously, none of the other thrombin inhibitors had any effects on the anti-Xa amidolytic assay. The antithrombin, ACT and TEG effects of hirudin correlated with its antithrombotic effects after 10 min stasis, with the ACT being the most sensitive assay. Ac-(D)Phe-Pro-boroArg-OH produced sustained maximal antithrombin and TEG effects, correlating with its 20 min stasis antithrombotic effects, while in the ACT results correlated more with its 10 min stasis antithrombotic effects (a steady increase with time over the 90 min period). D-MePhe-Pro-Arg-H also exhibited sustained maximal antithrombin and TEG effects, throughout the 90 min observation time after S.C. administration. However, in

the ACT, a time-dependent decline in this agent's anticoagulant activity was observed, correlating well with its effects after 20 min stasis. The assay that was most sensitive to all of the agents' effects without reaching the upper limit was the ACT, while the amidolytic antithrombin and TEG results often approached the upper limits of the assays, with the exception of hirudin.

When examining the time-dependent *ex vivo* anticoagulant effects of these agents, as studied in the plasma-based clotting assays, once again, it was observed that none of these agents had any effects on the PT. The results of heparin on the APTT, Heptest and TT correlated well with its antithrombotic effects after 20 min stasis. Hirudin produced minimal effects on the APTT and the Heptest, while its effects on the TT increased with time throughout the 90 min observation period post S.C. injection. The effects of Ac-(D)Phe-Pro-boroArg-OH on the APTT were always maximal, while the time-dependent anticoagulant effects in the Heptest and the TT paralleled the antithrombotic effects of this agent. D-MePhe-Pro-Arg-H produced sustained maximal effects in the APTT and the Heptest, while its effects in the TT correlated with its antithrombotic effects. Thus, the TT appeared to be the most sensitive plasma clotting assay for these agents, without exceeding the upper limits at the doses producing maximal antithrombotic effects. Another observation was that at 75 min post S.C. administration at 10 min stasis and 35 min post S.C. injection at 20 min stasis, all agents produced similar antithrombotic results. However, of all *ex vivo* analyses, only the TT assay results converged around the 75 time point.

To date, a comparison of the duration of action of the four agents investigated

after S.C. administration in any animal models is not available. The duration of *in vivo* antithrombotic actions may therefore have relevance to the prophylactic use of these agents.

I. Comparative Hemorrhagic Profile of Thrombin Inhibitors

The rabbit ear bleeding model used in these studies was introduced by Cade in 1959. This model has been extensively utilized to profile the hemorrhagic effects of various drugs and is based on the ability of superficial minor vessels to occlude after transection within minutes. The primary hemostatic plug is formed by platelets, which is then reinforced by fibrin deposition resulting from activation of coagulation. The clotting process is triggered by the exposure of blood to TF on the subendothelial sites of the transected vessels, resulting in thrombin formation which mediates platelet activation. Therefore, inhibitors of the coagulation cascade as well as platelet inhibitors are expected to have dose-dependent hemorrhagic effects in this model, which manifest by increased blood loss in a specified period of time. This model was used to compare the relative hemorrhagic effects of heparin, hirudin Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H, to allow for comparison of these effects with the antithrombotic effects, discussed previously, in the same species. During the course of the rabbit ear bleeding experiments blood samples were not obtained for concomitant *ex vivo* analysis to minimize blood loss and to avoid hemodynamic alterations in this model.

1. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After I.V. Administration

When the dose-dependent hemorrhagic effects of thrombin inhibitors were

examined in the rabbit ear blood loss model, heparin produced weak but steady hemorrhagic effects at all doses studied, 5 min after I.V. administration. The hemorrhagic effects of heparin after 20 min of I.V. injection were diminished. These weak bleeding effects of heparin may be attributed to the fact that heparin works through potentiating the anticoagulant effects of circulating antithrombin and HC-II. Therefore, the available amount of antithrombin and HC-II can be saturated by heparin and beyond a dose of 0.1 mg/kg I.V. heparin no additional anticoagulant effects can be achieved. Hirudin and D-MePhe-Pro-Arg-H produced dose-dependent hemorrhagic effects both after 5 and 20 min post I.V. administration and similar to heparin their effects were diminished after 20 min. Because of their mechanism of action, these direct thrombin inhibitors do not achieve a ceiling effect as seen with heparin. While Ac-(D)Phe-Pro-boroArg-OH also produced dose-dependent hemorrhagic effects after 5 and 20 min post I.V. injection, the effects of this agent after 20 min post I.V. administration were potentiated, in contrast to the bleeding effects of the other agents. This enhancement of the bleeding effects of Ac-(D)Phe-Pro-boroArg-OH with time may be due to vascular binding and subsequent slow release of this agent, or more likely, to non-anticoagulant effects of this agent and may involve vascular compromise. The observation substantiating the latter hypothesis is that at the 1 μmol/kg I.V. dose tested, 3 rabbits presented with respiratory depression within the first 5 min after I.V. injection, became cyanotic and expired within 30 min after the administration. Necroscopic examination of vital organs did not reveal any unusual findings. These rabbits were substituted to maintain the rabbit group size at 6 animals.

2. Dose-Dependent Hemorrhagic Effects of Thrombin Inhibitors After S.C. Administration

The hemorrhagic effects of thrombin inhibitors after S.C. injection were also examined. Heparin was found to have significant bleeding effects only at the highest dose administered (0.9 μmol/kg) and only after 45 min post S.C. administration. At 60 min post S.C. injection, this effect was diminished. These minimal bleeding effects after S.C. administration are also likely to be due to inability of all heparin fractions to pass the S.C. barrier. Hirudin produced dose-dependent hemorrhagic effects, 45 min post S.C. injection, but similar to the effects of heparin, these effects were also diminished 60 min post S.C. administration. In contrast to the relatively weak bleeding effects of hirudin and heparin, the dose-dependent effects of Ac-(D)Phe-Pro-boroArg-OH were maximal 45 min post S.C. injection and although they decreased 60 min post S.C. administration, these remained stronger than any effects produced by the other thrombin inhibitors. This observation supports the previous hypothesis that the hemorrhagic effects produced by Ac-(D)Phe-Pro-boroArg-OH may be due in part to additional non-thrombin related activities of this agent. D-MePhe-Pro-Arg-H exhibited a bleeding profile similar to that produced by hirudin, in that 45 min post S.C. injection it produced dose-dependent effects which were diminished 60 min post S.C. administration. Since Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H are of similar molecular weights and probably cross the S.C. barrier at similar rates, the limited effects of D-MePhe-Pro-Arg-H after S.C. injection further support the possibility of Ac-(D)Phe-Pro-boroArg-OH exhibiting hemorrhagic effects through non-thrombin mediated actions.

3. Time-Hemorrhagic Effects of Thrombin Inhibitors After I.V. and S.C. Administration

The time-dependent hemorrhagic effects of these thrombin inhibitors revealed a progressive agent specific decrease in the hemorrhagic activities. However, after S.C. administration, heparin, hirudin and Ac-(D)Phe-Pro-boroArg-OH produced increasing hemorrhagic effects with time with a maximal effect at 45 min post administration, after which these started to decline. In contrast to the S.C. hemorrhagic effects of heparin, hirudin and Ac-(D)Phe-Pro-boroArg-OH, S.C. injection of D-MePhe-Pro-Arg-H resulted in gradually decreasing bleeding effects from the first time point observed (15 min post S.C. administration). This suggests that D-MePhe-Pro-Arg-H is capable of crossing the S.C. barrier in less than 15 min post injection, further supporting the hypothesis that the maximal hemorrhagic effects produced by Ac-(D)Phe-Pro-boroArg-OH 45 min post S.C. administration are due to additional non-coagulation related effects of this agent.

J. Integration of Data on Antithrombotic and Hemorrhagic Effects of Thrombin Inhibitors

In comparing the dose-dependent antithrombotic and hemorrhagic effects of various thrombin inhibitors, it was found that even though overall hirudin was the strongest antithrombotic agent in the stasis thrombosis model after both I.V. and S.C. administration, it also had the strongest bleeding effects in the rabbit ear blood loss model, with the exception of the 60 min post S.C. injection results, where Ac-(D)Phe-Pro-boroArg-OH was the strongest agent. On the other hand, heparin produced strong antithrombotic effects, which were weaker than hirudin, after I.V. and S.C. administration, while producing the weakest hemorrhagic effects of all agents after both

I.V and S.C. administration. The effects of Ac-(D)Phe-Pro-boroArg-OH were consistently stronger than those produced by D-MePhe-Pro-Arg-H in both animal models after both I.V. and S.C. injection.

The comparison of the antithrombotic and hemorrhagic effects of each thrombin inhibitor can be best illustrated by comparing the ED_{50} value of the effects that each agent produced in the stasis model to the dose that resulted in bleeding of $2 \cdot 10^9$ RBC/L in the rabbit ear blood loss model. When comparing the ED_{50} values of each thrombin inhibitor derived in the stasis model after I.V. administration and 10 min stasis, and the doses the produced $2 \cdot 10^9$ RBC/L blood loss 5 min post I.V. injection, it was found that D-MePhe-Pro-Arg-H had the lowest ratio of the two respective parameters, with the bleeding dose being about 12-fold greater than the antithrombotic dose. Ac-(D)Phe-Pro-boroArg-OH closely followed with a 17-fold difference in its bleeding/antithrombotic values. Hirudin, on the other hand, producing less hemorrhagic effects than D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH at high doses, had a 37-fold difference of its respective hemorrhagic/antithrombotic indices. Heparin, producing minimal hemorrhagic effects at all doses had a ratio of >56-fold of the respective indices. These differences indicate that while hirudin is a more potent antithrombotic agent than heparin, as assessed in the rabbit stasis thrombosis model, heparin has a wider safety margin. This is most likely attributable to the fact that heparin mediates its actions through potentiation of a limiting amount of antithrombin and HC-II in plasma, which imposes a ceiling on its maximal effects, whether antithrombotic or hemorrhagic.

The relative antithrombotic/hemorrhagic effects of antithrombin agents after I.V.

administration depend on the time period studied. Thus, when the ED_{50} of each thrombin inhibitor in the stasis thrombosis model after I.V. injection and 20 min stasis is compared with the bleeding effects produced 20 min post I.V. administration, it was found that because D-MePhe-Pro-Arg-H exhibited a decrease in its hemorrhagic effects the ratio to the antithrombotic effects could be determined only as >3. The bleeding/antithrombotic index for Ac-(D)Phe-Pro-boroArg-OH was found to decrease from 17 to 12, which may be explained by increased non-thrombin related hemorrhagic effects of this agent with time. When comparing the hemorrhagic/antithrombotic effects of hirudin at these time points, the ratio decreased to 27, which could be attributed to sequestration of this drug and subsequent slow release, resulting in high enough circulating concentrations to produce an antiplatelet effect (through thrombin inhibition) but not sufficient for an antithrombotic effect. The bleeding/antithrombotic ratio of heparin remained high at >50, suggesting that this drug has the widest safety/efficacy window.

The ratios of the hemorrhagic and antithrombotic effects of various thrombin inhibitors after S.C. injection is not as clear, since in most cases these agents produced minimal hemorrhagic effects. Thus, for Ac-(D)Phe-Pro-boroArg-OH, the ratio of the ED_{50} from the 45 min post S.C. administration and 10 min stasis dose-response compared with the dose that results in $2 \cdot 10^9$ RBC/L bleeding was 7, and the ratio of the ED_{50} after 20 min stasis to the bleeding effects after 60 min post S.C. injection was 5. These ratios for the safety/efficacy of Ac-(D)Phe-Pro-boroArg-OH are lower than the ratios observed after I.V. administration. Hirudin had a safety/efficacy ratio of 22, when comparing the ED_{50} from the 45 min post S.C. injection and 10 min stasis antithrombotic dose-response

compared with the dose that results in $2 \cdot 10^9$ RBC/L 45 min post S.C. administration, which was also lower than the ratio produced after I.V. injection. The lower safety/efficacy ratios after S.C. administration as compared to those after I.V. injection, for both inhibitors may be attributable mostly to decreased antithrombotic effects after S.C. administration, as discussed earlier.

It is interesting to note that these experimental observations on the relative bleeding/antithrombotic effects of antithrombin agents are consistent with the recently available clinical data where increased risk of hemorrhage was found to be associated with the use of antithrombin agents such as hirudin (Antman and TIMI 9A Investigators, 1994, GUSTO IIa Investigators, 1994, GUSTO IIb Investigators, 1996). In the experimental studies, a dose-response for both the hemorrhagic and antithrombotic actions was evident. Such relationships in the clinical trials have not been established. These observations clearly suggest the need of individual dose-response investigation for different antithrombin agents.To obtain valid results, these studies should be optimally carried out in the same species.

K. Rat Laser-Induced Thrombosis Model

The comparative antithrombotic effects of thrombin inhibitors, as studied in the rabbit stasis thrombosis model, provide information on the antithrombotic activities of these agents in the venous system. Since the pathophysiology of thrombotic processes in the arterial and the venous systems differ, these compounds were also compared in a rat model of laser-induced arterial thrombosis as performed in the mesenteric arterioles. This model is standardized so that exposure of an arteriole to 2-3 sequential laser shots

results in thrombus formation that is either occluding or is of a length 1.5 x vessel diameter. The thrombi formed with this procedure are platelet-rich clots that do not stabilize unless continued laser exposure occurs. The laser exposures are directed towards the vessel wall. The laser injury is thermal in nature and after histologic examination of these vessels, it appears that only endothelial cells are affected (Dr. Klaus Breddin, personal communication). The injury is concentrated enough so that usually only the surface membrane of the endothelial cells is detached. However, in some instances the whole cell appears to be "dehydrated". This indicates that exposure of TF from sub-endothelial sites triggers local coagulation to generate enough thrombin to activate platelets, or it may be that coagulation is triggered by contact activation through exposed sub-endothelial matrices.

Heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H exhibited dose-dependent antithrombotic effects in this animal model. The order of antithrombotic potencies was heparin> Ac-(D)Phe-Pro-boroArg-OH> hirudin> D-MePhe-Pro-Arg-H. This order of potencies was different from those observed in the rabbit stasis thrombosis model. While D-MePhe-Pro-Arg-H was consistently the weakest antithrombotic agent in both the rabbit venous stasis model and the rat arterial thrombosis model, hirudin was the strongest antithrombotic agent in the rabbit stasis thrombosis model and in this rat model it ranked third. The fact that heparin was found to be the strongest antithrombotic agent in the rat arterial thrombosis model, in contrast to its effects in the rabbit venous thrombosis model suggest that heparin is more effective than hirudin in inhibiting the formation of thrombus in this model. On the other hand, when

coagulation is activated extrinsically with greater amounts of TF, as is the case with the rabbit stasis thrombosis procedure, the endogenous levels of antithrombin may not be sufficient or as effective as hirudin in inhibiting coagulation. Since Ac-(D)Phe-Pro-boroArg-OH was also found to be a stronger antithrombotic agent than hirudin in the rat model, and both heparin and Ac-(D)Phe-Pro-boroArg-OH exhibit both antithrombin and anti-Xa effects, it may be that inhibition of coagulation at multiple sites is more effective in preventing platelet activation due to generation of thrombin.

While D-MePhe-Pro-Arg-H dose-dependently prevented clot formation in the rat model in a dose-dependent fashion, a ceiling effect was reached at about a dose of 2 μmol/kg. This ceiling effect was of about 6 laser shots, which is lower than that of 8 laser shots observed with heparin and hirudin. Furthermore, at D-MePhe-Pro-Arg-H doses higher than 3.4 μmol/kg, the antithrombotic effects of this agent progressively decreased. It was visually observed that the blood flow markedly decreased at these higher doses, so that the thrombus formation could be an indirect effect of altered hemodynamics. Similar decreases in blood flow were observed with Ac-(D)Phe-Pro-boroArg-OH at doses above 0.2 μmol/kg, I.V. These effects by both D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH on the rat blood flow are indicative of the non-thrombin related effects that these agents may produce. Another interesting finding is that the ceiling effect of Ac-(D)Phe-Pro-boroArg-OH was about 10 laser shots which is higher than that produced by heparin and hirudin. Since Ac-(D)Phe-Pro-boroArg-OH is a direct thrombin and factor Xa inhibitor and is not limited by endogenous amounts of antithrombin as is heparin, the higher ceiling effect for this agent is not surprising.

Although the differences in the potency orders in the rabbit venous stasis thrombosis model and the rat laser-induced arterial thrombosis model are most likely due to the differences in the pathophysiologic processes, the possibility of species dependance cannot be excluded.

When the bleeding results of the I.V. administered heparin and antithrombin agents were compared with the observed antithrombotic actions in the rat laser model of arterial thrombosis, there was no relevance that could be established. Although the vascular sites are the primary trigger for the laser model and vascular compromise could also contribute to bleeding, the lack of any relationship between these two findings may be due to the species differences.

L. Species Dependence of Antithrombotic and Anticoagulant Effects of Thrombin Inhibitors

To compare the relative anticoagulant actions of newer agents, whole blood and plasma-based assays have been performed in rat, rabbit, dog, monkey and human blood and plasma (Schlam et al. 1975). In this dissertation, the *in vitro* studies are primarily carried out in the human and rabbit plasma and whole blood. In comparing the baseline coagulation profile of whole blood and plasma, it was evident that rabbit plasma and whole blood clotted faster in various assays. This may be due to the compositional variations in the coagulation pathways and number of blood cells. Interestingly, this trend was not followed when individual antithrombin agents were tested in several assays. In other words, each of the inhibitors exhibited a distinct assay-dependent profile in both the human and rabbit studies. These observations reinforce the notion that while

antithrombin agents primarily target thrombin in mediating their anticoagulant effects in plasma, these agents may exhibit complex interactions in different activation states and therefore exhibit variable responses which are both species and activation dependent.

Species dependent antithrombotic actions also play an important role in the determination of the antithrombotic potency of various antithrombin agents. In this investigation, a rabbit model of venous stasis thrombosis was employed to compare the relative antithrombotic actions of these agents. To investigate the comparative effects of these agents in arterial thrombosis, a laser model of rat arterial thrombosis was used. Although the model-dependent variations are predictable, the ED_{50} for each of the inhibitors were markedly higher in the rat laser model (5-10 fold) in contrast to the rabbit stasis thrombosis model. This observation may be partly dependent on the species used. In this research dissertation, the primary intent was to compare the relative potency of various thrombin inhibitors with heparin. In a consistent fashion such comparisons where carried out to determine the rank order of potency. The relevance of the ED_{50}s obtained in different models to projected human responses may be questionable. Regardless, such an exercise is of crucial value in the preclinical profile of a new antithrombin drug.

Beside the measurable anticoagulant actions of heparin and antithrombin agents, species dependent variations in the pharmacokinetic and pharmacodynamics effects may also contribute to the different responses noted during this research. Thus, it is clear that both the *in vitro* and *ex vivo* profile of various agents are not only dependent on the blood/plasma matrix but also on the endogenous transformations and interactions with the cellular and vascular sites.

M. Integration of *in vitro* Studies with Observed Pharmacologic Actions of Thrombin Inhibitors

The general order of potency of the antithrombotic effects of thrombin inhibitors in the rabbit stasis thrombosis model was hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H. This order correlated well in a number of *in vitro* assays. Specifically, the antithrombotic effects of hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H correlated with their published Ki values, the amidolytic thrombin potency evaluation results and the fibrinogen-based clotting assay results. The antithrombotic effects of all four thrombin inhibitors in the rabbit model correlated with their respective results in the extrinsic and intrinsic thrombin generation from fibrinogen deficient plasma results, as well as with the *in vitro* PT and TT clotting assays.

The order of potency of the four antithrombin agents in the rat laser model correlated with only one of the *in vitro* assay profiles of these agents, namely the APTT. This observation supports the hypothesis that platelet activation and clot formation in this model may not be due to intrinsic activation of coagulation.

The overall order of potency of the bleeding effects of antithrombin agents, as observed in the rabbit model of ear blood loss, was hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > heparin. The order of the three first agents followed the same trend with the order of their Ki values, the results of the amidolytic thrombin potency evaluation studies, the fibrinogen-based clotting results, the PT assay and the ECT test. However, when the antithrombotic effects of heparin are also considered, none of the *in vitro* assays correlate with the hemorrhagic results, indicating the necessity of *in vivo* evaluation of the hemorrhagic effects of antithrombin agents.

The above observations point to the usefulness of standardization of various thrombin inhibitors. Caution should be taken to compare various agents in the same system though, and with agents such as heparin which are not direct thrombin inhibitors, standardization is not possible in the same manner as with the direct antithrombin agents. Furthermore, it may be that some *in vitro* assays are not suitable for comparison of agents with different mechanisms of thrombin inhibition, as is the case with argatroban and its effects on the amidolytic assays. For the same reason, while the amidolytic protease generation assays provide useful information on the mechanisms of action of thrombin inhibitors and their relative efficacies and potencies, argatroban cannot be compared with the other agents in these systems in a manner that can be related to its effects in clot-based assays or to its *in vivo* antithrombotic effects. Therefore, apart from the Ki comparison of agents for thrombin, clot-based assays may be more applicable in comparing these thrombin inhibitors.

While concomitant *ex vivo* analyses were not carried out in the rat arterial thrombosis model due to animal weight and methodological limitations, the rabbit venous thrombosis *ex vivo* analyses indicate that the whole blood clotting assays (ACT and TEG) and the TT plasma clotting assay are the optimal tests for correlating antithrombotic with anticoagulant effects, since in the other tests (with the exception of the PT), while all agents produced dose-dependent anticoagulant effects, the relative extent of their *ex vivo* effects did not correlate with the relative extent of their antithrombotic effects. Similar observations have been recently reported (Carteaux et al. 1995). In this publication, the comparative antithrombotic effects of hirudin, heparin and Ro 46-6240 (low molecular

weight direct thrombin inhibitor) correlated with the *ex vivo* ACT effects but not with the *ex vivo* APTT results. It should be noted that the relative extent of *ex vivo* anticoagulant effects of hirudin in all tests utilized did not correlate to the relative extent of antithrombotic effects when compared to the other agents, pointing to the need for development of assays that correspond to antithrombotic effects and the importance of comparing thrombin inhibitors in a battery of assays both *in vitro* and *in vivo*.

In terms of therapeutic monitoring of an individual thrombin inhibitor, the ECT assay may be useful since the range of sensitivity of this test is wider than any other assay. Furthermore, this assay is not affected by the presence of indirect thrombin inhibitors, which makes this test ideal for thrombin inhibitor monitoring in situations where additional antithrombotic agents may have been used. However, the suitability of this assay for comparison of various anticoagulants and extrapolation of these relative *in vitro* effects to their relative *in vivo* antithrombotic effects is unclear at the time.

N. Significance of Studies in Understanding the Pharmacology of Thrombin Inhibitors

The relative potency of the thrombin inhibitors included in this investigation is usually expressed in terms of Ki or IC_{50} values which are carried out in defined *in vitro* systems, in which thrombin titration is used. Whether or not the assigned inhibitory constants translate into pharmacologic effects is not known. This investigation represents the first comprehensive study to simultaneously compare the biochemical and pharmacologic actions of heparin and antithrombin agents. An agent may show strong antithrombin potency, however, due to its complex pharmacodynamics and other

endogenous interactions, its behavior may not be predictable simply by assigning to it an antithrombin potency. For example, hirudin exhibited the strongest antithrombin potency, however, in the laser model of arterial thrombosis, its potency was relatively weaker in relation to thrombin inhibitors such as Ac-(D)Phe-Pro-boroArg-OH. This observation clearly suggests that beside the inhibition of thrombin, additional factors contribute to the observed pharmacologic actions of antithrombin agents.

Heparin is a complex polypharmacologic agent with multiple plasmatic and vascular sites of action. Although the overall anticoagulant activity of this drug is usually standardized in anticoagulant units (USP assays), the potency designation of antithrombin agents may not be directly comparable to that assigned to heparin and related drugs. Thus, an APTT elevation to 3 or 4 times the baseline level by a thrombin inhibitor may or may not be relevant to the overall anticoagulant/antithrombotic response observed with heparin. This investigation has provided clear evidence on this hypothesis. At equivalent levels of APTT, the relative antithrombotic actions of these agents are not the same. This is not just true for the comparison of heparin and antithrombin agents but thrombin inhibitors at equivalent prolongation of clotting tests exert different degrees of antithrombotic and/or hemorrhagic effects. This dissertation therefore provides evidence that the bioequivalence of these agents is not predictable by any of the *ex vivo* tests available at this time.

Significant difference in the pharmacodynamic effects of these agents are found regardless of the potency, molarity and gravimetric adjustments. Thus, each antithrombin agent exhibits its own unique biochemical and pharmacologic profile. The

data obtained in various studies is readily transformed into molar values which may have a closer relevance to the observed pharmacologic actions. An in-depth analysis of the dose and the time-dependent pharmacodynamics of each of these agents can therefore be useful in projecting some of the clinical effects of these agents. This dissertation therefore has addressed some of the pharmacologic and biochemical parameters in molar terms which can be applied for limited direct comparisons.

One of the most significant questions regarding the relative importance of thrombin in the mediation of thrombogenesis and its inhibition as the main target for controlling the thrombotic events has been objectively addressed in this dissertation. From the experimental evidence provided, it is clear that the sole inhibition of thrombin is not the primary determinant of the antithrombotic actions of antithrombin agents and heparin. In the case of thrombin inhibitors, additional plasmatic and non-plasmatic processes also contribute to the control of thrombogenesis by these agents. In the case of heparin, it is obvious that the antithrombin mediated inhibition of thrombin is only one of the multiple mechanisms for the pharmacologic action of this drug.

Another significant observation in this dissertation is related to the dichotomy between the *in vitro* and *in vivo* behavior of these agents. It is commonly believed that antithrombin agents are usually inert. If this was true, a steady decrease in the observed antithrombotic and bleeding effects after I.V. administration would have been noted. This certainly does not hold true for some of these agents, therefore, endogenous transformations and other cellular and vascular interactions contribute to their observed effects.

Therefore, a classification of thrombin inhibitors can be proposed on the basis of the spectrum of their pharmacologic actions, as depicted on Table 25. Antithrombin agents can be grouped into three major categories. Hirudin, hirulog and their analogues are clearly agents with plasmatic actions only, solely targeting the generated thrombin and its regulatory functions. On the other end, agents such as heparin and aptamers mediate diverse pharmacologic actions including vascular, anti-inflammatory, anti-platelet and anti-proliferative effects. Agents such as the tripeptide and peptidomimetic thrombin inhibitors possess vascular effects in addition to their plasmatic actions, placing these in a category between the two described above.

Table 25 -- Classification of Thrombin Inhibitors Based on their Pharmacologic Actions

Agents with plasmatic actions only	Hirudin, hirulog
Agents with dual mechanisms of action (plasmatic and vascular)	Peptides, peptidomimetics
Agents with additional regulatory effects (anti-proliferative, platelet interactions, anti-inflammatory)	Heparin, aptamers

An additional significant observation during this investigation relates to the fact that antithrombin agents are considered inhibitors which only target thrombin. However, their effects on other serine proteases and modified forms of thrombin are not taken into account. Many of the thrombin inhibitors such as D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH, significantly inhibit non-thrombin serine proteases and this may have a complex outcome. In addition, most of the thrombin inhibitors inhibit thrombomodulin bound thrombin, which can have significant implications in the management of

thrombotic and cardiovascular disorders. This dissertation has provided an additional insight on the potential clinical implications due to the non-specificity of these agents.

Another classification of thrombin inhibitors can thus be generated based on the spectrum of their serine protease activities, as depicted on Table 26. Hirudin, hirulog and their analogues, argatroban, HC-II and aptamers are monospecific agents that interact solely with thrombin. On the other hand, agents such as Ac-(D)Phe-Pro-boroArg-OH interact with almost every serine proteases involved in systems other than coagulation and including the fibrinolytic system. Agents with limited serine protease inhibitory activities but not restricted to thrombin are included in the narrow spectrum class of thrombin inhibitors. These agents include napsagatran, inogatran and antithrombin and beside their antithrombin activities, these also exhibit weaker inhibition of other coagulation factors.

Table 26 -- Classification of Thrombin Inhibitors Based on their Serine Protease Inhibitory Spectrum

Monospecific	Hirudin, hirulog, argatroban, HC-II, aptamers
Narrow spectrum	Napsagatran, inogatran, antithrombin
Broad spectrum	Ac-(D)Phe-Pro-boroArg-OH, peptide arginals

While preclinical screening of new antithrombin agents have mostly relied on thrombin titration methods to assign relative potency for antithrombin derived drugs, such potency designation has not been very useful in the prediction of the *in vivo* pharmacologic behavior of these drugs. One of the most important contributions of this research is based on the fact that it provides the first comprehensive database for the

comparative biochemical profile, *in vitro* anticoagulant actions and pharmacologic information relevant to their efficacy and safety. It is also for the first time that these agents are compared at a molar level. Table 27 shows the data on the comparative rank order of potencies of heparin and these antithrombin agents in several biochemically defined assays. It is interesting to note that heparin and hirudin exhibit the strongest potency profiles while the rank order for other agents varies depending on the type of assay used. Similarly, when heparin and these agents are tabulated in terms of their anticoagulant potency ranking, assay dependent rank order variations were obvious as shown on Table 28. While heparin and hirudin once again showed strong effects, Ac-(D)Phe-Pro-boroArg-OH exhibited relatively comparable effects. The rank order of other antithrombin agents varied with the type of test used. Of notable interest was the behavior of these agents in the ECT assay, where heparin failed to show any anticoagulant effects and hirudin remained the strongest anticoagulant agent tested. Interestingly, argatroban in this assay was found to have comparable effects to the peptide arginal derivatives.

The animal models of thrombosis and bleeding provided a useful preclinical approach to compare the *in vivo* effects of heparin, hirudin and other antithrombin agents and relate these results to the biochemical and anticoagulant studies. These results are tabulated on Table 29. These studies were carried out only on heparin, hirudin, Ac-(D)Phe-Pro-boroArg-OH and D-MePhe-Pro-Arg-H. While hirudin was found to be strongest in most of these *in vivo* studies, heparin and Ac-(D)Phe-Pro-boroArg-OH also exhibited comparable or stronger effects in some models. To a large extent, the order

Table 27 -- Comparative rank order of potencies of various antithrombin agents in various biochemical assays.

	1	2	3	4	5	6	7
Ki	hirudin	Ac-FPboroR	argatroban	FPR	Me-FPR	Boc-FPR	-
ATU potency	hirudin	Ac-FPboroR	Me-FPR	FPR	Boc-FPR	argatroban	-
Fibrinogen-based clotting	hirudin	Ac-FPboroR	Me-FPR	argatroban	FPR	Boc-FPR	-
Amidolytic anti-IIa in NHP	heparin	hirudin	Ac-FPboroR	FPR	Me-FPR	Boc-FPR	argatroban
Amidolytic anti-Xa in NHP	Heparin	Ac-FPboroR	-	-	-	-	-
Fibrinogen-deficient assay: thrombin generation	hirudin	heparin	Ac-FPboroR	Me-FPR	Ac-FPboroR	Boc-FPR	argatroban
Fibrinogen-deficient assay: factor Xa generation	heparin	Ac-FPboroR	FPR, Me-FPR, Boc-FPR	-	-	-	-

FPR = D-Phe-Pro-Arg-H
Me-FPR = D-MePhe-Pro-Arg-H
Boc-FPR = Boc-D-Phe-Pro-Arg-H
Ac-FPboroR = Ac-(D)Phe-Pro-boroArg-OH

Table 28 -- Comparative rank order of potencies of various antithrombin agents in *in vitro* anticoagulant assays.

	1	2	3	4	5	6	7
PT	hirudin	Ac-FPboroR	argatroban	Me-FPR	FPR	Boc-FPR	-
APTT	heparin	Ac-FPboroR	hirudin	argatroban	Boc-FPR	Me-FPR	FPR
TT	hirudin	heparin	Ac-FPboroR	Me-FPR	FPR	Boc-FPR	argatroban
ECT	hirudin	Ac-FPboroR	FPR	Me-FPR	argatroban	Boc-FPR	-
Heptest	heparin	hirudin	Ac-FPboroR	argatroban	Me-FPR	FPR	Boc-FPR
ACT	Ac-FPboroR, hirudin, heparin	Me-FPR, argatroban	-	-	-	-	-
TEG	Ac-FPboroR, hirudin, heparin	Me-FPR	argatroban	-	-	-	-

FPR = D-Phe-Pro-Arg-H
Me-FPR = D-MePhe-Pro-Arg-H
Boc-FPR = Boc-D-Phe-Pro-Arg-H
Ac-FPboroR = Ac-(D)Phe-Pro-boroArg-OH

Table 29 -- Comparative rank order of potencies of various antithrombin agents in *in vivo* pharmacologic models.

	1	2	3	4
Rabbit stasis thrombosis, I.V. 10 min stasis	hirudin	heparin	Ac-FPboroR	Me-FPR
Rabbit stasis thrombosis, I.V. 20 min stasis	heparin	hirudin	Ac-FPboroR	Me-FPR
Rabbit stasis thrombosis, S.C. 10 min stasis	hirudin	Ac-FPboroR	heparin	Me-FPR
Rabbit stasis thrombosis, S.C. 20 min stasis	hirudin	heparin	Ac-FPboroR	Me-FPR
Rat laser induced thrombosis	heparin	Ac-FPboroR	hirudin	Me-FPR
Rabbit hemorrhagic model, I.V., 5 and 20 min	hirudin	Ac-FPboroR	Me-FPR	heparin
Rabbit hemorrhagic model, S.C., 45 min	hirudin	Ac-FPboroR	Me-FPR, heparin	-
Rabbit hemorrhagic model, S.C., 60 min	Ac-FPboroR	Me-FPR, hirudin, heparin	-	-

FPR = D-Phe-Pro-Arg-H
Me-FPR = D-MePhe-Pro-Arg-H
Boc-FPR = Boc-D-Phe-Pro-Arg-H
Ac-FPboroR = Ac-(D)Phe-Pro-boroArg-OH

of potency in the rabbit model of thrombosis followed consistent patterns. This order followed a different ranking pattern for the rat laser model of thrombosis, where heparin was found to be strongest and hirudin was relatively weaker than both heparin and Ac-(D)Phe-Pro-boroArg-OH. Similarly, in the bleeding model, the order of hemorrhagic effects followed a different pattern, which was also route dependent. Heparin was found to be relatively weaker in comparison to all other antithrombin agents. These studies clearly warrant the necessity of evaluating the results of the *in vitro* and *in vivo* screening in a multiparametric integrated fashion. The data presented here is therefore of crucial importance in projecting the biochemical and pharmacological actions of newly developed antithrombin agents.

This dissertation therefore emphasizes the importance of pharmacologic profiling for new antithrombin agents as the direct antithrombin effects and other plasmatic or vascular actions of these agents can only be evaluated in valid pharmacologic studies. It is important to point out that some of the recent adjustments of clinical trials and the reported adverse events due to both the safety and efficacy compromise were largely based on incomplete preclinical pharmacological information.

O. Developmental Perspectives

The current developmental perspectives on various thrombin inhibitors are summarized in Table 30. Site-directed antithrombin agents such as recombinant hirudin, hirulog and synthetic tripeptides are currently undergoing various phases of clinical trials. These agents were initially developed with the premise that sole targeting of thrombin may be the most important step in the development of anticoagulants and antithrombotic

drugs to treat thrombotic and cardiovascular disorders (Lefkovits and Topol 1994, Verstraete and Zoldhelyi 1995). It is nearly five years that these agents have undergone clinical trials. Hirudin and hirulog have completed several phase II trials, whereas the peptide D-MePhe-Pro-Arg-H, marketed under the generic name efegatran sulfate, has recently entered a phase II trial. The primary focus of these trials has been in cardiovascular areas such as pretreatment of ischemic heart disorders, anticoagulation during coronary angioplasty, treatment of post-interventional abrupt closure and late reocclusion (restenosis) and adjunct usage during thrombolysis. It is too early to suggest that these agents have a definitive advantage over heparin. However, in heparin compromised patients, these new anticoagulants may be useful on a compassionate basis. The cost of hirudin therapy is relatively higher than heparin. The relative cost for the synthetic antithrombin agents may be lower than hirudin, however, the true cost analysis can only be valid after a comprehensive pharmacoeconomic analysis for specific prophylactic and therapeutic indications.

Several peptidomimetic antithrombin agents are also currently in clinical development for both cardiovascular and therapeutic indications. These include argatroban (Mitsubishi, Japan), inogatran (Astra, Sweden) and napsagatran (Roche), Switzerland). The results of some of these clinical trials are expected later this year. In addition, many other antithrombin agents are now in active preclinical development.

Of the various peptide derived antithrombin agents, efegatran is the only agent which is currently developed for cardiovascular indications. Many other agents developed by different companies were initially developed in various preclinical studies,

Table 30 -- Developmental Status of Site-Directed Thrombin Inhibitors

	Chemical Nature	Developmental Status
Hirudin and its analogues	Recombinant protein	Developed for both arterial and venous thrombosis
Hirulogs	Synthetic bifunctional oligopeptides	Several clinical studies are completed; additional studies are planned in various indications
Peptidomimetics	Synthetic heterocyclic derivatives (argatroban, inogatran, napsagatran)	Phase II clinical development in the USA; used in Japan
Peptides and their derivatives	Peptide arginals (efegatran)	Phase II clinical development
Aptamers	DNA and RNA derived oligonucleotides with thrombin-binding domains (defibrotide)	Preclinical stage; limited animal data available
Plasma derived antithrombin	Natural proteins and their recombinant equivalent products	Antithrombin (AT-III) is currently used. HC-II is still in developmental status
Transition state peptide analogues	Oligopeptides and synthetic organic agents (boronic acid derivatives)	Preclinical screening is being completed

but due to problematic developmental issues, their clinical development was discontinued.

Aptamers represent both DNA and RNA derived oligonucleotides with weaker anticoagulant effects. As such, these agents are not developed for the purpose of anticoagulation. However, some of these agents show antithrombin actions in the *in vitro* settings. A mammalian DNA derived product, defibrotide (Crinos, Italy), has been used as an anti-ischemic drug, however, its actions are not solely attributed to the inhibition of thrombin. This agent, like heparin, produces multiple pharmacologic effects on platelets, endothelial cells and also release of TFPI (Fareed et al. 1995).

Despite the isolation and characterization of various antithrombin proteins from human plasma and development of their recombinant equivalent, antithrombin obtained by the fractionation of human plasma remains the only thrombin inhibitor for clinical use (Menache 1991, Fourrier et al. 1993). Several products are currently available for the treatment of congenital thrombophilia and acquired hypercoagulable state including DIC. HC-II has also become available but is not currently used.

Many other synthetic and recombinant approaches have been used to develop antithrombin agents at this time. Of these, the development of the transition state analogues has been pursued. Despite developing very potent antithrombin agents using this approach, these agents have not been clinically developed due to toxic effects.

From the studies reported in this dissertation and the published data, it is clear that the currently available thrombin inhibitors are not solely specific for thrombin. With the exception of hirudin, most of the other thrombin inhibitors are capable of producing several additional effects including inhibition of non-thrombin serine proteases,

interacting with vascular sites and modulating various regulatory processes. Some of these effects are depicted in Fig. 61.

Most antithrombin agents have been designed and developed as specific inhibitors of thrombin. However, these agents exhibit variable interactions with the serine protease network, including the fibrinolytic, kallikrein and complement system. Since thrombin belongs to the serine protease family of enzymes, as do most of the coagulation factors and the fibrinolytic enzymes, developing an inhibitor with strong affinity for thrombin and none for the other enzymes remains a challenge to the pharmaceutical industry. While aptamers, hirudin and hirulog have inhibitory activities only towards thrombin due to their unique mode of action (they recognize and inhibit sites on thrombin other than the catalytic site, which are unique for thrombin), the other thrombin inhibitors, which are designed to bind to the catalytic site of thrombin have weaker inhibitory activities against other enzymes as well. Thus, at high doses, these agents may interfere with the fibrinolytic system and with APC. This interaction may have some safety compromise in patients who are treated with these drugs, especially at high dosages (Thèroux 1995).

Another important issue is the inhibition of thrombomodulin-bound thrombin by the low molecular weight thrombin inhibitors. While thrombomodulin-bound thrombin is devoid of procoagulant effects, it retains its anticoagulant effects since it is still capable of activating protein C (which functions both as an anticoagulant by inactivating factor Va and factor VIIIa and as a pro-fibrinolytic by inactivating PAI-1 and PAI-3). The importance of thrombomodulin-bound thrombin inhibition remains to be clarified. However, as reported in clinical trial results with hirudin, the lack of dose-dependency

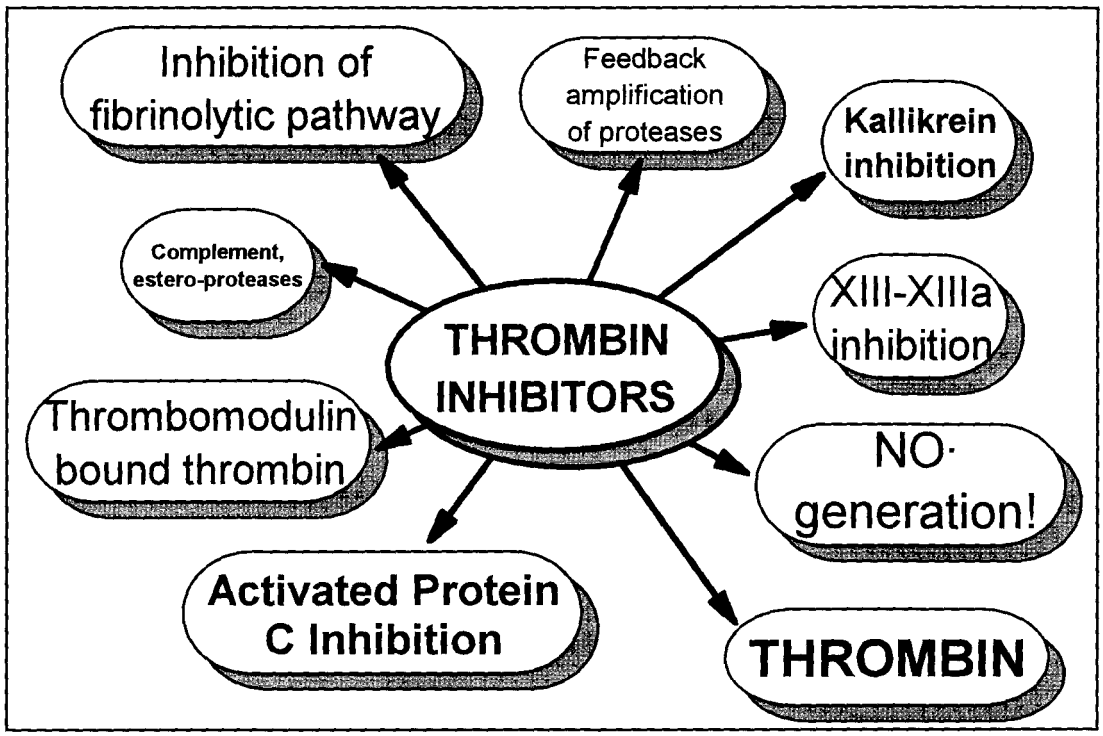


Fig. 61. Sites of Actions of Thrombin Inhibitors. Beside the inhibition of thrombin, these agents are also capable of inhibiting some of the regulatory actions of thrombin, such as the activation of protein C through thrombomodulin bound thrombin, stabilization of clot through factor XIIIa and primary hemostatic activation of platelets. The non-thrombin mediated activities of these agents include the inhibition of fibrinolytic and kallikrein related enzymes, feedback amplification of proteases (thrombin and non-thrombin), modulation of complement esteroproteases and potential modulation of nitric oxide pathways.

after a threshold dose of hirudin, indicate that thrombomodulin-bound thrombin plays an significant role in the overall anticoagulant response (Thèroux 1995).

Some of the thrombin inhibitors also possess direct vasodilatory effects, independent of their activity on thrombin. These effects are believed to be mediated through metabolic products containing guanidino groups which result in the increase of nitric oxide generation (Fareed and Callas 1995). Depending on the specific indication for which the thrombin inhibitor is developed, this vascular effect may be desirable. Nevertheless, this type of effect should be investigated and considered.

The pathophysiologic role of thrombin extends beyond coagulation and platelet activation. Thrombin also has direct effects on other cell types and functions, ranging from mitogenesis and contraction of smooth muscle cells to adhesion and metastasis of tumor cells to neurite growth regulation to endothelial cell stimulation (Tapparelli et al. 1993). Although the functions of thrombin in such processes as atherogenesis and cancer metastasis have not been fully characterized, a thrombin inhibitor will interfere with all of thrombin's bioregulatory functions. These observations may have some impact on the development of thrombin inhibitors in cancer and cardiovascular indications.

Ever since their introduction, most of the clinical indications for these new anticoagulants have been in the interventional cardiovascular areas (Herrman and Serruys 1994). Initial focus has been on the prevention of abrupt closure during coronary angioplasty (Topol et al. 1993, Topol 1995). These agents have also been tried for the post-PTCA prevention of both the early and late reocclusion (Suzuki et al. 1995). In addition, some of the thrombin inhibitors have been used for the treatment of unstable

angina (Topol 1995). More recently, some of these agents have been tested for their efficacy in stenting (Stemberger et al. 1996, van Beusekom et al. 1994, Buchwald et al. 1993). Although several reports on the experimental use of these antithrombin agents have been made available on their use in cardiovascular surgery in animal models, only isolated reports in human studies are available (Riess et al. 1995). Since a known antagonist to neutralize the effects of antithrombin agents is not available, concerns over the use of these agents have been expressed (Edmunds 1995). Currently, no pharmacologic antagonists exist for hirudin or any of the other direct thrombin inhibitors, making the managing of precipitated hemorrhagic effects problematic. Current hirudin reversal strategies employed include prothrombin complex concentrate to neutralize the circulating antithrombin agents (Irani et al. 1995) or dialysis to remove an antithrombin agent from the blood (Fareed et al. 1991). Strategies that are under development of effective neutralizing agents are production of mutant thrombin molecules that are devoid of clotting activity but for which antithrombin agents retain a high affinity, thus saturating circulating levels of these agents (Fischer et al. 1996).

Thrombin inhibitors are attractive to both clinicians and surgeons as substitute anticoagulants in heparin compromised patients in particular. For patients exhibiting heparin induced thrombocytopenia and requiring anticoagulation, several trials are currently in progress to test the efficacy of new antithrombin agents (Edmunds 1995, Chamberlin et al. 1995).

Although thrombin inhibitors are potent anticoagulants and unlike heparin do not require any endogenous cofactors for their actions, their development as surgical

anticoagulants has been rather slow due to the non-availability of a pharmacologic antagonist. Only when such an antagonist is available and clinically validated, these agents may find their way into surgical applications.

There is a growing interest in the use of these agents in the prevention and treatment of DVT (Bridey et al. 1995). A recent study (Eriksson et al. 1996) reported on the successful use of recombinant hirudin in the prophylaxis of post-orthopaedic surgery. In this study unfractionated heparin (5000 IU s.c. t.i.d.) was compared with hirudin (15 mg s.c. b.i.d.), which was found to be superior. Several additional trials are currently being conducted on different antithrombin agents.

Current clinical trends also point to polypharmacologic approaches for the treatment and management of cardiovascular disorders. Combination modalities of anticoagulants with other drug classes such as antiplatelet and fibrinolytic drugs has been employed (Théroux et al. 1995, Lee 1995, Cannon and Braunwald 1995). While clinically developed thrombin inhibitors are reportedly specific for thrombin inhibition, these agents exhibit interactions with other drugs. Antiplatelet drugs such as aspirin and ticlopidine have additive or synergistic actions when used in conjunction with thrombin inhibitors (O'Donnell et al. 1995). Similar interactions would be expected when thrombin inhibitors would be used with heparin and low molecular weight heparins or antithrombin III concentrates (Biemond et al. 1994). On the other hand, adjunct usage of thrombin inhibitors with thrombolytic agents, at an optimized dose, could result in the facilitation of thrombolysis or, if the thrombin inhibitor dose is not adjusted, in thrombolytic compromise. Some of these agents may also directly inhibit activated

protein C, or inhibit activated protein C formation by neutralizing thrombomodulin-bound thrombin (Théroux et al. 1995, Callas et al 1995, Callas and Fareed 1995). Furthermore, thrombin inhibitors may neutralize the coagulant actions of factor VIIa and prothrombin complex concentrates (Biemond et al. 1994). Therefore, it is desirable that the interactions of thrombin inhibitors are well studied and considered when designing drug combination protocols for clinical applications, as such interactions may have both safety and efficacy implications.

The development of thrombin inhibitors has added a new dimension in the management of thrombotic and vascular disorders. While the major clinical indications for these agents include interventional cardiovascular procedures and DVT prophylaxis, little is known for their use in the area of surgical and therapeutic anticoagulation. It is projected that many of the newer thrombin inhibitors, such as hirulog and hirudin, will be developed as anticoagulants and may be substituted for heparin. Synthetic tripeptides with broader serine protease inhibitory spectrum offer unique tools to control thrombogenesis at different levels and may prove to be more useful in chronic indications, including prophylactic usage. The usefulness of various thrombin inhibitors in a given medical or surgical indication can be proven only in properly designed clinical trials.

CHAPTER VI

SUMMARY

A. *In Vitro* Studies

1. The molecular profile was investigated in the case of the peptide arginals, Ac-(D)Phe-Pro-boroArg-OH and unfractionated heparin. The specifications on hirudin and argatroban indicated these agents to be >90% pure and homogeneous. The peptide arginals and Ac-(D)Phe-Pro-boroArg-OH exhibited hydrate form transformations. As expected, heparin was determined to be polycomponent and exhibited similar molecular weight profile in both the UV and RI analyses.

2. The relative antithrombin potency (ATU) for each thrombin inhibitor as measured by an amidolytic method was found to widely vary in the range of 2,000-200,000 ATU/mg (1,000-156,000 ATU/nmol). These unit potency values were not consistent with the published Ki and IC_{50} values. This may be due to differences in the experimental methods, the choice of thrombin preparations and the mathematical analyses of the generated data.

3. When the thrombin titration was carried out on the natural substrate of thrombin, fibrinogen, against the commercially available human thrombin preparation, all of the thrombin inhibitors exhibited different degrees of antithrombin potency. However, heparin did not exhibit any antithrombin potency in these assays. The rank

order for the relative antithrombin actions of these agents is given as follows: hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > argatroban > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H.

4. The amidolytic antithrombin potencies were also measured for heparin and various antithrombin agents in normal human plasma and normal rabbit plasma based assays. In these assays, heparin was found to be the strongest inhibitor (IC_{50} of 6.1-9.6 nM). Species dependent variations were noted. The various thrombin inhibitors exhibited the following rank order of potency: heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban.

5. In the amidolytic anti-Xa assays performed in the normal human plasma and normal rabbit plasma assays, except for heparin and Ac-(D)Phe-Pro-boroArg-OH, none of the other thrombin inhibitors produced any anti-Xa effects. Heparin in both systems was found to be an efficient inhibitor of factor Xa.

6. The antiprotease profile for each of the individual thrombin inhibitors and heparin/antithrombin complex was investigated utilizing chymotrypsin, trypsin, activated protein C, glandular kallikrein, tPA, urokinase and plasmin. Aprotinin was also included as a reference inhibitor. None of the thrombin inhibitors produced any inhibition of chymotrypsin. With the exception of hirudin and argatroban, all of the thrombin inhibitors produced varying degrees of inhibition of these stated enzymes with IC_{50} values in the μM range. These studies clearly indicate that different thrombin inhibitors are capable of producing measurable inhibition of non-thrombin serine proteases of the fibrinolytic and kallikrein system, at concentrations which are least one order of

magnitude greater than those observed for the inhibition of thrombin.

7. The thrombin and factor Xa generation studies were carried out in fibrinogen deficient plasma to compare the relative ability of heparin and these thrombin inhibitors on the generation of these enzymes after extrinsic and intrinsic activation. All agents produced varying degrees of the inhibition of thrombin and factor Xa generation, following the order of hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban for extrinsic and intrinsic thrombin generation and with the following order for extrinsic factor Xa generation: heparin > Ac-(D)Phe-Pro-boroArg-OH > D-Phe-Pro-Arg-H, D-MePhe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H.

8. The influence of these agents on the generation of factor Xa and thrombin was also carried out in activated and native prothrombin complex concentrates. After extrinsic activation, varying degrees of inhibition of both of these enzymes was noted. Activation and system variations were evident and each inhibitor showed an individual profile. In comparison to heparin, thrombin inhibitors produced relatively stronger anti-Xa than antithrombin effects. Ac-(D)Phe-Pro-boroArg-OH was found to be the strongest inhibitor in most of these assays.

9. Each of the thrombin inhibitors and heparin exhibited a distinct inhibitory profile in the norman human plasma based clotting assays, such as the PT, APTT, TT, Heptest and ECT. The relative anticoagulant potency was also both agent and assay dependent. In each test, the following potency rank orders were observed:

PT: hirudin, Ac-(D)Phe-Pro-boroArg-OH > argatroban > D-MePhe-Pro-Arg-H > D-Phe-

Pro-Arg-H > Boc-D-Phe-Pro-Arg-H

APTT: heparin > Ac-(D)Phe-Pro-boroArg-OH > hirudin > argatroban > Boc-D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H

TT: hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H > argatroban

Heptest: heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > argatroban > D-MePhe-Pro-Arg-H > D-Phe-Pro-Arg-H > Boc-D-Phe-Pro-Arg-H

ECT: hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-Phe-Pro-Arg-H > D-MePhe-Pro-Arg-H > argatroban > Boc-D-Phe-Pro-Arg-H

10. The relative anticoagulant effects of heparin and various inhibitors were also investigated in the whole blood ACT and TEG assays. In both assays, a concentration-dependent anticoagulant effect was evident in the ACT (rank order: hirudin, heparin, Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H, argatroban) and the TEG (rank order: hirudin, heparin, Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > argatroban).

11. For the comparative anticoagulant effects in the normal rabbit plasma based PT, APTT, TT and Heptest, heparin, hirudin, D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH exhibited distinct profiles. The relative anticoagulant potency was also both agent and assay dependent. In each test, the following rank orders were observed:

PT: hirudin, Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H

APTT: heparin > Ac-(D)Phe-Pro-boroArg-OH > hirudin > D-MePhe-Pro-Arg-H

TT: hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H

Heptest: heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H

12. Heparin and various thrombin inhibitors also produced varying degrees of anticoagulant actions in the ACT and TEG assays performed in the native rabbit whole blood. The relative anticoagulant effects were both assay and agent dependent. Thus, each method provided a different degree of anticoagulant potency.

B. *In Vivo* Studies

1. Recombinant TF was found to produce dose-dependent thrombogenic effects in a rabbit jugular vein stasis thrombosis model. The apparent ED_{50} for producing the thrombogenic response at 10 min was determined to be 0.34 pmol/kg. At 20 min, the ED_{50} was about 4 pmol/kg. The relative thrombogenic response of rTF at 10 and 20 min did not appear to be significantly different. At dosages of up to 1 pmol/kg I.V. administration, rTF did not produce any alterations in the clot based tests such as the PT, APTT, TT and Heptest. However, in the ACT and TEG tests, at doses ranging from 0.1-1 pmol/kg, a dose-dependent anticoagulant effect was noted. The mechanism for this rTF produced effect on ACT and TEG was not explored.

2. Heparin, hirudin, D-MePhe-Pro-Arg-H and Ac-(D)Phe-Pro-boroArg-OH produced dose-dependent antithrombotic effects at both the 10 and 20 min end points. The order of potency for these agents was found to be hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H at the 10 min end point and heparin > hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H at the 20 min end point. The *ex vivo* analyses of the blood revealed sizeable antithrombin activities. However, the relative anti-Xa activities were markedly lower. The ACT and TEG test also showed individual anticoagulant profiles for each of these agents. In the plasmatic clotting tests,

while the PT remained unaffected, agent specific anticoagulant effects were noted in the APTT, TT and Heptest. Ac-(D)Phe-Pro-boroArg-OH produced the strongest anticoagulant effects.

3. In the S.C. studies, a dose-dependent and agent specific antithrombotic action was noted with all four agents. The order of potency was found to be hirudin > Ac-(D)Phe-Pro-boroArg-OH > heparin > D-MePhe-Pro-Arg-H and hirudin > heparin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H at both the 10 and 20 min end points respectively. Dose-dependent antithrombin effects were also evident with all four agents. However, relatively weaker anti-Xa activities were observed. The *ex vivo* ACT and TEG analyses also revealed agent and dose-dependent effects. The *ex vivo* analyses of plasma revealed a dose-dependent prolongation of the APTT, TT and Heptest. However, no effects were observed in the PT assay. On a molar basis, hirudin was found to be the strongest antithrombotic agent in these studies.

4. At a fixed I.V. dosage, each of these agents produced a time-dependent antithrombotic action. Regardless of the antithrombotic potency, each of these agents showed varying duration of the antithrombotic and *ex vivo* effects as measured by various tests. The duration of action followed the order of hirudin > D-MePhe-Pro-Arg-H > heparin > Ac-(D)Phe-Pro-boroArg-OH. With the exception of the PT, the other *ex vivo* tests showed time-dependent agent specific effects.

5. In the S.C. studies, fixed doses of each of these inhibitors also produced time-dependent antithrombotic and assay-dependent *ex vivo* effects on various parameters. In contrast to the I.V. studies, the subcutaneously administered agents produced sustained

antithrombotic actions. Heparin and hirudin exhibited relatively longer duration of actions in contrast to the peptides used.

6. Heparin and antithrombin agents also produced dose-dependent antithrombotic actions in a rat model of laser-induced arterial thrombosis. The antithrombotic potency in this model was determined to be heparin > Ac-(D)Phe-Pro-boroArg-OH > hirudin > D-MePhe-Pro-Arg-H.

7. The hemorrhagic actions of heparin and thrombin inhibitors were investigated in a rabbit model of ear blood loss at 5 and 20 min post agent administration. Each of these agents produced a dose-dependent hemorrhagic effect. The hemorrhagic potential for these agents is graded in the following order: hirudin > Ac-(D)Phe-Pro-boroArg-OH > D-MePhe-Pro-Arg-H > heparin. The duration of the hemorrhagic action for each of these agents was also investigated at a fixed dosage. The hemorrhagic actions of these agents followed different time courses and were cleared within 60 min.

8. Subcutaneously administered heparin and antithrombin agents were also found to produce hemorrhagic effects in the rabbit ear blood loss model at 45 and 60 min circulation times. Hirudin and Ac-(D)Phe-Pro-boroArg-OH produced a dose-dependent effect. However, heparin and D-MePhe-Pro-Arg-H produced relatively weaker effects. At a fixed dosage, the time course of the hemorrhagic actions of D-MePhe-Pro-Arg-H decreased with time, whereas hirudin and Ac-(D)Phe-Pro-boroArg-OH showed biphasic responses. The hemorrhagic actions of these agents peaked at 45 min. While the effect produced by hirudin reverted to baseline, the action of Ac-(D)Phe-Pro-boroArg-OH remained elevated at 90 min.

CHAPTER VII

CONCLUSIONS

Antithrombin agents represent a chemically and functionally diverse variety of pharmacologic agents with different degrees of specificity for the inhibition of thrombin. As a class, these agents have been found to be distinct from heparin in terms of their mechanism of action and pharmacodynamic effects. Independent of the antithrombin potency for each of the individual agents as measured by either the Ki or the IC_{50} based titration of the antithrombin actions, these agents exhibit several endogenous effects which are not completely attributable to their relative inhibitory action on thrombin. Furthermore, each of the thrombin inhibitors exhibits a distinct anti-serine protease and protease generation inhibitory profile which also contributes to its biochemical and pharmacologic identity. This research also provided experimental evidence that sole targeting of thrombin is not enough in the control of thrombogenesis and additional endogenous factors which contribute to these processes. Furthermore, indiscriminate inhibition of thrombin may compromise the regulatory roles of thrombin, such as the activation of protein C, clot stabilization through factor XIIIa, hemostatic process mediated through platelets and other cellular functions. It is concluded that beside distinct differences amongst various thrombin inhibitors, these agents also exhibit non-thrombin mediated biochemical and pharmacologic actions which may not be related to

their actions on thrombin. Even in the case of highly specific thrombin inhibitors such as hirudin, several distinct actions of this agent are responsible for some of the additional biochemical and pharmacologic effects which can contribute to its therapeutic index. Thus, a comprehensive profile for each agent is a prerequisite for an objective development of these new anticoagulant and antithrombotic drugs. Furthermore, for the clinical development in specific vascular and thrombotic indications, these drugs should not be developed mimicking the approaches which have been used for heparin, rather each agent should be considered as a distinct pharmacologic agent with its own profile and be developed as such. Biochemical and pharmacologic modelling approaches can be used to mimic specific thrombotic and cardiovascular disorders where each of these new antithrombin agents can be profiled. On the basis of these investigations, some projections can be made. Such an approach may be more cost-effective and will minimize the necessity for large scale, costly and time consuming clinical trials where the safety issues are not always clear.

APPENDIX I

PRODUCT SPECIFICATION SHEETS

BPN 4724-97

CONT. FROM p. TO p

DATE 2/5/92 DIRECTED BY

SUBJECT Ac-(D)Phe-Pro-boroArg-OH·HCl

REFERENCES Ac-(D)Phe-Pro-boroArg-OH·BSA (3-E10-111) was converted to the HCl salt. 130 mg of 3E10-111 was remained and labeled. The remaining sample, 6.4g, was dissolved

OBJECT in ~150 ml of water and passed through a column containing 40 ml of Biorad AG-1-X8 (Cl form). Fractions containing the desired product were evaporated & then dried overnight in vacuo with P_2O_5 & KOH. The product was triturated with ether, isolated, and dried under high vac to yield 4.8g.

Product assigned IN# P8714-24. A sample was submitted for proton nmr and in addition, a sample was given Margaret Krueller for HPLC analysis.

SERSJ 5 DUP714_07FEB92,5,1
rted on 7-FEB-1992 at 14:27

Injection Report

Acquired on 7-FEB-1992 at 10:02

VAX MULTICHROM - ESDM02

Analyst Name : MAK
Lims Id :
Comment :
Method Title :
Sample Name : DUP714-24
Sample Id :
Sample Type : Sample Amount=1.00000
Bottle No : 3

PEAK INFORMATION

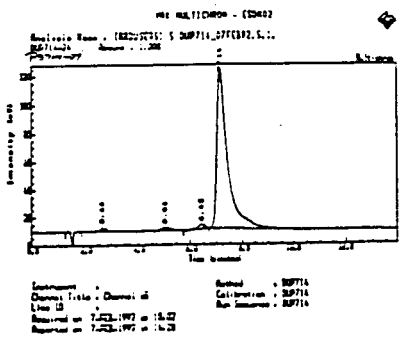
RT mins	Area uVs	Area %	AREA	Peak name
2.638	69280	2.05	0.000	
5.058	61035	1.81	0.000	
6.427	49023	1.45	0.000	
7.147	3196721	94.69	3196721.000	DUP714 ←
Totals				
Unknowns	0	0.00	N/A	
	3376059	100.00	3196721.000	
	3376059	100.00	3196721.000	

ANALYSIS SUMMARY

Method..... DUP714
Run sequence..... DUP714
Calibration..... DUP714
External standard calibration using area
Calibration last modified on 7-FEB-1992 at 08:55

Uncalibrated peaks use user factor (0.0000)

NO WRITING UNDERNEATH



NO WRITING UNDERNEATH

0.5g of DuP714-24 were given to John Blaskai (SP) for Anti Flu test. 0.5g were labeled + retained. The email was given Bill Kaiko for further testing 3/19/92.

EXPERIMENTER Laurence J. Mersinger DATE JUN 18 1992

WITNESS John D. Williams DATE 6/19/92

BASF Hauptlaboratorium

18.10.88/gr
ZEB/? - A 30
Tel.: 4 23 04

Reaktionsnotiz	~~Verfahrensnotiz~~	~~Herstellvorschrift~~	PRODAS Ref.-Nr.
Labornotiz	~~Laborbericht~~		Nr. 5238

Bearbeiter; Dr. Kurfürst

Thema: Vergleich von rekombinantem Hirudin (BASF) mit natürlichem (Pentapharm)

Formelschema incl. wichtige Details wie Ausbeute, Reagenzien, Reaktionsbedingungen, Lit.-Seite ...

Erläuterungen: Problemstellung, Zielsetzung, Ergebnis, Besonderheiten, Vorschrift, optimale Fahrweise

Das im Labor Dr. Lang renaturierte und chromatographisch aufgereinigte Hirudin wird von einer Kationenaustauschersäule in 2 aktiven Peaks in einem Verhältnis von P1:P2 = 1:2 eluiert. Ein Vergleich der beiden Hirudinspezies miteinander und mit natürlichem Hirudin (Pentapharm) zeigt, daß P2 in allen untersuchten Parametern identisch zum natürlichen Hirudin ist. Demgegenüber zeigt P1 sowohl bei den Ionenaustauschchromatographien als auch bei der isoelektrischen Fokussierung ein saureres Verhalten (vgl. Tabelle 2). Weiter zeigen Experimente zum einen, daß P2 bei Anwesenheit von Ameisensäure in P1 überführt werden kann (Fig. 1). Zum anderen, daß bei Verwendung von geringeren Konzentrationen Ameisensäure während der Grobreinigung das Verhältnis von P2 zu P1 im Endprodukt zugunsten von P2 verschoben werden kann (Fig. 2 + Fig. 3). Diese Ergebnisse und die Sequenzierung des gesamten Hirudinmoleküls deuten darauf hin, daß es sich bei der Hirudinspezies P1 um ein aktives Hirudin handelt, das an der Position 47 (Lysin) vermutlich formyliert ist. An der Aufklärung der Hirudinmodifikation wird gearbeitet.

○kont. ⊗diskont. ⊗Labor ○Technikus ○Betrieb

Anlagen: ... S. Text, 1. Tabelle(n), 1. Grafik(en)

Lit. (intern, extern)
L.J. 20 942
21 594

Verteiler:
D. [illegible] (ZHB/C)

Unterschrift

Anlage zur Labormotiz Nr. 5238 vom 18.10.88 (Dr. Kurfürst)

Tabelle 2:

Vergleich der Hirudinspezies

	P1	P2	Pentapharm* (natürliches Hirudin)
spez. Aktivität (U/mg)	8200	7900	7200
Mono Q (Retentionszeit)	14,7	14,0	14,0
RP (Retentionszeit)	29,0	28,9	28,9
IEF (pI)	3,5	3,7	3,7
Absorption/Fluoreszenz	276 nm (300-310 nm)	276 nm	276 nm
Fluoreszenz/exc. 280 nm	307 nm	307 nm	307 nm
CD-Spektrum	identisch		
N-term. Sequenz	VVYTDCTESG		
C-term. Sequenz	QL		
Proteinsequenzierung	Fremdpeak zwischen His und Ala (an der Stelle 47 Lys)		identisch mit natürlichem Hirudin

* Da sich das natürliche Hirudin in allen untersuchten Analysenmethoden dem P identisch zeigte, stellt sich die Frage, ob das Hirudin der Firma Pentaphar nicht bereits in desulfatisierter Form vorliegt.

[redacted] **PENTAPHARM AG** 12.20 **BASEL**

Analysen-Zertifikat Certificat d'Analyse Certificate of Analysis

Produkt, Produit, Product: A P R O T I N I N 6120 KIU/mg

Lot 5388/074

Prüfungs-Resultate, Résultats de l'examen, Examination Results:

Aspect:		white, solid lyophilisate
Solubility in water at 20 mg/ml		clearly soluble
pH of the aqueous solution (0,2%)		7,35
Activity		6,8 TIU/mg = 6'120 KIU/mg
Water content	(K.Fischer)	7,79 %
Preservative		none
Protein content (UV)		0,88 mg/mg
SDS - PAG - Electrophoresis		on single band
Heparin neutralizing activity		< 3 U/90'000 KIU

Befund, Conclusion, Conclusion:

Basel/Schweiz,
Bâle/Suisse,
Basle/Switzerland, November 22, 1989

Für die verantwortliche Abteilung:
Pour le service responsable:
On behalf of the responsible department:

Code: A 29772

O. Ries.

APPENDIX II

BLOOD DONATION CONSENT FORM

LOYOLA UNIVERSITY MEDICAL CENTER
MAYWOOD, ILLINOIS
SPECIAL HEMATOLOGY (PATHOLOGY)
HEMOSTASIS RESEARCH (PATHOLOGY)

INFORMED CONSENT

Patient's Name:_____ Date:_____

Project Title: Consent Form for Volunteer Blood Donors

VOLUNTEER INFORMATION

A sample of your blood is to be drawn from a vein for the purpose of being used as a control for procedures performed in the Hematology/Hemostasis Research Laboratories. The amount of blood drawn from you, the type of test performed on this sample (as a control) is marked on the following lists:

TEST	AMOUNT OF BLOOD REQUIRED
___ Platelet Aggregation Study	9 ml citrated blood (Butterfly Syringe method)
___ Osmotic Fragility Studies	5 ml blood (Plastic syringe)
___ Autohemolysis	5 ml blood (syringe method)
___ NBT Test (White cell function test)	10 ml heparinized blood (Vacutainer method)
___ Leucocyte Chemotaxis	10 ml heparinized blood (Syringe method)
___ Whole Blood Viscosity Studies	10 ml heparinized blood (Syringe method)
___ For the Preparation of Normal Human Pool Plasma and other Blood Components	10 ml citrated blood (Syringe method)

For these tests there is no commercially available control at this time. For the proper interpretation of these tests, fresh normal human blood is needed. The drawing of your blood from the vein is a well established procedure which has very little risk. Slight pain and a small amount of bleeding may occur. Occasionally, a blood clot may form in the vein, but this is rare and seldom produces permanent damage.

(over)

VOLUNTEER INFORMATION (continued)

No direct benefit is expected to any one individual, but it may eventually improve the quality of tests done on blood in the future, and may identify certain diseases more accurately. There is no alternative procedure at this time which would permit us to obtain the amount of human blood we need for the test.

CONSENT

I have fully explained to _____
Name: Volunteer
the nature and purpose of the above described procedure and the risks that are involved in its performance. I have answered and will answer all questions to the best of my ability.

_____
(Signature: Special Hematology Personnel)

I have been fully informed of the above described procedure with its possible benefits and risks. I give permission for my participation in this study. I know that _____ or his/her associates will be available to answer any questions I may have. If, at any time, I feel my questions have not been adequately answered, I may request to speak with a member of the Medical Center Institutional Review Board. I understand that I am free to withdraw this consent and discontinue participation in this project at any time without prejudice to my care. I have received a copy of this informed consent document.

_____
(Signature: Volunteer)

_____
Signature: Witness to Signatures)

APPENDIX III

LETTERS OF PERMISSION

STATE OF NEW YORK
DEPARTMENT OF HEALTH

Wadsworth Center The Governor Nelson A. Rockefeller Empire State Plaza P.O. Box 509 Albany, New York 12201-0509

Barbara A. DeBuono, M.D., M.P.H.
Commissioner

Karen Schimke
Executive Deputy Commissioner

February 5, 1996

Ms. Demetra Callas
Department of Pathology
Loyola University Medical Center
2160 South First Ave.
Maywood, IL 60153

Fax: 708-216-6660

Dear Ms. Callas:

As far as I am concerned, I have no objections to you using figures from a previous publication by D.H. Bing and myself (Thromb. Hemost. 14: 234-240, 1988). However, I do not know if the publisher of the journal require written permission for reproduction of such materials.

Sincerely,

John W. Fenton, II, Ph.D., FACB
Phone: 518-486-2562/2119
Fax: 518-474-7992

JWF/lmj

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

February 13, 1996

Mr. Chris Hall
Thieme Medical Publishers
381 Park Ave
New York, NY

Dear Mr. Hall,

Thank you for your assistance regarding my inquiry about reproducing a figure published in Seminars in Thrombosis and Hemostasis. I am a graduate student in the Ph.D. program of the Department of Pharmacology at Loyola University Chicago, in the final stages of my training, under the guidance of Dr. Jawed Fareed. The figure I would like to get permission to reproduce is Fig. 1 ("A 3-dimensional model of human α-thrombin") published in Seminars in Thrombosis and Hemostasis, 14(3):235, 1988, in the article authored by Dr. Fenton. My intention is to reproduce this figure in my dissertation in the literature review section, for visual representation and clarification of the structure of thrombin. If there is any other information that you need, do not hesitate to contact me.

Sincerely

Demetra Callas

Demetra Callas

Phone: 708-216-5587
Fax: 708-216-6660
E-mail: dcallas@wpo.it.luc.edu

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Kim Hall
Permissions Coordinator

Feb. 14 1996
Date

APPENDIX IV

DATA TABLES

Table 8a -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

D-Phe-Pro-Arg-H		
ng/ml	nM	% Residual Thrombin Activity*
21.28	4.25	7.7 ± 0.9
15.96	3.19	9.6 ± 1.8
13.83	2.76	11.0 ± 3.6
12.77	2.55	11.9 ± 3.0
11.70	2.34	13.9 ± 2.9
10.64	2.13	15.4 ± 0.9
10.11	2.02	16.5 ± 4.4
9.57	1.91	17.8 ± 6.9
9.31	1.86	18.8 ± 2.4
9.04	1.81	20.2 ± 4.9
8.78	1.75	21.4 ± 2.9
8.51	1.70	22.7 ± 4.5
8.24	1.65	23.3 ± 4.5
7.98	1.59	25.6 ± 3.4
7.71	1.54	25.8 ± 3.8
6.91	1.38	26.1 ± 3.7
6.38	1.28	31.3 ± 3.6
5.85	1.17	33.8 ± 5.4
5.59	1.12	35.0 ± 3.3
5.32	1.06	39.1 ± 1.2
5.05	1.01	41.4 ± 5.0
4.79	0.96	46.5 ± 6.7
4.52	0.90	51.3 ± 5.6
3.99	0.80	53.3 ± 5.2
3.72	0.74	57.3 ± 5.7
3.46	0.69	61.9 ± 3.9
3.19	0.64	64.6 ± 3.5
2.66	0.53	66.5 ± 5.7
2.39	0.48	72.2 ± 6.4
2.13	0.43	72.9 ± 3.0
1.86	0.37	77.3 ± 6.1
1.60	0.32	85.0 ± 3.3
1.06	0.21	89.2 ± 2.3
0.80	0.16	91.8 ± 5.4
0.53	0.11	94.7 ± 6.8
0.27	0.05	98.8 ± 6.1
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 8b -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

D-MePhe-Pro-Arg-H		
ng/ml	nM	% Residual Thrombin Activity*
21.28	41.35	10.4 ± 0.5
19.15	37.21	10.9 ± 0.8
17.02	33.08	11.8 ± 1.1
15.96	31.01	12.8 ± 1.2
14.89	28.94	13.2 ± 2.5
13.83	26.87	15.7 ± 0.6
12.77	24.81	18.9 ± 3.8
11.70	22.74	17.7 ± 0.8
11.17	21.71	18.2 ± 2.2
10.64	20.67	19.7 ± 3.3
10.11	19.64	22.7 ± 1.9
9.57	18.61	23.5 ± 1.7
9.04	17.57	26.4 ± 2.7
8.51	16.54	26.1 ± 5.2
7.98	15.50	29.1 ± 3.1
7.45	14.47	30.7 ± 3.9
6.91	13.44	30.3 ± 4.5
6.38	12.40	37.7 ± 3.0
5.85	11.37	43.1 ± 4.2
5.32	10.34	46.0 ± 1.3
4.79	9.30	51.7 ± 8.5
4.26	8.27	57.6 ± 0.7
3.72	7.24	63.2 ± 10.4
3.19	6.20	69.1 ± 7.9
2.66	5.17	73.4 ± 2.0
2.13	4.13	76.6 ± 5.8
1.60	3.10	81.2 ± 1.9
1.06	2.07	88.3 ± 6.3
0.96	1.86	89.8 ± 5.2
0.85	1.65	89.6 ± 5.0
0.74	1.45	90.8 ± 3.1
0.64	1.24	91.6 ± 2.7
0.53	1.03	95.4 ± 1.8
0.43	0.83	95.7 ± 3.5
0.21	0.41	98.7 ± 4.3
0.11	0.21	98.0 ± 0.7
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 8c -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

Boc-D-Phe-Pro-Arg-H		
ng/ml	nM	% Residual Thrombin Activity*
1063.83	1928.63	12.5 ± 1.4
957.45	1735.76	12.9 ± 1.8
851.06	1542.90	14.2 ± 1.0
744.68	1350.04	14.2 ± 1.0
638.30	1157.18	16.2 ± 2.5
531.91	964.31	17.1 ± 1.2
425.53	771.45	18.2 ± 4.8
319.15	578.59	19.2 ± 4.2
212.77	385.73	24.4 ± 6.0
106.38	192.86	30.8 ± 3.4
95.74	173.58	32.8 ± 0.5
85.11	154.29	31.7 ± 2.5
74.47	135.00	32.9 ± 2.6
63.83	115.72	35.2 ± 3.8
53.19	96.43	36.2 ± 3.5
47.87	86.79	38.1 ± 4.3
42.55	77.15	35.7 ± 2.5
37.23	67.50	37.1 ± 0.9
31.91	57.86	38.5 ± 2.0
29.26	53.04	36.6 ± 0.4
26.60	48.22	39.2 ± 5.6
23.94	43.39	40.3 ± 5.7
21.28	38.57	40.3 ± 2.3
18.62	33.75	43.9 ± 7.8
15.96	28.93	44.2 ± 4.3
13.30	24.11	52.7 ± 3.0
10.64	19.29	55.2 ± 1.8
9.57	17.36	56.5 ± 2.2
8.51	15.43	64.4 ± 4.8
7.45	13.50	68.1 ± 1.9
6.38	11.57	72.4 ± 6.4
5.32	9.64	77.0 ± 1.4
4.26	7.71	80.7 ± 3.0
3.19	5.79	86.5 ± 4.7
2.13	3.86	88.5 ± 5.1
1.06	1.93	97.0 ± 6.1
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 8d -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

Ac-(D)Phe-Pro-boroArg-OH		
ng/ml	nM	% Residual Thrombin Activity*
6.38	12.85	8.4 ± 4.2
5.85	11.78	9.1 ± 4.8
5.32	10.71	15.8 ± 4.4
5.05	10.17	20.0 ± 16.8
4.79	9.64	18.7 ± 5.8
4.52	9.10	17.9 ± 4.2
4.26	8.57	19.8 ± 2.0
3.99	8.03	23.4 ± 3.4
3.72	7.49	32.8 ± 0.9
3.46	6.96	39.8 ± 2.9
3.19	6.42	39.5 ± 3.3
2.93	5.89	44.4 ± 1.6
2.66	5.35	49.0 ± 3.3
2.39	4.82	53.2 ± 5.0
2.13	4.28	59.0 ± 3.7
1.86	3.75	60.8 ± 3.7
1.60	3.21	65.9 ± 3.0
1.33	2.68	74.4 ± 1.3
1.06	2.14	77.2 ± 1.4
0.96	1.93	78.1 ± 1.3
0.85	1.71	81.5 ± 1.3
0.74	1.50	86.2 ± 3.4
0.64	1.28	91.3 ± 4.8
0.53	1.07	88.3 ± 1.4
0.43	0.86	94.9 ± 5.1
0.32	0.64	94.8 ± 5.3
0.21	0.43	97.3 ± 4.8
0.11	0.21	97.0 ± 5.0
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 8e -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

Argatroban		
ng/ml	nM	% Residual Thrombin Activity*
2127.66	3999.36	9.3 ± 0.7
1861.70	3499.44	10.7 ± 0.7
1489.36	2799.55	12.8 ± 1.0
1276.60	2399.62	14.2 ± 1.0
1063.83	1999.68	17.3 ± 0.9
957.45	1799.71	19.2 ± 1.1
851.06	1599.74	20.6 ± 1.0
744.68	1399.78	22.6 ± 2.7
638.30	1199.81	24.3 ± 1.0
531.91	999.84	27.5 ± 1.0
478.72	899.86	28.9 ± 1.6
425.53	799.87	32.0 ± 2.4
372.34	699.89	35.1 ± 2.2
319.15	599.90	38.6 ± 2.8
265.96	499.92	40.2 ± 2.5
212.77	399.94	47.6 ± 3.7
159.57	299.95	51.2 ± 3.2
106.38	199.97	61.3 ± 3.1
101.06	189.97	65.2 ± 4.2
95.74	179.97	69.2 ± 3.1
85.11	159.97	71.9 ± 0.8
74.47	139.98	73.1 ± 2.8
63.83	119.98	74.3 ± 4.6
58.51	109.98	77.5 ± 4.7
53.19	99.98	81.7 ± 1.1
47.87	89.99	84.8 ± 2.5
42.55	79.99	85.0 ± 1.6
37.23	69.99	85.2 ± 2.0
31.91	59.99	89.0 ± 1.6
26.60	49.99	91.4 ± 4.1
21.28	39.99	91.9 ± 1.7
15.96	30.00	95.3 ± 5.8
10.64	20.00	97.6 ± 3.9
5.32	10.00	98.7 ± 2.3
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 8f -- Antithrombin Activity of Antithrombin Agents, as Studied in the Thrombin Titration Assay.

Hirudin		
ng/ml	nM	% Residual Thrombin Activity*
58.51	8.40	5.5 ± 0.2
50.53	7.26	7.2 ± 1.4
47.87	6.88	8.4 ± 1.0
45.21	6.49	12.0 ± 3.5
42.55	6.11	14.5 ± 0.1
41.49	5.96	16.6 ± 2.1
40.43	5.81	17.7 ± 2.5
39.36	5.65	20.0 ± 2.0
38.30	5.50	21.8 ± 2.9
37.23	5.35	26.1 ± 3.4
36.17	5.19	25.8 ± 3.6
35.11	5.04	26.0 ± 1.1
34.04	4.89	31.8 ± 4.7
32.98	4.74	35.1 ± 7.8
31.91	4.58	34.8 ± 1.9
30.85	4.43	37.0 ± 1.3
29.79	4.28	39.0 ± 2.6
28.72	4.13	41.7 ± 2.3
27.66	3.97	46.9 ± 6.9
26.60	3.82	48.0 ± 3.9
24.47	3.51	48.5 ± 4.4
22.34	3.21	50.9 ± 1.4
21.28	3.06	49.8 ± 0.9
20.21	2.90	51.5 ± 1.4
19.15	2.75	58.6 ± 2.4
18.09	2.60	58.2 ± 0.7
17.02	2.44	63.6 ± 3.4
15.96	2.29	67.9 ± 4.1
14.89	2.14	74.3 ± 1.0
13.83	1.99	75.0 ± 4.6
11.70	1.68	76.5 ± 0.8
9.57	1.38	80.9 ± 2.5
8.51	1.22	84.3 ± 0.3
6.38	0.92	86.9 ± 1.3
5.32	0.76	87.7 ± 1.1
4.26	0.61	91.2 ± 1.0
3.19	0.46	93.1 ± 3.2
1.06	0.15	98.9 ± 5.9
0.00	0.00	100.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 9a -- 10 U TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

D-Phe-Pro-Arg-H			D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
ng/ml	nM	sec*	nM	sec*	nM	sec*
800.000	-	-	-	-	1450.3	>300.0
400.000	-	-	-	-	725.2	124.8 ± 76.9
200.000	399.5	>300.0	388.7	>300.0	362.6	59.0 ± 32.5
100.000	199.8	115.8 ± 1.3	194.3	210.0 ± 75.7	181.3	36.5 ± 26.1
50.000	99.9	27.9 ± 7.2	97.2	33.9 ± 19.0	90.6	20.7 ± 7.2
25.000	49.9	18.1 ± 5.4	48.6	28.7 ± 19.6	45.3	18.6 ± 6.1
12.500	25.0	17.9 ± 5.3	24.3	24.6 ± 15.8	22.7	18.2 ± 4.5
6.250	12.5	17.2 ± 4.7	12.1	16.6 ± 4.1	-	-
3.125	6.2	19.3 ± 7.1	6.1	17.8 ± 4.3	-	-
0.000	0.0	18.7 ± 1.7	0.0	18.7 ± 1.7	0.0	18.7 ± 1.7

* Each value represents the mean ± SD of three independent determinations.

Table 9b -- 10 U TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

Ac-(D)Phe-Pro-boroArg-OH		
ng/ml	nM	sec*
40.000	80.5	>300.0
20.000	40.3	44.0 ± 10.0
10.000	20.1	25.1 ± 5.9
5.000	10.1	17.5 ± 3.1
2.500	5.0	22.3 ± 10.5
1.250	2.5	18.7 ± 4.6
0.000	0.0	18.7 ± 1.7

* Each value represents the mean ± SD of three independent determinations.

Table 9c -- 10 U TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

	Argatroban		Hirudin	
ng/ml	nM	sec*	nM	sec*
500.000	939.8	>300.0	71.8	>300.0
250.000	469.9	294.9 ± 8.8	35.9	42.9 ± 34.0
125.000	235.0	165.6 ± 39.0	18.0	24.8 ± 5.8
62.500	117.5	83.1 ± 18.7	9.0	24.6 ± 12.1
31.250	58.7	40.0 ± 15.4	4.5	21.7 ± 8.4
15.625	29.4	30.8 ± 13.4	2.2	17.1 ± 3.4
7.813	14.7	21.3 ± 5.1	-	-
3.906	7.3	17.5 ± 2.5	-	-
1.953	3.7	19.4 ± 2.2	-	-
0.000	0.0	18.7 ± 1.7	0.0	18.7 ± 1.7

* Each value represents the mean ± SD of three independent determinations.

Table 9d -- 10 U TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

	Heparin	
ng/ml	nM	sec*
100000	9090.9	32.6 ± 12.1
50000	4545.5	27.0 ± 0.9
10000	909.1	22.8 ± 5.5
0	0.0	18.7 ± 1.7

* Each value represents the mean ± SD of three independent determinations.

Table 9e -- 10 U Ca^{++}TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-OH		Boc-D-Phe-Pro-Arg-H	
ng/ml	nM	sec*	nM	sec*	nM	sec*
1600.00	-	-	-	-	2900.7	242.3 ± 71.8
800.000	-	-	1554.6	>300.0	1450.3	72.7 ± 3.6
400.000	799.0	>300.0	777.3	174.1 ± 54.8	725.2	29.3 ± 5.4
200.000	399.5	21.5 ± 3.5	388.7	16.6 ± 7.7	362.6	13.2 ± 3.1
100.000	199.8	12.2 ± 1.7	194.3	10.6 ± 0.5	181.3	9.3 ± 0.1
50.000	99.9	10.4 ± 3.0	97.2	8.4 ± 0.4	90.6	7.7 ± 1.1
25.000	49.9	8.7 ± 1.5	48.6	8.2 ± 0.5	45.3	9.5 ± 2.0
12.500	25.0	8.4 ± 0.7	24.3	8.1 ± 0.6	22.7	8.1 ± 0.3
6.250	12.5	8.5 ± 0.4	12.1	7.6 ± 0.5	-	-
3.125	6.2	8.5 ± 0.4	6.1	7.7 ± 0.3	-	-
0.000	0.0	7.6 ± 0.1	0.0	7.6 ± 0.1	0.0	7.6 ± 0.1

* Each value represents the mean ± SD of three independent determinations.

Table 9f -- 10 U Ca^{++}TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

Ac-(D)Phe-Pro-boroArg-OH		
ng/ml	nM	sec*
80.000	161.0	>300.0
40.000	80.5	34.8 ± 22.0
20.000	40.3	25.4 ± 25.2
10.000	20.1	9.0 ± 0.3
5.000	10.1	8.5 ± 0.6
2.500	5.0	8.3 ± 0.1
1.250	2.5	8.1 ± 0.6
0.000	0.0	7.6 ± 0.1

* Each value represents the mean ± SD of three independent determinations.

Table 9g -- 10 U Ca^{++}TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

	Argatroban		Hirudin	
ng/ml	nM	sec*	nM	sec*
1000.000	1879.7	>300.0	143.6	>300.0
500.000	939.8	221.7 ± 34.3	71.8	169.9 ± 123.3
250.000	469.9	109.3 ± 22.7	35.9	15.3 ± 7.9
125.000	235.0	59.2 ± 22.6	18.0	19.4 ± 18.0
62.500	117.5	33.8 ± 17.3	9.0	16.9 ± 14.1
31.250	58.7	21.6 ± 15.0	4.5	15.1 ± 12.1
15.625	29.4	7.7 ± 3.5	2.2	8.7 ± 0.7
7.813	14.7	6.2 ± 3.1	-	-
3.906	7.3	7.5 ± 0.7	-	-
1.953	3.7	8.1 ± 0.3	-	-
0.000	0.0	7.6 ± 0.1	0.0	7.6 ± 0.1

* Each value represents the mean ± SD of three independent determinations.

Table 9h -- 10 U Ca^{++}TT of Various Thrombin Inhibitors in the Human Fibrinogen Based System.

	Heparin	
ng/ml	nM	sec*
100000	9090.9	15.9 ± 11.2
50000	4545.5	9.6 ± 0.9
10000	909.1	8.7 ± 0.6
0	0.0	7.6 ± 0.1

* Each value represents the mean ± SD of three independent determinations.

Table 10a -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NHP.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
ng/ml	nM	% Inhibition*	nM	% Inhibition*	nM	% Inhibition*
6666.67	13317.35	97.3 ± 0.3	12955.05	96.4 ± 0.2	12086.05	93.3 ± 0.0
3333.33	6658.68	96.0 ± 0.4	6477.52	94.5 ± 0.9	6043.03	89.5 ± 0.1
1666.67	3329.34	94.5 ± 0.5	3238.76	92.3 ± 0.5	3021.51	83.1 ± 0.3
666.67	1331.74	92.4 ± 0.7	1295.50	89.4 ± 1.3	1208.61	75.4 ± 3.1
333.33	665.87	90.1 ± 0.9	647.75	85.6 ± 1.4	604.30	67.8 ± 3.3
166.67	332.93	87.1 ± 1.3	323.88	82.6 ± 1.2	302.15	61.0 ± 2.5
83.33	166.47	85.5 ± 1.5	161.94	79.6 ± 1.3	151.08	54.0 ± 2.9
41.67	83.23	82.0 ± 2.0	80.97	75.2 ± 2.6	75.54	42.3 ± 6.7
20.83	41.62	67.3 ± 10.2	40.48	58.7 ± 9.5	37.77	25.8 ± 7.4
10.42	20.81	36.3 ± 10.7	20.24	31.3 ± 10.2	18.88	11.4 ± 4.5
5.21	10.40	16.6 ± 2.8	10.12	13.9 ± 3.4	9.44	5.5 ± 1.2
2.60	5.20	8.8 ± 1.8	5.06	7.1 ± 1.3	4.72	2.9 ± 1.1
1.30	2.60	5.9 ± 1.9	2.53	4.5 ± 0.6	2.36	2.6 ± 0.8
0.00	0.00	0.0 ± 0.0	0.00	0.0 ± 0.0	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 10b -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NHP.

Ac-D-Phe-Pro-boroArg-OH		
ng/ml	nM	% Inhibition*
666.67	1341.92	97.1 ± 0.4
333.33	670.96	96.5 ± 0.2
166.67	335.48	95.4 ± 0.2
66.67	134.19	94.6 ± 0.4
33.33	67.10	90.7 ± 1.1
16.67	33.55	63.1 ± 9.5
8.33	16.77	28.3 ± 3.4
4.17	8.39	11.9 ± 1.4
2.08	4.19	3.6 ± 0.5
0.00	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 10c -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NHP.

	Argatroban		Hirudin	
ng/ml	nM	% Inhibition*	nM	% Inhibition*
6666.67	12531.33	84.8 ± 2.9	957.44	90.7 ± 1.2
3333.33	6265.66	79.1 ± 3.9	478.72	90.6 ± 1.1
1666.67	3132.83	67.9 ± 5.5	239.36	90.5 ± 1.3
666.67	1253.13	49.6 ± 10.6	95.74	89.9 ± 1.0
333.33	626.57	26.3 ± 6.4	47.87	86.0 ± 1.9
166.67	313.28	8.4 ± 4.6	23.94	57.8 ± 16.7
83.33	156.64	0.0 ± 0.5	11.97	22.8 ± 5.2
41.67	-	-	5.98	8.3 ± 0.8
20.83	-	-	2.99	2.5 ± 1.1
0.00	0.00	0.0 ± 0.0	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 10d -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NHP.

	Heparin	
ng/ml	nM	% Inhibition*
66666.67	6060.61	70.1 ± 1.9
33333.33	3030.30	68.6 ± 1.3
16666.67	1515.15	68.9 ± 2.7
6666.67	606.06	71.0 ± 2.2
3333.33	303.03	70.9 ± 1.5
1666.67	151.52	72.3 ± 1.0
666.67	60.61	78.4 ± 1.6
333.33	30.30	79.9 ± 1.7
166.67	15.15	68.5 ± 2.3
83.33	7.58	40.4 ± 2.3
41.67	3.79	17.4 ± 0.8
20.83	1.89	5.5 ± 0.6
10.42	0.95	2.6 ± 1.6
0.00	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 10e -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NRP.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
ng/ml	nM	% Inhibition*	nM	% Inhibition*	nM	% Inhibition*	nM	% Inhibition*
6666.7	-	-	-	-	-	-	606.1	77.1 ± 6.3
2666.7	5182.0	95.4 ± 0.4	-	-	383.0	93.1 ± 0.5	242.4	75.8 ± 2.5
1333.3	2591.0	93.6 ± 0.7	-	-	191.5	92.6 ± 0.7	121.2	77.2 ± 2.5
666.7	1295.5	91.1 ± 0.8	1342.0	98.9 ± 0.0	95.7	91.1 ± 0.9	60.6	79.6 ± 0.9
333.3	647.8	86.4 ± 4.7	671.0	98.3 ± 0.5	47.9	81.9 ± 4.9	30.3	78.6 ± 4.0
166.7	323.9	86.7 ± 0.9	335.0	97.8 ± 0.1	23.9	47.4 ± 7.3	15.2	70.5 ± 6.6
83.3	161.9	85.1 ± 0.7	168.0	97.1 ± 0.0	12.0	23.3 ± 5.9	7.6	57.9 ± 6.3
41.3	80.3	82.3 ± 0.5	83.9	95.6 ± 0.6	5.9	13.5 ± 5.6	3.8	31.5 ± 8.0
20.7	40.2	75.8 ± 3.9	41.9	85.5 ± 2.7	3.0	7.2 ± 1.9	1.9	17.1 ± 2.0
10.7	20.7	53.7 ± 10.8	21.0	47.3 ± 4.0	1.5	4.7 ± 2.5	1.0	9.2 ± 2.0
5.3	10.4	22.3 ± 19.4	10.5	21.4 ± 2.4	0.8	3.5 ± 2.1	-	-
2.7	5.2	17.7 ± 6.8	5.2	11.9 ± 0.9	0.4	4.7 ± 2.7	-	-
1.3	-	-	2.6	6.3 ± 1.4	-	-	-	-
0.7	-	-	1.3	5.4 ± 0.8	-	-	-	-
0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 11a -- Inhibition of the Amidolytic Activity of Factor Xa by Various Agents in NHP.

Ac-D-Phe-Pro-boroArg-OH		
ng/ml	nM	% Inhibition*
540.54	1088.04	72.1 ± 2.5
270.27	544.02	58.3 ± 2.8
135.14	272.01	40.6 ± 5.0
54.05	108.80	26.3 ± 3.6
27.03	54.40	12.9 ± 1.2
13.51	27.20	8.4 ± 2.1
6.76	13.60	2.5 ± 0.4
3.38	6.80	0.0 ± 0.7
0.00	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 11b -- Inhibition of the Amidolytic Activity of Factor Xa by Various Agents in NHP.

Heparin		
ng/ml	nM	% Inhibition*
54054.05	4914.00	45.6 ± 9.5
27027.03	2457.00	49.4 ± 10.3
13513.51	1228.50	56.7 ± 9.3
5405.41	491.40	66.5 ± 5.3
2702.70	245.70	75.2 ± 3.2
1351.35	122.85	87.6 ± 2.2
540.54	49.14	89.1 ± 1.2
270.27	24.57	90.4 ± 0.7
135.14	12.29	81.4 ± 4.1
67.57	6.14	55.8 ± 7.4
33.78	3.07	26.6 ± 2.5
16.89	1.54	10.9 ± 0.9
8.45	0.77	5.0 ± 1.3
4.22	0.38	0.0 ± 2.7
0.00	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 11c -- Inhibition of the Amidolytic Activity of Thrombin by Various Agents in NRP.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
ng/ml	nM	% Inhibition*	nM	% Inhibition*	nM	% Inhibition*	nM	% Inhibition*
6666.7	-	-	-	-	-	-	606.1	77.1 ± 6.3
2666.7	5182.0	95.4 ± 0.4	-	-	383.0	93.1 ± 0.5	242.4	75.8 ± 2.5
1333.3	2591.0	93.6 ± 0.7	-	-	191.5	92.6 ± 0.7	121.2	77.2 ± 2.5
666.7	1295.5	91.1 ± 0.8	1341.9	98.9 ± 0.0	95.7	91.1 ± 0.9	60.6	79.6 ± 0.9
333.3	647.8	86.4 ± 4.7	671.0	98.3 ± 0.5	47.9	81.9 ± 4.9	30.3	78.6 ± 4.0
166.7	323.9	86.7 ± 0.9	335.5	97.8 ± 0.1	23.9	47.4 ± 7.3	15.2	70.5 ± 6.6
83.3	161.9	85.1 ± 0.7	167.7	97.1 ± 0.0	12.0	23.3 ± 5.9	7.6	57.9 ± 6.3
41.7	80.3	82.3 ± 0.5	83.9	95.6 ± 0.6	5.9	13.5 ± 5.6	3.8	31.5 ± 8.0
20.8	40.2	75.8 ± 3.9	41.9	85.5 ± 2.7	3.0	7.2 ± 1.9	1.9	17.1 ± 2.0
10.4	20.7	53.7 ± 10.8	21.0	47.3 ± 4.0	1.5	4.7 ± 2.5	1.1	9.2 ± 2.0
5.2	10.4	22.3 ± 19.4	10.5	21.4 ± 2.4	0.8	3.5 ± 2.1	-	-
2.6	5.2	17.7 ± 6.8	5.2	11.9 ± 0.9	0.4	4.7 ± 2.7	-	-
1.3	-	-	2.6	6.3 ± 1.4	-	-	-	-
0.7	-	-	1.3	5.4 ± 0.8	-	-	-	-
0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 12a -- Inhibition of the Amidolytic Activity of Chymotrypsin by Various Inhibitors.

Aprotinin		
μg/ml	μM	% Inhibition*
30.71	4.72	87.6 ± 0.5
15.36	2.36	82.1 ± 0.4
7.68	1.18	71.4 ± 1.2
3.84	0.59	34.3 ± 3.5
1.92	0.29	28.6 ± 0.7
0.96	0.15	11.6 ± 0.5
0.48	0.07	5.0 ± 0.4
0.24	0.04	3.9 ± 0.3
0.12	0.02	0.9 ± 0.6
0.00	0.00	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 12b -- Inhibition of the Amidolytic Activity of Trypsin by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Aprotinin	
μg/ml	μM	% Inhibition*	μM	% Inhibition*	μM	% Inhibition*	μM	% Inhibition*	μM	% Inhibition*
13.333	26.635	95.3 ± 1.1	25.910	97.7 ± 0.1	24.172	99.1 ± 0.2	26.838	99.4 ± 0.3	2.048	98.7 ± 0.9
6.667	13.317	93.8 ± 1.0	12.955	95.9 ± 0.1	12.086	98.8 ± 0.2	13.419	99.4 ± 0.2	1.024	92.2 ± 10.0
3.333	6.659	91.8 ± 1.2	6.478	93.1 ± 1.5	6.043	97.7 ± 0.1	6.710	99.2 ± 0.1	0.512	66.8 ± 27.5
1.667	3.329	88.6 ± 0.6	3.239	88.6 ± 0.1	3.022	97.0 ± 0.6	3.355	99.4 ± 0.2	0.256	29.5 ± 12.6
0.833	1.665	86.1 ± 3.0	1.619	86.5 ± 1.3	1.511	94.2 ± 3.5	1.677	99.1 ± 0.2	0.128	30.9 ± 17.3
0.417	0.832	79.8 ± 0.4	0.810	86.3 ± 3.8	0.755	95.7 ± 0.7	0.839	99.0 ± 0.3	0.064	24.4 ± 6.9
0.208	0.416	44.0 ± 12.5	0.405	79.1 ± 9.7	0.378	81.9 ± 6.1	0.419	98.6 ± 0.9	0.032	19.9 ± 1.5
0.104	0.208	26.3 ± 3.0	0.202	54.1 ± 22.8	0.189	38.0 ± 3.8	0.210	82.9 ± 22.6	0.016	20.1 ± 6.6
0.052	0.104	17.5 ± 9.8	0.101	28.6 ± 21.1	0.094	26.9 ± 6.3	0.105	54.1 ± 53.1	-	-
0.026	0.052	16.4 ± 12.2	0.051	17.9 ± 17.8	0.047	19.2 ± 9.4	0.052	27.6 ± 15.1	-	-
0.013	0.026	12.9 ± 10.4	0.025	9.9 ± 5.9	0.024	19.2 ± 0.1	0.026	20.9 ± 22.1	-	-
0.007	0.013	7.7 ± 14.8	0.013	16.3 ± 7.5	0.012	18.0 ± 5.7	0.013	11.9 ± 17.4	-	-
0.003	0.007	8.7 ± 4.0	0.006	7.4 ± 3.5	0.006	15.3 ± 6.6	0.007	14.4 ± 7.8	-	-
0.002	0.003	7.1 ± 15.6	0.003	4.0 ± 5.1	0.003	6.8 ± 1.3	0.003	12.1 ± 26.3	-	-
0.001	0.002	14.1 ± 2.8	0.002	6.5 ± 15.6	0.001	9.7 ± 4.5	0.002	15.7 ± 11.4	-	-
0.000	0.001	3.3 ± 4.7	0.001	13.4 ± 0.5	0.001	12.4 ± 2.5	0.001	9.3 ± 14.4	-	-
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 12c -- Inhibition of the Amidolytic Activity of APC by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Aprotinin	
μg/ml	μM	% Inh*	μM	% Inh*	μM	% Inh*	μM	% Inh*	μM	% Inh*
125.000	-	-	-	-	-	-	-	-	19.195	55.0 ± 1.2
62.500	124.850	96.4 ± 0.6	121.454	93.3 ± 0.8	113.307	86.4 ± 1.3	125.805	97.9 ± 0.3	9.598	36.6 ± 1.4
31.250	62.425	93.9 ± 0.6	60.727	86.4 ± 1.5	56.653	72.0 ± 7.2	62.903	96.1 ± 0.2	4.799	25.8 ± 1.9
15.625	31.213	89.2 ± 0.7	30.363	76.4 ± 4.3	28.327	58.4 ± 9.3	31.451	92.6 ± 1.1	2.399	13.8 ± 1.4
7.813	15.606	80.2 ± 1.5	15.182	63.1 ± 3.4	14.163	49.6 ± 5.3	15.726	86.4 ± 0.2	1.200	10.1 ± 0.3
3.906	7.803	66.8 ± 3.2	7.591	47.4 ± 4.5	7.082	32.5 ± 4.7	7.863	73.6 ± 1.3	0.600	3.7 ± 0.4
1.953	3.902	48.1 ± 3.4	3.795	31.3 ± 2.9	3.541	16.7 ± 2.9	3.931	53.0 ± 0.9	0.300	1.9 ± 0.9
0.977	1.951	28.9 ± 4.1	1.898	17.4 ± 2.5	1.770	9.3 ± 1.4	1.966	30.0 ± 2.7	-	-
0.488	0.975	15.2 ± 4.6	0.949	10.4 ± 1.1	0.885	4.2 ± 2.8	0.983	12.9 ± 2.4	-	-
0.244	0.488	5.5 ± 4.9	0.474	6.8 ± 3.2	0.443	3.9 ± 1.7	0.491	5.5 ± 1.5	-	-
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 12d -- Inhibition of the Amidolytic Activity of Glandular Kallikrein by Thrombin Inhibitors.

Ac-(D)Phe-Pro-boroArg-OH			Aprotinin	
μg/ml	μM	% Inhibition*	μM	% Inhibition*
66.667	-	-	5.119	95.0 ± 4.5
33.333	67.096	79.8 ± 4.6	2.559	96.8 ± 3.8
16.667	33.548	68.8 ± 7.5	1.280	87.7 ± 8.4
8.333	16.774	49.2 ± 6.9	0.640	72.5 ± 2.9
4.167	8.387	34.9 ± 9.5	0.320	42.0 ± 2.8
2.083	4.194	20.6 ± 2.3	0.160	21.0 ± 4.6
1.042	2.097	11.4 ± 3.2	0.080	8.3 ± 3.3
0.521	1.048	3.0 ± 3.5	0.040	1.6 ± 4.3
0.260	0.524	4.3 ± 1.6	0.020	2.1 ± 6.2
0.000	0.000	0.0 ± 2.3	0.010	0.5 ± 2.8

* Each value represents the mean ± SD of three independent determinations.

Table 12e -- Inhibition of the Amidolytic Activity of Glandular Kallikrein by Thrombin Inhibitors.

Heparin		
μg/ml	μM	% Inhibition*
66.667	6.231	44.6 ± 27.8
33.333	3.115	38.6 ± 30.9
16.667	1.558	28.7 ± 33.6
8.333	0.779	13.7 ± 22.3
4.167	0.389	6.1 ± 14.4
2.083	0.195	1.3 ± 8.0
1.042	0.097	0.0 ± 7.7
0.000	0.000	0.0 ± 1.0

* Each value represents the mean ± SD of three independent determinations.

Table 12f -- Inhibition of the Amidolytic Activity of t-PA by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	66.587	56.2 ± 2.9	64.775	64.2 ± 2.1	60.430	94.1 ± 1.0	67.096	99.5 ± 0.8
16.667	33.293	39.7 ± 1.2	32.388	50.0 ± 4.0	30.215	86.8 ± 1.8	33.548	99.5 ± 0.8
8.333	16.647	25.2 ± 2.0	16.194	30.7 ± 2.7	15.108	68.4 ± 2.5	16.774	98.8 ± 0.7
4.167	8.323	13.3 ± 3.2	8.097	17.7 ± 4.8	7.554	44.8 ± 7.0	8.387	48.9 ± 7.1
2.083	4.162	9.4 ± 4.6	4.048	10.8 ± 0.9	3.777	22.6 ± 5.5	4.194	20.3 ± 3.4
1.042	2.081	2.5 ± 5.7	2.024	6.7 ± 1.8	1.888	12.2 ± 5.5	2.097	6.8 ± 1.7
0.521	1.040	3.3 ± 7.4	1.012	4.6 ± 1.3	0.944	5.9 ± 2.7	1.048	5.4 ± 4.4
0.260	0.520	2.6 ± 4.1	0.506	3.3 ± 4.1	0.472	5.9 ± 2.3	0.524	1.2 ± 5.2
0.130	0.260	2.1 ± 3.6	0.253	3.2 ± 3.8	0.236	2.4 ± 3.6	0.262	0.4 ± 5.2
0.065	0.130	1.9 ± 5.6	0.127	1.5 ± 2.9	0.118	0.0 ± 4.0	0.131	0.0 ± 7.9

* Each value represents the mean ± SD of three independent determinations.

Table 12g -- Inhibition of the Amidolytic Activity of Urokinase by Thrombin Inhibitors.

	Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	%Inhibition*	μM	%Inhibition*
33.333	60.430	76.9 ± 1.2	67.096	98.6 ± 1.3
16.667	30.215	61.8 ± 3.2	33.548	97.8 ± 1.2
8.333	15.108	47.1 ± 0.9	16.774	95.6 ± 1.2
4.167	7.554	35.4 ± 2.8	8.387	92.0 ± 0.6
2.083	3.777	23.6 ± 5.6	4.194	83.4 ± 2.6
1.042	1.888	15.2 ± 4.6	2.097	62.9 ± 4.1
0.521	0.944	8.0 ± 2.2	1.048	31.1 ± 1.8
0.260	0.472	5.7 ± 4.0	0.524	14.8 ± 1.8
0.130	0.236	2.5 ± 9.0	0.262	6.5 ± 1.1
0.065	0.118	5.2 ± 4.5	0.131	4.0 ± 1.6
0.033	0.059	2.6 ± 3.4	0.066	3.3 ± 3.2
0.016	0.030	1.6 ± 2.9	0.033	3.1 ± 2.0

* Each value represents the mean ± SD of three independent determinations.

Table 12h -- Inhibition of the Amidolytic Activity of Plasmin by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	66.587	93.6 ± 4.3	64.775	96.6 ± 4.1	60.430	99.4 ± 1.0
16.667	33.293	88.8 ± 4.6	32.388	94.3 ± 3.7	30.215	99.2 ± 1.3
8.333	16.647	80.2 ± 2.1	16.194	88.3 ± 4.6	15.108	97.9 ± 1.7
4.167	8.323	66.3 ± 3.0	8.097	79.0 ± 6.4	7.554	95.9 ± 1.4
2.083	4.162	52.1 ± 2.2	4.048	68.0 ± 4.6	3.777	90.8 ± 1.3
1.042	2.081	35.2 ± 2.2	2.024	50.7 ± 4.0	1.888	82.8 ± 1.6
0.521	1.040	22.1 ± 1.7	1.012	34.1 ± 2.0	0.944	71.4 ± 2.8
0.260	0.520	11.7 ± 2.9	0.506	22.0 ± 2.8	0.472	55.3 ± 5.5
0.130	0.260	7.2 ± 0.4	0.253	13.1 ± 2.3	0.236	35.7 ± 6.1
0.065	0.130	3.5 ± 1.6	0.127	6.2 ± 0.6	0.118	19.6 ± 3.3
0.033	0.065	1.7 ± 0.7	0.063	2.6 ± 1.2	0.059	12.0 ± 2.1
0.016	0.033	0.1 ± 2.4	0.032	0.0 ± 1.8	0.030	3.3 ± 3.0

* Each value represents the mean ± SD of three independent determinations.

Table 12i -- Inhibition of the Amidolytic Activity of Plasmin by Thrombin Inhibitors.

	Ac-(D)Phe-Pro-boroArg-OH		Heparin		Aprotinin	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
66.667	-	-	6.231	22.2 ± 10.2	-	-
33.333	67.096	100.0 ± 0.0	3.115	21.3 ± 9.6	5.119	99.0 ± 8.4
16.667	33.548	100.0 ± 0.0	1.558	20.2 ± 14.9	2.559	99.0 ± 1.4
8.333	16.774	100.0 ± 0.0	0.779	17.2 ± 16.8	1.280	99.1 ± 1.4
4.167	8.387	100.0 ± 0.0	0.389	18.0 ± 16.8	0.640	99.3 ± 1.5
2.083	4.194	100.0 ± 0.0	0.195	19.3 ± 17.8	0.320	98.9 ± 1.1
1.042	2.097	99.8 ± 0.4	0.097	4.1 ± 7.2	0.160	85.8 ± 1.9
0.521	1.048	97.2 ± 0.3	0.049	8.1 ± 5.4	0.080	58.4 ± 4.5
0.260	0.524	92.5 ± 0.9	0.024	6.2 ± 2.1	0.040	26.1 ± 3.8
0.130	0.262	78.9 ± 3.1	0.012	1.8 ± 2.5	0.020	13.8 ± 4.4
0.065	0.131	53.8 ± 2.6	0.006	2.0 ± 2.0	0.010	3.4 ± 3.8
0.033	0.066	30.2 ± 2.2	0.003	2.3 ± 0.9	0.005	1.4 ± 3.4
0.016	0.033	15.3 ± 1.0	0.002	0.5 ± 1.9	0.002	1.9 ± 1.3
0.008	0.016	7.4 ± 1.0	0.001	1.1 ± 1.3	0.001	1.7 ± 2.7
0.004	0.008	1.4 ± 1.0	0.000	1.4 ± 0.0	0.001	0.2 ± 1.7

* Each value represents the mean ± SD of three independent determinations.

Table 13a -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by Streptokinase.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
7.813	15.606	71.7 ± 3.0	15.182	73.9 ± 3.0	14.163	80.4 ± 2.0
3.906	7.803	47.8 ± 2.0	7.591	54.3 ± 2.0	7.082	65.2 ± 2.0
1.953	3.902	41.3 ± 2.0	3.795	47.8 ± 2.0	3.541	52.2 ± 1.5
0.977	1.951	15.2 ± 1.5	1.898	17.4 ± 1.5	1.770	39.1 ± 1.5
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13b -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by Streptokinase.

	Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
7.813	15.726	91.3 ± 1.0	1.122	28.3 ± 1.0	0.730	47.8 ± 1.0
3.906	7.863	87.0 ± 1.5	0.561	19.6 ± 1.5	0.365	34.8 ± 0.5
1.953	3.931	78.3 ± 2.0	0.281	19.6 ± 1.0	0.183	34.8 ± 0.5
0.977	1.966	63.0 ± 1.5	0.140	8.7 ± 0.5	0.091	26.1 ± 1.0
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13c -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by Streptokinase.

	Aprotinin	
μg/ml	μM	%Inhibition*
25.000	3.839	47.8 ± 1.0
12.500	1.920	37.0 ± 2.0
6.250	0.960	23.9 ± 1.5
3.125	0.480	21.7 ± 1.0
0.000	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13d -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by t-PA.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
0.781	1.561	68.2 ± 2.0	1.518	72.7 ± 2.0	1.416	90.9 ± 0.5
0.391	0.780	45.5 ± 1.5	0.759	59.1 ± 1.5	0.708	90.9 ± 2.0
0.195	0.390	45.5 ± 2.0	0.380	45.5 ± 2.0	0.354	81.8 ± 1.0
0.098	0.195	45.5 ± 1.0	0.190	45.5 ± 1.0	0.177	72.7 ± 1.5
0.049	0.098	45.5 ± 1.0	0.095	22.7 ± 1.0	0.089	63.6 ± 1.0
0.024	0.049	0.0 ± 0.0	0.047	0.0 ± 0.0	0.044	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13e -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by t-PA.

	Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	% Inhibition*	μM	% Inhibition*	μM	%Inhibition*
0.781	1.573	90.9 ± 0.5	0.112	18.2 ± 2.0	0.073	36.4 ± 1.0
0.391	0.786	90.9 ± 0.0	0.056	18.2 ± 1.0	0.037	36.4 ± 1.5
0.195	0.393	90.9 ± 1.0	0.028	18.2 ± 1.5	0.018	36.4 ± 1.0
0.098	0.197	81.8 ± 1.0	0.014	18.2 ± 1.0	0.009	31.8 ± 2.0
0.049	0.098	68.2 ± 1.5	0.007	9.1 ± 0.5	0.005	9.1 ± 0.5
0.024	0.049	0.0 ± 0.0	0.004	0.0 ± 0.0	0.002	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13f -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: Pure Plasminogen Lysed by t-PA.

	Aprotinin	
μg/ml	μM	%Inhibition*
25.000	3.839	54.5 ± 2.0
12.500	1.920	36.4 ± 1.0
6.250	0.960	31.8 ± 1.0
3.125	0.480	31.8 ± 1.5
1.563	0.240	18.2 ± 1.5
0.781	0.120	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13g -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by Streptokinase.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
7.813	15.606	68.9 ± 4.0	15.182	81.1 ± 4.0	14.163	85.1 ± 3.0
3.906	7.803	58.1 ± 3.0	7.591	64.9 ± 4.0	7.082	73.0 ± 3.0
1.953	3.902	41.9 ± 3.0	3.795	48.6 ± 3.0	3.541	51.4 ± 2.5
0.977	1.951	27.0 ± 2.0	1.898	33.8 ± 2.0	1.770	27.0 ± 2.0
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13h -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by Streptokinase.

	Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
7.813	15.726	98.6 ± 1.0	1.122	24.3 ± 1.0	0.730	25.7 ± 2.0
3.906	7.863	97.3 ± 1.0	0.561	16.2 ± 1.5	0.365	21.6 ± 1.0
1.953	3.931	89.2 ± 2.0	0.281	8.1 ± 1.0	0.183	16.2 ± 1.5
0.977	1.966	82.4 ± 2.0	0.140	5.4 ± 1.0	0.091	8.1 ± 1.0
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13i -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by Streptokinase.

	Aprotinin	
	μM	%Inhibition*
25.000	3.839	48.6 ± 1.5
12.500	1.920	27.0 ± 1.0
6.250	0.960	18.9 ± 0.5
3.125	0.480	16.2 ± 0.5
0.000	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13j -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by t-PA.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
7.813	15.606	83.3 ± 2.0	15.182	79.2 ± 3.0	-	-
3.906	7.803	66.7 ± 2.0	7.591	66.7 ± 2.5	-	-
1.953	3.902	58.3 ± 2.5	3.795	58.3 ± 3.0	-	-
0.977	1.951	50.0 ± 2.0	1.898	41.7 ± 2.0	1.770	79.2 ± 2.0
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13k -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by t-PA.

	Ac-(D)Phe-Pro-boroArg-OH		Hirudin	
μg/ml	μM	%Inhibition*	μM	%Inhibition*
7.813	-	-	1.122	33.3 ± 1.0
3.906	-	-	0.561	25.0 ± 1.5
1.953	3.931	87.5 ± 2.0	0.281	25.0 ± 1.5
0.977	1.966	87.5 ± 2.0	0.140	16.7 ± 1.0
0.000	0.000	0.0 ± 0.0	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 13l -- Inhibition of Plasminogenolysis by Thrombin Inhibitors: NHP-Derived Plasminogen Lysed by t-PA.

	Aprotinin	
μg/ml	μM	%Inhibition*
25.000	3.839	75.0 ± 2.0
12.500	1.920	50.0 ± 2.0
6.250	0.960	41.7 ± 1.5
3.125	0.480	25.0 ± 1.0
0.000	0.000	0.0 ± 0.0

* Each value represents the mean ± SD of three independent determinations.

Table 14a -- Inhibition of Thrombin Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Argatroban	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	66.587	97.4 ± 1.5	64.775	97.5 ± 1.0	62.657	79.4 ± 5.4
26.667	53.269	96.7 ± 1.6	51.820	96.9 ± 1.3	50.125	75.3 ± 8.9
20.000	39.952	95.3 ± 1.1	38.865	95.7 ± 1.6	37.594	71.4 ± 12.1
13.333	26.635	93.9 ± 2.3	25.910	93.7 ± 2.4	25.063	63.9 ± 19.8
10.000	19.976	92.1 ± 2.0	19.433	91.9 ± 2.9	18.797	59.6 ± 21.7
8.333	16.647	90.8 ± 2.2	16.194	90.5 ± 3.1	15.664	51.9 ± 26.2
6.667	13.317	88.9 ± 2.7	12.955	88.4 ± 3.5	12.531	46.4 ± 31.3
6.000	11.986	88.1 ± 2.5	11.660	86.4 ± 4.3	11.278	43.1 ± 31.4
5.333	10.654	85.4 ± 3.6	10.364	85.5 ± 2.9	10.025	40.7 ± 30.3
4.667	9.322	83.8 ± 4.4	9.069	82.9 ± 2.8	8.772	38.6 ± 28.4
4.000	7.990	81.1 ± 5.5	7.773	75.5 ± 2.5	7.519	36.9 ± 26.8
3.333	6.659	72.1 ± 9.0	6.478	53.1 ± 10.8	6.266	29.3 ± 24.4
3.000	5.993	56.9 ± 11.6	5.830	50.8 ± 2.4	5.639	20.2 ± 20.8
2.667	5.327	49.1 ± 14.2	5.182	42.9 ± 4.5	5.013	14.5 ± 14.5
2.333	4.661	43.7 ± 9.8	4.534	33.9 ± 3.8	4.386	12.0 ± 14.9
2.000	3.995	38.1 ± 12.8	3.887	23.5 ± 11.6	3.759	10.9 ± 15.4
1.833	3.662	33.7 ± 10.1	3.563	22.9 ± 5.8	3.446	8.9 ± 12.9
1.667	3.329	33.2 ± 8.1	3.239	28.8 ± 2.7	3.133	18.3 ± 14.7
1.500	2.996	34.2 ± 9.0	2.915	25.0 ± 2.2	2.820	11.0 ± 13.4
1.333	2.663	24.0 ± 13.5	2.591	18.9 ± 4.1	2.506	10.0 ± 12.5
1.167	2.331	21.2 ± 12.1	2.267	15.3 ± 2.2	2.193	8.1 ± 12.4
1.000	1.998	18.0 ± 7.6	1.943	9.9 ± 1.8	1.880	6.1 ± 12.1
0.833	1.665	10.9 ± 6.8	1.619	4.7 ± 3.3	1.566	5.6 ± 11.7
0.667	1.332	6.3 ± 4.9	1.296	2.2 ± 1.8	1.253	4.7 ± 11.2

* All values represent the mean ± SD of three independent determinations.

Table 14b -- Inhibition of Thrombin Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Boc-D-Phe-Pro-Arg-H		
μg/ml	μM	%Inhibition*
16.667	30.215	95.9 ± 2.5
13.333	24.172	95.5 ± 2.3
10.000	18.129	95.0 ± 2.4
6.667	12.086	92.7 ± 2.2
5.000	9.065	89.4 ± 2.0
4.167	7.554	83.5 ± 4.5
3.333	6.043	70.9 ± 9.6
2.667	4.834	17.1 ± 12.4
2.000	3.626	2.4 ± 5.0

* All values represent the mean ± SD of three independent determinations.

Table 14c -- Inhibition of Thrombin Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Ac-(D)Phe-Pro-boroArg-OH		
μg/ml	μM	%Inhibition*
3333.330	6709.610	98.3 ± 2.1
2500.000	5032.210	98.3 ± 2.2
1666.670	3354.800	98.3 ± 2.3
833.330	1677.400	97.8 ± 2.5
333.330	670.960	93.3 ± 3.3
250.000	503.220	90.2 ± 4.5
166.670	335.480	48.0 ± 23.8
83.330	167.740	0.1 ± 0.7

* All values represent the mean ± SD of three independent determinations.

Table 14d -- Inhibition of Thrombin Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Hirudin		
μg/ml	μM	%Inhibition*
33.333	4787.210	80.3 ± 8.3
26.667	3829.770	80.5 ± 8.1
20.000	2872.330	80.3 ± 8.3
13.333	1914.880	80.7 ± 8.1
10.000	1436.160	80.5 ± 8.0
6.667	957.440	80.3 ± 8.3
5.000	718.080	80.1 ± 8.3
4.167	598.400	80.2 ± 8.2
3.333	478.720	80.1 ± 8.3
2.500	359.040	79.8 ± 8.4
1.667	239.360	79.7 ± 8.7
0.833	119.680	79.5 ± 8.5
0.333	47.870	78.5 ± 9.4
0.250	35.900	76.3 ± 12.1
0.167	23.940	54.2 ± 46.7
0.083	11.970	0.6 ± 3.6

* All values represent the mean ± SD of three independent determinations.

Table 14e -- Inhibition of Thrombin Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Heparin		
μg/ml	μM	%Inhibition*
333.333	30303.000	96.7 ± 2.9
250.000	22727.300	96.5 ± 3.0
166.667	15151.500	98.0 ± 1.9
133.333	12121.200	98.4 ± 1.7
100.000	9090.900	97.8 ± 2.5
66.667	6060.600	98.8 ± 1.3
33.333	3030.300	98.6 ± 1.6
26.667	2424.200	99.0 ± 1.8
20.000	1818.200	99.1 ± 1.2
13.333	1212.100	98.8 ± 1.8
10.000	909.100	98.0 ± 1.9
6.667	606.100	94.7 ± 1.4
5.000	454.500	90.1 ± 3.1
4.167	378.800	81.8 ± 9.3
3.333	303.000	72.5 ± 14.2
2.667	242.400	54.7 ± 25.5
2.000	181.800	20.0 ± 7.8
1.667	151.500	5.7 ± 8.5
1.333	121.200	4.0 ± 7.1
1.000	90.900	3.0 ± 5.6

* All values represent the mean ± SD of three independent determinations.

Table 14f -- Inhibition of Factor Xa Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Argatroban	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	66.587	88.2 ± 6.1	64.775	91.0 ± 4.5	62.657	36.1 ± 6.0
26.667	53.269	86.7 ± 7.0	51.820	88.4 ± 6.2	50.125	33.6 ± 6.2
20.000	39.952	81.0 ± 9.4	38.865	84.4 ± 8.3	37.594	31.8 ± 6.5
13.333	26.635	76.4 ± 8.0	25.910	76.4 ± 13.3	25.063	28.2 ± 5.1
10.000	19.976	69.8 ± 10.8	19.433	71.2 ± 13.7	18.797	27.2 ± 6.8
8.333	16.647	64.9 ± 12.4	16.194	66.6 ± 13.2	15.664	26.4 ± 7.1
6.667	13.317	58.0 ± 12.9	12.955	61.9 ± 15.4	12.531	24.8 ± 7.0
6.000	11.986	57.9 ± 9.0	11.660	59.6 ± 14.9	11.278	23.8 ± 7.0
5.333	10.654	50.2 ± 12.4	10.364	57.4 ± 15.0	10.025	23.9 ± 7.4
4.667	9.322	49.1 ± 13.8	9.069	53.0 ± 18.8	8.772	22.6 ± 7.2
4.000	7.990	46.8 ± 13.4	7.773	51.6 ± 16.7	7.519	21.8 ± 7.6
3.333	6.659	29.0 ± 29.3	6.478	44.3 ± 20.6	6.266	21.5 ± 7.5
3.000	5.993	38.6 ± 13.4	5.830	46.9 ± 16.2	5.639	21.0 ± 8.1
2.667	5.327	36.5 ± 16.7	5.182	45.3 ± 16.2	5.013	20.9 ± 6.6
2.333	4.661	32.0 ± 12.3	4.534	43.1 ± 16.5	4.386	20.0 ± 7.7
2.000	3.995	30.9 ± 12.6	3.887	41.4 ± 16.0	3.759	19.6 ± 7.7
1.833	3.662	21.2 ± 19.5	3.563	40.2 ± 15.9	3.446	21.4 ± 7.7
1.667	3.329	31.6 ± 5.6	3.239	32.2 ± 7.4	3.133	16.5 ± 7.3
1.500	2.996	30.8 ± 5.1	2.915	31.0 ± 6.9	2.820	16.0 ± 6.5
1.333	2.663	29.2 ± 7.7	2.591	30.6 ± 6.4	2.506	15.4 ± 7.0
1.167	2.331	27.5 ± 6.1	2.267	29.0 ± 6.6	2.193	15.5 ± 6.7
1.000	1.998	25.5 ± 5.7	1.943	26.0 ± 6.2	1.880	15.4 ± 6.5
0.833	1.665	23.6 ± 6.8	1.619	24.7 ± 5.2	1.566	15.1 ± 6.1
0.667	1.332	21.5 ± 5.7	1.296	23.2 ± 5.6	1.253	14.7 ± 5.7
0.500	0.999	19.3 ± 4.6	0.972	21.0 ± 4.1	0.940	13.6 ± 5.5
0.333	0.666	17.0 ± 3.9	0.648	19.8 ± 4.2	0.627	11.6 ± 5.6
0.250	0.499	16.7 ± 4.4	0.486	17.5 ± 4.0	0.470	10.4 ± 4.6
0.167	0.333	15.1 ± 4.5	0.324	16.1 ± 4.3	0.313	7.9 ± 3.0
0.083	0.166	14.1 ± 3.1	0.162	8.4 ± 3.0	0.157	4.3 ± 1.7
0.033	0.067	6.8 ± 3.0	0.065	0.6 ± 1.8	0.063	0.4 ± 0.9

* All values represent the mean ± SD of three independent determinations.

Table 14g -- Inhibition of Factor Xa Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Boc-D-Phe-Pro-Arg-H		
μg/ml	μM	%Inhibition*
16.667	30.215	96.7 ± 0.2
13.333	24.172	95.6 ± 0.5
10.000	18.129	94.0 ± 0.6
6.667	12.086	89.5 ± 2.4
5.000	9.065	86.1 ± 4.0
4.167	7.554	82.0 ± 6.2
3.333	6.043	78.5 ± 4.8
2.667	4.834	68.5 ± 5.3
2.000	3.626	61.8 ± 5.3
1.333	2.417	51.9 ± 7.6
1.000	1.813	44.5 ± 6.0
0.833	1.511	42.4 ± 5.7
0.667	1.209	35.1 ± 6.4
0.583	1.058	36.2 ± 7.0
0.500	0.906	32.3 ± 7.3
0.417	0.755	28.1 ± 5.2
0.333	0.604	22.3 ± 6.9
0.250	0.453	18.3 ± 8.7
0.167	0.302	12.7 ± 8.7
0.083	0.151	4.5 ± 5.0

* All values represent the mean ± SD of three independent determinations.

Table 14h -- Inhibition of Factor Xa Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Ac-(D)Phe-Pro-boroArg-OH		
μg/ml	μM	%Inhibition*
3333.330	6709.610	99.6 ± 0.4
2500.000	5032.210	99.4 ± 0.5
1666.670	3354.800	99.1 ± 0.8
833.330	1677.400	96.1 ± 0.7
333.330	670.960	79.3 ± 1.1
250.000	503.220	71.2 ± 6.3
166.670	335.480	60.4 ± 8.5
83.330	167.740	40.0 ± 12.0
33.330	67.100	17.8 ± 7.0
26.670	53.680	9.2 ± 4.2
20.000	40.260	1.5 ± 2.5

* All values represent the mean ± SD of three independent determinations.

Table 14i -- Inhibition of Factor Xa Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Hirudin		
μg/ml	μM	%Inhibition*
33.333	4787.210	24.9 ± 5.5
26.667	3829.770	26.2 ± 8.0
20.000	2872.330	26.7 ± 8.6
13.333	1914.880	26.9 ± 8.6
10.000	1436.160	26.7 ± 9.0
6.667	957.440	26.8 ± 9.0
5.000	718.080	25.6 ± 7.0
4.167	598.400	26.8 ± 9.8
3.333	478.720	27.0 ± 9.6
2.500	359.040	25.3 ± 7.5
1.667	239.360	24.8 ± 7.8
0.833	119.680	23.7 ± 8.1
0.333	47.870	4.4 ± 8.6

* All values represent the mean ± SD of three independent determinations.

Table 14j -- Inhibition of Factor Xa Generation After Extrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Heparin		
μg/ml	μM	%Inhibition*
333.333	30303.000	91.5 ± 7.0
250.000	22727.300	94.8 ± 3.2
166.667	15151.500	95.2 ± 2.9
133.333	12121.200	95.2 ± 2.3
100.000	9090.900	94.7 ± 1.9
66.667	6060.600	95.4 ± 2.1
33.333	3030.300	95.3 ± 2.4
26.667	2424.200	95.4 ± 2.0
20.000	1818.200	95.5 ± 2.1
13.333	1212.100	95.5 ± 2.3
10.000	909.100	95.6 ± 2.8
6.667	606.100	95.0 ± 2.2
5.000	454.500	94.8 ± 2.2
4.167	378.800	94.0 ± 1.4
3.333	303.000	93.0 ± 2.2
2.667	242.400	90.4 ± 2.3
2.000	181.800	87.0 ± 4.8
1.667	151.500	27.2 ± 47.1
1.333	121.200	52.8 ± 46.2
1.000	90.900	48.5 ± 42.7
0.667	60.600	42.1 ± 38.3
0.583	53.000	35.8 ± 31.8
0.500	45.500	32.9 ± 29.1
0.417	37.900	29.5 ± 26.3
0.333	30.300	25.4 ± 22.8
0.250	22.700	17.7 ± 15.7
0.167	15.200	7.8 ± 6.9

* All values represent the mean ± SD of three independent determinations.

Table 14k -- Inhibition of Thrombin Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Argatroban	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	-	-	-	-	62.657	64.2 ± 11.0
26.667	-	-	51.820	100.0 ± 0.0	50.125	64.6 ± 13.7
20.000	-	-	38.865	99.9 ± 0.2	37.594	54.9 ± 16.5
13.333	-	-	25.910	99.1 ± 1.6	25.063	60.8 ± 15.9
10.000	19.976	100.0 ± 0.0	19.433	98.6 ± 2.4	18.797	57.4 ± 12.4
8.333	16.647	99.3 ± 1.3	16.194	97.7 ± 4.0	15.664	55.5 ± 17.0
6.667	13.317	98.4 ± 2.8	12.955	96.8 ± 5.5	12.531	51.6 ± 20.7
6.000	11.986	98.5 ± 2.6	11.660	96.8 ± 5.5	11.278	50.9 ± 23.5
5.333	10.654	97.6 ± 4.1	10.364	95.9 ± 7.0	10.025	47.3 ± 20.0
4.667	9.322	97.0 ± 5.2	9.069	91.4 ± 15.0	8.772	50.1 ± 19.9
4.000	7.990	96.1 ± 6.8	7.773	92.5 ± 13.0	7.519	50.1 ± 18.1
3.333	6.659	94.0 ± 10.3	6.478	90.8 ± 15.2	6.266	40.3 ± 13.2
3.000	5.993	92.2 ± 13.5	5.830	89.6 ± 16.9	5.639	36.1 ± 22.4
2.667	5.327	90.5 ± 16.4	5.182	84.1 ± 25.8	5.013	31.8 ± 18.9
2.333	4.661	87.3 ± 21.9	4.534	79.3 ± 32.6	4.386	25.3 ± 17.7
2.000	3.995	82.2 ± 30.0	3.887	77.2 ± 34.6	3.759	22.0 ± 17.3
1.833	3.662	80.0 ± 32.7	3.563	75.7 ± 35.1	3.446	20.6 ± 16.5
1.667	3.329	80.0 ± 32.2	3.239	74.6 ± 37.2	3.133	13.3 ± 6.9
1.500	2.996	76.8 ± 33.5	2.915	71.9 ± 39.5	2.820	11.1 ± 3.9
1.333	2.663	75.1 ± 36.3	2.591	64.3 ± 38.6	2.506	7.7 ± 2.6
1.167	2.331	69.3 ± 36.2	2.267	61.3 ± 42.4	2.193	6.1 ± 2.1
1.000	1.998	57.8 ± 33.6	1.943	50.8 ± 40.4	1.880	2.1 ± 0.6
0.833	1.665	52.9 ± 35.0	1.619	42.5 ± 40.3	1.566	1.0 ± 1.2
0.667	1.332	35.9 ± 24.3	1.296	27.5 ± 27.4	1.253	0.3 ± 0.7
0.500	0.999	20.3 ± 12.4	0.972	16.2 ± 18.8	-	-
0.333	0.666	5.9 ± 6.6	0.648	2.7 ± 5.0	-	-
0.250	0.499	2.8 ± 3.9	-	-	-	-

* All values represent the mean ± SD of three independent determinations.

Table 14l -- Inhibition of Thrombin Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Boc-D-Phe-Pro-Arg-H		
μg/ml	μM	%Inhibition*
13.333	24.172	100.0 ± 0.0
10.000	18.129	99.1 ± 1.5
6.667	12.086	97.8 ± 3.8
5.000	9.065	97.2 ± 4.8
4.167	7.554	95.7 ± 7.1
3.333	6.043	92.7 ± 11.3
2.667	4.834	85.5 ± 19.9
2.000	3.626	67.2 ± 48.1
1.333	2.417	61.6 ± 50.8
1.000	1.813	58.0 ± 49.2
0.833	1.511	50.2 ± 43.0
0.667	1.209	13.4 ± 11.0
0.583	1.058	13.8 ± 10.7
0.500	0.906	1.9 ± 2.0

* All values represent the mean ± SD of three independent determinations.

Table 14m -- Inhibition of Thrombin Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Ac-(D)Phe-Pro-boroArg-OH		
μg/ml	μM	%Inhibition*
166.670	335.480	100.0 ± 0.1
83.330	167.740	83.4 ± 12.5
33.330	67.100	0.0 ± 0.4

* All values represent the mean ± SD of three independent determinations.

Table 14n -- Inhibition of Thrombin Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Hirudin		
μg/ml	μM	%Inhibition*
33.333	4787.210	79.6 ± 3.7
26.667	3829.770	79.2 ± 4.1
20.000	2872.330	79.6 ± 3.9
13.333	1914.880	79.5 ± 3.7
10.000	1436.160	79.6 ± 3.9
6.667	957.440	79.4 ± 3.9
5.000	718.080	79.3 ± 3.8
4.167	598.400	79.3 ± 4.0
3.333	478.720	79.3 ± 3.8
2.500	359.040	78.9 ± 4.2
1.667	239.360	79.1 ± 4.3
0.833	119.680	78.9 ± 3.9
0.333	47.870	45.4 ± 41.0
0.250	35.900	20.5 ± 31.4
0.167	23.940	0.0 ± 0.8

* All values represent the mean ± SD of three independent determinations.

Table 14o -- Inhibition of Thrombin Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Heparin		
μg/ml	μM	%Inhibition*
333.333	30303.000	75.1 ± 16.5
250.000	22727.300	73.8 ± 16.4
166.667	15151.500	73.2 ± 16.2
133.333	12121.200	73.0 ± 17.8
100.000	9090.900	74.3 ± 17.8
66.667	6060.600	74.0 ± 15.3
33.333	3030.300	71.8 ± 7.5
26.667	2424.200	71.6 ± 2.7
20.000	1818.200	71.4 ± 2.6
13.333	1212.100	69.9 ± 6.1
10.000	909.100	71.0 ± 7.2
6.667	606.100	73.2 ± 4.8
5.000	454.500	76.5 ± 1.7
4.167	378.800	77.1 ± 1.9
3.333	303.000	78.3 ± 4.2
2.667	242.400	77.9 ± 5.6
2.000	181.800	78.0 ± 4.9
1.667	151.500	51.8 ± 45.7
1.333	121.200	52.2 ± 45.3
1.000	90.900	51.9 ± 44.9
0.667	60.600	51.4 ± 44.3
0.583	53.000	50.8 ± 44.8
0.500	45.500	50.6 ± 44.4
0.417	37.900	47.3 ± 42.9
0.333	30.300	29.1 ± 34.2
0.250	22.700	18.0 ± 35.8
0.167	15.200	0.0 ± 2.0

* All values represent the mean ± SD of three independent determinations.

Table 14p -- Inhibition of Factor Xa Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Argatroban	
μg/ml	μM	%Inhibition*	μM	%Inhibition*	μM	%Inhibition*
33.333	66.587	99.2 ± 1.4	-	-	62.657	97.0 ± 4.0
26.667	53.269	99.2 ± 1.4	-	-	50.125	98.0 ± 2.1
20.000	39.952	99.2 ± 1.4	-	-	37.594	94.4 ± 5.0
13.333	26.635	99.1 ± 1.5	-	-	25.063	95.6 ± 4.9
10.000	19.976	99.1 ± 1.5	-	-	18.797	96.8 ± 5.6
8.333	16.647	99.1 ± 1.6	-	-	15.664	97.1 ± 5.1
6.667	13.317	99.0 ± 1.7	-	-	12.531	95.9 ± 7.1
6.000	11.986	99.0 ± 1.7	-	-	11.278	97.7 ± 4.0
5.333	10.654	98.9 ± 1.9	-	-	10.025	95.3 ± 8.1
4.667	9.322	98.9 ± 1.9	-	-	8.772	97.1 ± 5.0
4.000	7.990	98.8 ± 2.0	-	-	7.519	94.3 ± 9.8
3.333	6.659	98.8 ± 2.1	-	-	6.266	96.2 ± 6.6
3.000	5.993	98.7 ± 2.2	-	-	5.639	96.0 ± 7.0
2.667	5.327	98.7 ± 2.3	-	-	5.013	96.1 ± 6.7
2.333	4.661	98.5 ± 2.7	-	-	4.386	96.1 ± 6.7
2.000	3.995	98.3 ± 2.9	3.887	100.0 ± 0.0	3.759	95.7 ± 7.5
1.833	3.662	97.9 ± 3.2	3.563	99.9 ± 0.1	3.446	95.5 ± 7.8
1.667	3.329	98.0 ± 3.5	3.239	100.0 ± 0.0	3.133	86.6 ± 15.0
1.500	2.996	97.8 ± 3.8	2.915	99.8 ± 0.3	2.820	86.4 ± 14.7
1.333	2.663	96.7 ± 3.9	2.591	99.5 ± 0.5	2.506	84.8 ± 14.5
1.167	2.331	96.1 ± 3.9	2.267	99.2 ± 0.8	2.193	84.9 ± 14.7
1.000	1.998	96.6 ± 4.7	1.943	98.7 ± 1.1	1.880	84.6 ± 15.0
0.833	1.665	95.1 ± 4.7	1.619	97.9 ± 1.7	1.566	84.1 ± 14.7
0.667	1.332	94.1 ± 5.6	1.296	97.2 ± 1.4	1.253	83.4 ± 14.2
0.500	0.999	93.4 ± 5.4	0.972	96.0 ± 1.6	0.940	81.1 ± 13.0
0.333	0.666	92.3 ± 5.9	0.648	93.8 ± 1.7	0.627	78.2 ± 13.3
0.250	0.499	91.2 ± 5.6	0.486	91.5 ± 1.1	0.470	73.9 ± 13.7
0.167	0.333	89.1 ± 5.5	0.324	86.4 ± 0.8	0.313	66.0 ± 12.6
0.083	0.166	81.1 ± 5.2	0.162	69.3 ± 5.5	0.157	47.4 ± 15.0
0.033	0.067	43.2 ± 12.7	0.065	28.2 ± 3.1	0.063	17.5 ± 13.7
0.025	0.050	25.1 ± 13.9	0.049	17.5 ± 2.4	0.047	11.4 ± 12.5
0.017	0.033	10.7 ± 7.5	0.032	5.5 ± 1.1	0.031	5.2 ± 6.8
0.008	0.017	1.8 ± 2.5	0.016	0.1 ± 0.3	0.016	0.4 ± 2.1

* All values represent the mean ± SD of three independent determinations.

Table 14q -- Inhibition of Factor Xa Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Boc-D-Phe-Pro-Arg-H		
μg/ml	μM	%Inhibition*
2.000	3.626	100.0 ± 0.0
1.333	2.417	99.8 ± 0.3
1.000	1.813	99.2 ± 1.5
0.833	1.511	98.6 ± 2.5
0.667	1.209	97.7 ± 4.0
0.583	1.058	97.7 ± 4.0
0.500	0.906	96.7 ± 4.6
0.417	0.755	94.4 ± 7.7
0.333	0.604	91.8 ± 7.3
0.250	0.453	85.4 ± 5.1
0.167	0.302	73.1 ± 4.8
0.083	0.151	46.8 ± 5.9
0.033	0.060	11.9 ± 6.1
0.025	0.045	2.8 ± 1.5
0.017	0.030	0.6 ± 1.2

* All values represent the mean ± SD of three independent determinations.

Table 14r -- Inhibition of Factor Xa Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Ac-(D)Phe-Pro-boroArg-OH		
μg/ml	μM	%Inhibition*
83.330	167.740	100.0 ± 0.0
33.330	67.100	89.0 ± 8.5
26.670	53.680	77.7 ± 25.6
20.000	40.260	60.7 ± 27.6
13.330	26.840	22.1 ± 11.3
6.670	13.420	1.0 ± 1.5

* All values represent the mean ± SD of three independent determinations.

Table 14s -- Inhibition of Factor Xa Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Hirudin		
μg/ml	μM	%Inhibition*
0.833	119.680	100.0 ± 0.0
0.333	47.870	97.1 ± 2.0
0.250	35.900	80.7 ± 9.6
0.167	23.940	56.9 ± 21.1
0.083	11.970	5.9 ± 5.4
0.033	4.790	0.9 ± 1.1

* All values represent the mean ± SD of three independent determinations.

Table 14t -- Inhibition of Factor Xa Generation After Intrinsic Stimulation of Fibrinogen-Deficient Human Plasma.

Heparin		
μg/ml	μM	%Inhibition*
333.333	30303.000	93.6 ± 4.4
250.000	22727.300	93.7 ± 3.9
166.667	15151.500	93.6 ± 4.1
133.333	12121.200	93.5 ± 4.0
100.000	9090.900	93.8 ± 3.8
66.667	6060.600	93.8 ± 3.9
33.333	3030.300	93.7 ± 3.9
26.667	2424.200	93.9 ± 3.8
20.000	1818.200	93.8 ± 3.9
13.333	1212.100	94.2 ± 4.0
10.000	909.100	94.2 ± 4.1
6.667	606.100	94.6 ± 3.9
5.000	454.500	94.5 ± 4.4
4.167	378.800	94.9 ± 4.4
3.333	303.000	94.8 ± 4.5
2.667	242.400	95.2 ± 4.3
2.000	181.800	95.0 ± 4.3
1.667	151.500	96.1 ± 5.6
1.333	121.200	95.3 ± 4.1
1.000	90.900	95.6 ± 3.9
0.667	60.600	96.3 ± 3.4
0.583	53.000	97.0 ± 2.9
0.500	45.500	97.4 ± 2.9
0.417	37.900	97.4 ± 3.2
0.333	30.300	96.5 ± 4.5
0.250	22.700	93.6 ± 7.8
0.167	15.200	86.0 ± 14.1
0.083	7.600	55.3 ± 25.3
0.033	3.000	19.3 ± 13.2
0.025	2.300	7.4 ± 7.4
0.017	1.500	1.7 ± 1.9
0.008	0.800	0.1 ± 0.2

* All values represent the mean ± SD of three independent determinations.

Table 15a -- Inhibition of Thrombin Generation from KONYNE® by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	%In	μM	%In	μM	%In	μM	%In
1.500	-	-	2.915	53.6	2.719	59.8	-	-
1.000	1.998	44.8	1.943	49.8	1.813	53.3	2.013	80.5
0.500	0.999	29.5	0.972	38.2	0.906	35.5	0.999	68.3
0.250	0.499	15.9	0.486	24.2	0.453	18.7	0.499	57.7
0.125	0.250	2.3	0.243	14.5	0.227	8.0	0.250	50.2
0.063	0.125	2.9	0.121	8.7	0.113	5.6	0.125	39.6
0.031	0.062	0.0	0.061	1.6	0.057	5.2	0.062	28.3
0.016	0.031	1.6	0.030	1.8	0.028	1.3	0.031	19.2
0.000	0.000	0.0	0.000	0.0	0.000	0.0	0.000	0.0

Table 15b -- Inhibition of Thrombin Generation from KONYNE® by Thrombin Inhibitors.

Argatroban		
μg/ml	μM	%In
5.000	9.398	62.6
2.500	4.699	42.0
1.250	2.350	38.0
0.625	1.175	35.0
0.313	0.587	23.0
0.156	0.294	14.0
0.078	0.147	11.0
0.039	0.073	8.0
0.020	0.037	8.0
0.010	0.018	1.0
0.000	0.000	0.0

Table 15c -- Inhibition of Thrombin Generation from KONYNE® by Thrombin Inhibitors.

	Hirudin		Heparin	
μg/ml	μM	%In	μM	%In
20.000	2.872	36.7	-	-
15.000	-	-	1.402	43.2
10.000	1.436	35.1	0.935	39.1
5.000	-	-	0.467	25.3
2.500	0.359	44.6	0.234	9.8
1.000	0.144	43.4	0.093	0.0
0.500	0.072	40.2	-	-
0.250	0.036	28.2	-	-
0.125	0.018	19.7	-	-
0.063	0.009	6.1	-	-
0.031	0.004	2.9	-	-
0.000	0.000	0.0	0.000	0.0

Table 15d -- Inhibition of Factor Xa Generation from KONYNE® by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	% In	μM	% In	μM	% In	μM	% In
1.500	2.996	50.6	-	-	2.719	75.3	-	-
1.000	1.998	48.0	1.943	58.6	1.813	70.7	2.013	99.3
0.500	0.999	34.3	-	-	0.906	49.8	0.999	98.1
0.250	0.499	0.0	0.486	58.3	0.453	21.7	0.499	94.1
0.125	0.250	0.0	0.243	14.5	0.227	1.6	0.250	89.3
0.063	0.125	0.0	0.121	0.0	0.113	0.9	0.125	74.7
0.031	0.062	0.0	0.061	0.0	0.057	1.6	0.062	8.5
0.016	0.031	0.0	0.030	0.0	0.028	1.4	0.031	0.0
0.000	0.000	0.0	0.000	0.0	0.000	0.0	0.000	0.0

Table 15e -- Inhibition of Factor Xa Generation from KONYNE® by Thrombin Inhibitors.

Argatroban		
μg/ml	μM	%In
10.000	18.797	56.0
5.000	9.398	52.0
2.500	4.699	50.0
1.250	2.350	49.0
0.625	1.175	49.0
0.313	0.587	45.0
0.156	0.294	39.0
0.078	0.147	33.0
0.039	0.073	22.0
0.020	0.037	13.0
0.010	0.018	0.0

Table 15f -- Inhibition of Factor Xa Generation from KONYNE® by Thrombin Inhibitors.

	Hirudin		Heparin	
μg/ml	μM	%In	μM	%In
20.000	2.872	51.7	-	-
15.000	2.154	52.8	1.402	69.1
10.000	1.436	53.1	0.935	60.1
5.000	-	-	0.467	28.1
2.500	0.359	48.7	0.234	14.1
1.000	0.144	46.5	0.093	0.0
0.500	0.072	19.6	0.047	0.9
0.250	0.036	0.0	0.023	0.0
0.125	0.018	5.9	-	-
0.063	0.009	6.2	-	-
0.031	0.004	2.3	-	-
0.016	0.002	0.0	-	-

Table 15g -- Inhibition of Thrombin Generation from FEIBA® by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	%In	μM	%In	μM	%In	μM	%In
1.500	2.996	69.4	2.915	1.1	2.719	0.2	-	-
1.000	1.998	10.9	1.943	3.1	1.813	0.0	96.8	99.3
0.500	0.999	7.6	0.972	16.2	0.906	1.9	81.7	98.1
0.250	0.499	5.1	0.486	0.0	0.453	0.0	28.0	94.1
0.125	0.250	7.0	0.243	4.9	0.227	0.0	13.9	89.3
0.063	0.125	5.1	0.121	2.1	0.113	0.0	10.3	74.7
0.031	0.062	5.0	0.061	4.0	0.057	0.0	11.3	8.5
0.016	0.031	6.6	0.030	3.2	0.028	5.3	13.2	0.0

Table 15h -- Inhibition of Thrombin Generation from FEIBA® by Thrombin Inhibitors.

Argatroban		
μg/ml	μM	%In
10.000	18.797	49.0
5.000	9.398	14.0
2.500	4.699	11.0
1.250	2.350	8.0
0.625	1.175	3.0
0.313	0.587	0.0
0.156	0.294	1.0
0.078	0.147	0.0

Table 15i -- Inhibition of Thrombin Generation from FEIBA® by Thrombin Inhibitors.

	Hirudin		Heparin	
μg/ml	μM	%In	μM	%In
20.000	2.872	93.8	1.869	56.9
15.000	2.154	91.7	1.402	34.2
10.000	1.436	90.4	0.935	5.2
5.000	0.718	83.7	0.467	0.0
2.500	0.359	0.0	-	-
1.000	0.144	2.4	-	-
0.500	0.072	5.5	-	-
0.250	0.036	2.9	-	-
0.125	0.018	4.2	-	-
0.063	0.009	10.6	-	-
0.031	0.004	4.7	-	-
0.016	0.002	0.0	-	-

Table 15j -- Inhibition of Factor Xa Generation from FEIBA® by Thrombin Inhibitors.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	%In	μM	%In	μM	%In	μM	%In
1.500	2.996	66.4	2.915	85.4	2.719	73.3	-	-
1.000	1.998	0.0	1.943	65.7	1.813	33.2	2.013	99.8
0.500	0.999	0.0	0.972	0.5	0.906	2.6	1.006	99.1
0.250	0.499	0.0	0.486	0.0	0.453	0.0	0.503	94.4
0.125	0.250	0.0	0.243	1.5	0.227	0.0	0.252	2.8

Table 15k -- Inhibition of Factor Xa Generation from FEIBA® by Thrombin Inhibitors.

Argatroban		
μg/ml	μM	%In
10.000	18.797	91.0
5.000	9.398	90.0
2.500	4.699	85.0
1.250	2.350	78.0
0.625	1.175	66.0
0.313	0.587	33.0
0.156	0.294	1.0
0.078	0.147	1.0
0.039	0.073	2.0
0.020	0.037	0.0

Table 15l -- Inhibition of Factor Xa Generation from FEIBA® by Thrombin Inhibitors.

	Hirudin		Heparin	
μg/ml	μM	%In	μM	%In
20.000	2.872	88.4	1.869	0.0
15.000	2.154	82.5	1.402	2.3
10.000	1.436	87.8	0.935	2.6
5.000	0.718	84.7	0.467	0.0
2.500	0.359	0.0	0.234	0.0

Table 16a -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based PT assay.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
100.00	199.76	>300.0	-	-	181.29	>300.0	-	-
50.00	99.88	186.5± 2.0	97.16	>300.0	90.65	74.5± 4.7	-	-
25.00	49.94	75.9± 2.2	48.58	162.1± 36.3	45.32	26.8± 0.2	-	-
10.00	19.98	26.1± 0.6	19.43	49.4± 1.4	18.13	30.4± 0.8	-	-
5.00	9.99	17.5± 0.3	9.72	23.4± 0.5	9.06	18.8± 0.3	10.06	>300.0
2.50	4.99	14.8± 0.5	4.86	17.1± 0.8	4.53	14.8± 0.3	5.03	39.9± 2.1
1.25	2.50	13.0± 0.6	2.43	14.4± 0.5	2.27	12.3± 1.2	2.52	22.0± 1.5
0.63	1.25	13.3± 0.5	1.21	13.2± 0.3	1.13	12.6± 0.2	1.26	16.7± 1.4
0.31	0.62	12.6± 0.3	0.61	12.6± 0.3	0.57	12.6± 0.2	0.63	15.2± 1.7
0.16	0.31	12.0± 1.8	0.30	12.4± 1.0	0.28	12.5± 0.9	0.31	13.3± 1.3
0.00	0.00	12.0± 1.8	0.00	12.0± 1.8	0.00	12.0± 1.8	0.00	12.0± 1.8

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16b -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based PT assay.

	Argatroban		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)
100.00	187.97	>300.0	-	-
50.00	93.98	219.9± 4.0	-	-
25.00	46.99	139.9± 3.0	-	-
10.00	18.80	89.3± 3.0	0.93	20.5± 3.6
5.00	9.40	58.3± 2.0	0.47	13.8± 0.6
2.50	4.70	37.9± 1.0	0.23	12.3± 0.3
1.25	2.35	26.4± 0.7	0.12	12.1± 0.3
0.63	1.17	20.4± 0.5	0.06	11.8± 0.3
0.31	0.59	15.9± 0.3	0.03	12.3± 0.9
0.16	0.29	13.7± 0.3	0.01	12.2± 1.2
0.00	0.00	11.6± 0.3	0.00	12.0± 1.8

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16c -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based PT assay.

Hirudin		
μg/ml	μM	Clotting Time (sec)
25.00	3.59	>300.0
12.50	1.80	18.4± 1.0
6.25	0.90	15.7± 1.0
3.13	0.45	13.9± 1.0
1.56	0.22	12.9± 0.5
0.78	0.11	12.4± 0.5
0.39	0.06	12.3± 0.5
0.20	0.03	11.9± 0.3
0.00	0.00	11.4± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16d -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based APTT assay.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
50.00	-	-	-	-	90.65	>300.0	-	-
25.00	49.94	>300.0	48.58	>300.0	45.32	191.2± 9.8	-	-
10.00	19.98	145.8± 1.6	19.43	186.0± 3.0	18.13	198.1± 4.7	-	-
5.00	9.99	88.4± 1.0	9.72	110.7± 1.1	9.06	112.0± 3.8	-	-
2.50	4.99	64.7± 1.8	4.86	75.5± 0.6	4.53	70.1± 3.3	-	-
1.25	2.50	49.5± 0.7	2.43	59.5± 3.5	2.27	51.4± 0.5	2.52	>300.0
0.63	1.25	40.7± 1.5	1.21	45.7± 0.3	1.13	42.4± 1.0	1.26	218.0± 14.0
0.31	0.62	38.3± 1.3	0.61	40.3± 0.6	0.57	37.7± 0.3	0.63	90.1± 8.0
0.16	0.31	34.7± 0.3	0.30	36.2± 0.6	0.28	35.0± 0.3	0.31	68.3± 1.8
0.00	0.00	34.7± 0.3	0.00	34.7± 0.3	0.00	34.7± 0.3	0.00	34.7± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16e -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based APTT assay.

	Argatroban		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)
50.00	93.98	>300.0	-	-
25.00	46.99	216.6± 5.0	-	-
10.00	18.80	157.9± 4.0	-	-
5.00	9.40	123.2± 4.0	0.47	>300.0
2.50	4.70	94.4± 3.0	0.23	98.0± 3.9
1.25	2.35	79.0± 3.0	0.12	51.0± 1.3
0.63	1.17	63.4± 2.0	0.06	35.7± 0.3
0.31	0.59	53.4± 2.0	0.03	36.4± 4.6
0.16	0.29	46.8± 1.0	0.01	32.7± 0.6
0.00	0.00	29.4± 0.5	0.00	34.7± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16f -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based APTT assay.

Hirudin		
μg/ml	μM	Clotting Time (sec)
25.00	3.59	>300.0
12.50	1.80	105.9± 3.0
6.25	0.90	89.3± 3.0
3.13	0.45	74.9± 3.0
1.56	0.22	63.0± 2.0
0.78	0.11	56.0± 2.0
0.39	0.06	48.5± 1.0
0.20	0.03	42.7± 1.0
0.00	0.00	29.4± 0.5

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16g -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based TT assay.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
10.00	19.98	>300.0	-	-	18.13	>300.0	-	-
5.00	9.99	207.4± 41.7	9.72	>300.0	9.06	190.5± 37.6	-	-
2.50	4.99	96.5± 2.0	4.86	161.3± 20.4	4.53	75.2± 2.0	-	-
1.25	2.50	32.5± 2.3	2.43	91.4± 4.8	2.27	23.2± 2.0	-	-
0.63	1.25	8.7± 3.0	1.21	48.0± 6.0	1.13	10.2± 0.8	1.26	>300.0
0.31	0.62	9.3± 0.5	0.61	16.0± 0.7	0.57	7.0± 0.3	0.63	106.9± 4.7
0.16	0.31	7.2± 0.4	0.30	8.0± 1.5	0.28	6.5± 0.3	0.31	20.2± 3.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16h -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based TT assay.

	Hirudin		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)
5.00	0.72	>300.0	0.47	>300.0
2.50	0.36	288.4± 16.3	0.23	137.0± 2.0
1.25	0.18	204.1± 24.0	0.12	78.0± 0.2
0.63	0.09	93.2± 4.7	0.06	6.7± 0.4
0.31	0.04	54.8± 1.0	0.03	6.0± 0.3
0.16	0.02	8.2± 2.3	0.01	5.4± 0.6

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16i -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based TT assay.

Argatroban		
μg/ml	μM	Clotting Time (sec)
50.00	93.98	>300.0
25.00	46.99	239.9± 5.0
12.50	23.50	181.9± 3.0
6.25	11.75	118.2± 3.0
3.13	5.87	79.5± 2.0
1.56	2.94	64.9± 2.0
0.78	1.47	44.6± 1.0
0.39	0.73	33.7± 1.0
0.20	0.37	24.2± 1.0
0.10	0.18	12.2± 1.0

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16j -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based ECT assay.

D-Phe-Pro-Arg-H			D-MePhe-Pro-Arg-H			Boc-D-Phe-Pro-Arg-H		
μg/ml	μM	Clotting Time (sec)	μg/ml	μM	Clotting Time (sec)	μg/ml	μM	Clotting Time (sec)
0.00	0.00	43.1 ± 0.0	0.00	0.00	41.5 ± 1.5	0.00	0.00	40.9 ± 2.7
0.01	0.02	51.7 ± 1.3	0.01	0.02	46.9 ± 4.0	0.05	0.09	50.7 ± 1.3
0.05	0.10	75.4 ± 6.6	0.05	0.10	64.6 ± 6.3	0.10	0.18	57.0 ± 2.2
0.10	0.20	114.0 ± 9.7	0.10	0.19	92.5 ± 13.4	0.50	0.91	100.7 ± 7.4
0.25	0.50	233.4 ± 18.6	0.25	0.49	178.0 ± 33.3	1.00	1.81	149.1 ± 15.8
0.50	1.00	361.0 ± 75.1	0.50	0.97	306.7 ± 81.3	2.00	3.63	225.1 ± 27.0
0.75	1.50	587.7 ± 34.6	0.75	1.46	437.0 ± 100.4	3.00	5.44	295.8 ± 29.0
1.00	2.00	768.9 ± 71.7	1.00	1.94	553.5 ± 121.1	4.00	7.25	356.0 ± 37.3
1.25	2.50	887.2 ± 24.8	1.25	2.43	694.1 ± 161.6	5.00	9.06	406.6 ± 51.5
-	-	-	1.50	2.91	719.0 ± 102.8	6.00	10.88	585.5 ± ERR
-	-	-	1.75	3.40	798.4 ± 124.9	7.50	13.60	575.1 ± 120.2
-	-	-	-	-	-	10.00	18.13	663.7 ± 194.7
-	-	-	-	-	-	12.50	22.66	742.8 ± 104.2

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16k -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based ECT assay.

Ac-(D)Phe-Pro-boroArg-OH			Argatroban			Hirudin		
μg/ml	μM	Clotting Time (sec)	μg/ml	μM	Clotting Time (sec)	μg/ml	μM	Clotting Time (sec)
0.00	0.00	42.1 ± 3.8	0.00	0.00	43.7 ± 2.4	0.00	0.00	43.1 ± 3.5
0.01	0.02	48.8 ± 2.7	0.01	0.02	71.6 ± 30.9	0.10	0.01	59.3 ± 4.0
0.01	0.02	52.6 ± 2.9	0.05	0.09	73.5 ± 1.0	0.25	0.04	73.8 ± 9.2
0.05	0.10	104.5 ± 14.8	0.10	0.19	96.0 ± 3.6	0.50	0.07	105.5 ± 21.0
0.10	0.20	188.7 ± 19.9	0.25	0.47	147.6 ± 9.4	1.00	0.14	173.5 ± 38.9
0.20	0.40	378.1 ± 29.4	0.50	0.94	213.7 ± 13.3	2.00	0.29	325.5 ± 70.5
0.30	0.60	577.9 ± 42.5	0.75	1.41	282.4 ± 12.0	3.00	0.43	457.3 ± 148.4
0.40	0.81	755.3 ± 50.9	1.00	1.88	329.0 ± 9.3	4.00	0.57	632.4 ± 183.7
-	-	-	1.25	2.35	373.7 ± 4.0	5.00	0.72	698.1 ± 96.0
-	-	-	1.50	2.82	420.2 ± 2.5	6.00	0.86	796.4 ± 143.0
-	-	-	1.75	3.29	454.5 ± 6.7	-	-	-
-	-	-	2.00	3.76	475.9 ± 39.1	-	-	-
-	-	-	3.00	5.64	600.8 ± 9.4	-	-	-
-	-	-	4.00	7.52	694.3 ± 11.5	-	-	-
-	-	-	5.00	9.40	793.6 ± 0.0	-	-	-

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16l -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based Heptest assay.

	D-Phe-Pro-Arg-H		D-MePhe-Pro-Arg-H		Boc-D-Phe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
50.00	99.88	>300.0	-	-	-	-	-	-
25.00	49.94	240.5± 6.2	-	-	45.32	>300.0	-	-
10.00	19.98	90.5± 5.1	19.43	>300.0	18.13	44.2± 7.3	-	-
5.00	9.99	34.8± 3.2	9.72	65.4± 7.2	9.06	75.1± 0.7	-	-
2.50	4.99	25.5± 0.7	4.86	38.7± 2.9	4.53	33.0± 0.8	5.03	>300.0
1.25	2.50	21.8± 2.1	2.43	28.4± 0.7	2.27	24.8± 1.0	2.52	125.4± 0.8
0.63	1.25	19.1± 1.2	1.21	22.2± 0.3	1.13	20.1± 0.7	1.26	59.0± 0.3
0.31	0.62	17.8± 0.5	0.61	19.2± 0.3	0.57	17.4± 0.5	0.63	37.3± 2.0
0.16	0.31	16.6± 0.6	0.30	16.9± 0.5	0.28	17.1± 0.8	0.31	24.3± 0.5
0.00	0.00	16.6± 0.6	0.00	16.6± 0.6	0.00	15.6± 0.3	0.00	16.6± 0.6

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16m -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based Heptest assay.

	Argatroban		Hirudin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)
25.0	-	-	3.59	>300.0
12.5	23.50	>300.0	1.80	127.4± 3.0
6.3	11.75	221.7± 4.0	0.90	77.3± 2.0
3.1	5.87	131.7± 3.0	0.45	30.7± 2.0
1.6	2.94	89.9± 3.0	0.22	26.1± 1.0
0.8	1.47	63.3± 2.0	0.11	21.8± 0.7
0.4	0.73	44.8± 2.0	0.06	21.4± 0.5
0.2	0.37	34.7± 1.0	0.03	20.8± 0.5
0.0	0.00	20.7± 0.5	0.00	20.7± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16n -- Comparative anticoagulant effects of thrombin inhibitors in the NHP based Heptest assay.

Heparin		
μg/ml	μM	Clotting Time (sec)
10.00	2.34	>300.0
5.00	0.93	274.5± 14.2
2.50	0.47	123.7± 0.7
1.25	0.23	84.0± 0.2
0.63	0.12	57.0± 2.3
0.31	0.06	28.9± 0.9
0.16	0.03	19.5± 1.0
0.08	0.01	17.0± 0.0
0.00	0.00	16.3± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16o -- Comparative anticoagulant effects of thrombin inhibitors after supplementation into normal human whole blood, in the ACT assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Argatroban		Hirudin		Heparin	
μM	ng/ml	Clotting Time (sec)	ng/ml	Clotting Time (sec)	μg/ml	Clotting Time (sec)	μg/ml	Clotting Time (sec)	μg/ml	Clotting Time (sec)
1.00	514.6	156.0 ± 27.9	496.8	307 ± 23.8	532	183.3 ± 25.0	6963	366.0 ± 19.7	10700	326.3 ± 21.0
0.10	51.46	134.3 ± 2.5	49.68	139.7 ± 11.6	53.2	145.7 ± 65.0	696.3	184.3 ± 10.6	1070	160.7 ± 32.8
0.01	5.146	128.7 ± 2.1	4.968	132.3 ± 6.7	5.32	139.0 ± 2.8	69.63	136.0 ± 5.7	107	127.3 ± 7.4
0.00	0	130.0 ± 4.2	-	-	-	-	-	-	-	-

Each clotting time value represents the mean ± SD of three independent determinations.

Table 16p -- Comparative anticoagulant effects of thrombin inhibitors after supplementation into normal human whole blood, in the TEG assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Argatroban		Hirudin		Heparin	
μM	ng/ml	TEG R Value (mm)	ng/ml	TEG R Value (mm)	μg/ml	TEG R Value (mm)	μg/ml	TEG R Value (mm)	μg/ml	TEG R Value (mm)
1.00	-	-	-	-	532	31.2 ± 1.6	-	-	-	-
0.10	51.46	38	49.68	43.3 ± 17.7	53.2	25.2 ± 2.5	696.3	42.8 ± 3.9	1070	46.5 ± 3.0
0.01	5.146	22	4.968	26.3 ± 2.5	5.32	25.7 ± 1.0	69.63	27.0 ± 4.2	107	26.7 ± 3.3

Each TEG R value represents the mean ± SD of three independent determinations.

Table 17a -- Comparative anticoagulant effects of thrombin inhibitors in the NRP based PT assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
100.00	194.33	>300.0	-	-	-	-	9.35	16.5 ± 0.7
40.00	77.73	32.2 ± 4.1	-	-	5.745	>300.0	3.74	10.7 ± 1.4
20.00	38.87	15.9 ± 2.0	-	-	2.872	48.3 ± 25.6	1.87	8.8 ± 1.0
10.00	19.43	11.0 ± 1.4	20.13	23.7 ± 5.5	1.436	14.1 ± 1.2	0.94	7.4 ± 0.9
5.00	9.72	8.8 ± 0.3	10.06	13.8 ± 3.2	0.718	10.3 ± 2.9	0.47	7.1 ± 0.4
2.50	4.86	7.9 ± 0.4	5.03	10.3 ± 0.6	0.359	8.7 ± 0.5	0.23	7.0 ± 0.5
1.25	2.43	7.4 ± 0.1	2.52	8.3 ± 0.3	0.180	8.3 ± 0.6	0.12	6.6 ± 0.3
0.62	1.21	6.7 ± 0.1	1.26	7.5 ± 0.5	0.089	7.4 ± 0.5	0.06	6.8 ± 0.6
0.31	0.60	6.6 ± 0.7	0.63	7.0 ± 0.2	0.045	7.3 ± 0.2	0.03	6.8 ± 0.3
0.16	0.31	6.9 ± 0.2	0.32	6.6 ± 0.3	0.023	6.8 ± 0.2	0.02	6.7 ± 0.1
0.08	0.16	6.8 ± 0.3	0.16	6.8 ± 0.2	0.011	6.9 ± 0.4	-	-
0.04	0.08	6.5 ± 0.6	0.08	6.5 ± 0.3	0.006	7.1 ± 0.2	-	-
0.02	-	-	0.04	7.0 ± 0.1	-	-	-	-
0.01	-	-	0.02	6.7 ± 0.4	-	-	-	-
0.00	0.00	6.9 ± 0.3	0.00	6.8 ± 0.4	0.000	7.3 ± 0.1	0.00	7.0 ± 0.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 17b -- Comparative anticoagulant effects of thrombin inhibitors in the NRP based APTT assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
40.00	-	-	-	-	5.745	>300.0	-	-
20.00	38.87	>300.0	-	-	2.872	167.2 ± 21.1	-	-
10.00	19.43	273.2 ± 46.4	-	-	1.436	130.6 ± 45.8	0.94	>300.0
5.00	9.72	195.7 ± 35.5	-	-	0.718	101.6 ± 40.5	0.47	132.7 ± 36.2
2.50	4.86	101.5 ± 6.1	-	-	0.359	72.7 ± 19.3	0.23	76.8 ± 16.7
1.25	2.43	74.9 ± 7.7	2.52	>300.0	0.180	64.0 ± 21.9	0.12	57.7 ± 11.3
0.62	1.21	54.6 ± 1.8	1.26	263.5 ± 63.3	0.089	56.2 ± 15.3	0.06	52.2 ± 8.6
0.31	0.60	52.8 ± 7.6	0.63	124.7 ± 7.9	0.045	45.7 ± 5.7	0.03	44.4 ± 8.9
0.16	0.31	42.2 ± 2.9	0.32	82.1 ± 14.1	0.023	39.2 ± 4.7	0.02	37.7 ± 6.0
0.08	0.16	39.8 ± 3.1	0.16	63.6 ± 7.7	0.011	39.8 ± 3.9	-	-
0.04	0.08	36.9 ± 5.7	0.08	50.2 ± 8.6	0.006	34.9 ± 3.6	-	-
0.02	-	-	0.04	44.0 ± 8.9	-	-	-	-
0.01	-	-	0.02	37.4 ± 4.7	-	-	-	-
0.00	0.00	39.4 ± 5.8	0.00	37.5 ± 4.3	0.000	36.1 ± 6.0	0.00	38.2 ± 4.3

Each clotting time value represents the mean ± SD of three independent determinations.

Table 17c -- Comparative anticoagulant effects of thrombin inhibitors in the NRP based TT assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
20.00	38.87	>300.0	-	-	-	-	-	-
10.00	19.43	298.6 ± 2.4	-	-	-	-	-	-
5.00	9.72	176.8 ± 29.3	-	-	0.718	>300.0	0.47	>300.0
2.50	4.86	106.8 ± 19.9	-	-	0.359	172.1 ± 26.0	0.23	59.3 ± 14.9
1.25	2.43	59.0 ± 10.5	2.52	>300.0	0.180	100.7 ± 4.4	0.12	26.3 ± 10.0
0.62	1.21	44.7 ± 4.9	1.26	131.7 ± 7.0	0.089	66.7 ± 2.5	0.06	18.3 ± 2.6
0.31	0.60	29.2 ± 9.1	0.63	71.6 ± 3.8	0.045	41.0 ± 8.7	0.03	13.7 ± 0.6
0.16	0.31	17.9 ± 6.2	0.32	37.5 ± 6.5	0.023	23.6 ± 6.1	0.02	11.8 ± 2.5
0.08	0.16	13.6 ± 6.3	0.16	20.7 ± 4.5	0.011	13.8 ± 5.5	-	-
0.04	0.08	11.5 ± 3.2	0.08	16.2 ± 7.5	0.006	11.3 ± 3.1	-	-
0.02	-	-	0.04	12.8 ± 4.6	-	-	-	-
0.01	-	-	0.02	11.0 ± 2.0	-	-	-	-
0.00	0.00	12.3 ± 3.2	0.00	10.9 ± 3.0	0.000	11.9 ± 3.2	0.00	11.9 ± 3.0

Each clotting time value represents the mean ± SD of three independent determinations.

Table 17d -- Comparative anticoagulant effects of thrombin inhibitors in the NRP based Heptest assay.

	D-MePhe-Pro-Arg-H		Ac-(D)Phe-Pro-boroArg-OH		Hirudin		Heparin	
μg/ml	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)	μM	Clotting Time (sec)
10.00	19.43	>300.0	-	-	-	-	-	-
5.00	9.72	215.3 ± 32.0	-	-	0.718	>300.0	0.47	>300.0
2.50	4.86	133.9 ± 44.2	5.03	>300.0	0.359	169.8 ± 8.0	0.23	80.5 ± 55.6
1.25	2.43	83.7 ± 4.5	2.52	290.6 ± 16.3	0.180	76.9 ± 9.9	0.12	45.5 ± 7.1
0.62	1.21	56.6 ± 20.5	1.26	133.4 ± 17.6	0.089	54.1 ± 16.5	0.06	31.3 ± 2.7
0.31	0.60	38.4 ± 9.0	0.63	64.6 ± 2.8	0.045	38.0 ± 14.9	0.03	26.0 ± 1.6
0.16	0.31	29.6 ± 4.9	0.32	43.3 ± 5.9	0.023	30.3 ± 3.8	0.02	22.6 ± 2.5
0.08	0.16	25.5 ± 5.0	0.16	29.0 ± 10.3	0.011	26.7 ± 2.7	-	-
0.04	0.08	21.8 ± 6.2	0.08	22.6 ± 8.0	0.006	21.4 ± 2.2	-	-
0.02	-	-	0.04	18.7 ± 6.6	-	-	-	-
0.01	-	-	0.02	17.6 ± 7.1	-	-	-	-
0.00	0.00	22.0 ± 2.5	0.00	17.9 ± 7.3	0.000	23.0 ± 2.3	0.00	21.3 ± 2.1

Each clotting time value represents the mean ± SD of three independent determinations.

Table 17e -- Comparative anticoagulant effects of thrombin inhibitors after supplementation into normal whole rabbit blood, as assessed by the ACT assay.

	μg/ml	nM	Clotting Time Difference from Baseline (sec)
D-MePhe-Pro-Arg-H	0.2	389	92 ± 28
Ac-(D)Phe-Pro-boroArg-OH	0.1	201	179 ± 58
Hirudin	1.0	144	215 ± 32
Heparin	2.0	187	235 ± 4

Each clotting time value represents the mean ± SD of three independent determinations.

Table 17f -- Comparative anticoagulant effects of thrombin inhibitors after supplementation into normal whole rabbit blood, as assessed by the TEG assay.

	μg/ml	nM	R Value Difference from Baseline (mm)
D-MePhe-Pro-Arg-H	0.2	389	32.0 ± 8.0
Ac-(D)Phe-Pro-boroArg-OH	0.1	201	40.3 ± 23.6
Hirudin	1.0	144	30.0 ± 13.0
Heparin	2.0	187	>200

Each TEG R value represents the mean ± SD of three independent determinations.

Table 19a -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after I.V. injection, 5 min prior to r-TF injection, after 10 and 20 min stasis.

D-Me-Phe-Pro-Arg-H			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.00	3.2±0.2	3.6±0.3
50	0.10	3.0±0.4	3.6±0.3
100	0.19	2.4±0.3	2.8±0.2
250	0.49	0.6±0.4	2.2±0.4
500	0.97	0.8±0.3	2.3±0.4
1000	1.94	0.2±0.2	0.8±0.4

* Each value represents the mean ± SEM of 6 independent determinations.

Table 19b -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after I.V. injection, 5 min prior to r-TF injection, after 10 and 20 min stasis.

Ac-D-Phe-Pro-boroArg-OH			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.00	3.2±0.2	3.6±0.3
5	0.01	3.6±0.3	3.4±0.3
12.5	0.03	2.8±0.2	2.8±0.2
25	0.05	0.6±0.3	1.8±0.4
50	0.10	0.0±0.0	0.2±0.2

* Each value represents the mean ± SEM of 6 independent determinations.

Table 19c -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after I.V. injection, 5 min prior to r-TF injection, after 10 and 20 min stasis.

Hirudin			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.000	3.2±0.2	3.6±0.3
10	0.001	3.0±0.4	3.4±0.3
25	0.004	1.3±0.4	2.7±0.2
50	0.007	1.6±0.3	2.2±0.2
100	0.014	0.6±0.3	1.4±0.3
250	0.036	0.2±0.2	0.0±0.0

* Each value represents the mean ± SEM of 6 independent determinations.

Table 19d -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after I.V. injection, 5 min prior to r-TF injection, after 10 and 20 min stasis.

Heparin			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.000	3.2±0.2	3.6±0.3
25	0.002	4.0±0.0	3.8±0.3
50	0.005	2.5±0.3	2.8±0.3
100	0.009	1.8±0.3	2.0±0.3
250	0.023	0.0±0.0	0.6±0.4

* Each value represents the mean ± SEM of 6 independent determinations.

Table 19e -- Time dependance of antithrombotic effects of thrombin inhibitors in the rabbit model of jugular vein stasis thrombosis, after I.V. injection and 10 minutes stasis.

	Clot Scores After 10 min Stasis*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
5	0.2±0.2	0.0±0.0	0.2±0.2	0.0±0.0
30	0.2±0.2	2.6±0.3	-	1.5±0.4
60	2.0±0.0	3.0±0.3	1.3±0.2	3.8±0.2
90	3.3±0.5	-	3.0±0.4	-

D-MePhe-Pro-Arg-H was injected at 1.94 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 0.10 μmol/kg, hirudin at 0.04 μmol/kg and heparin at 0.02 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

Table 19f -- Time dependance of antithrombotic effects of thrombin inhibitors in the rabbit model of jugular vein stasis thrombosis, after I.V. injection and 20 minutes stasis.

	Clot Score After 20 min Stasis*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
5	0.8±0.4	0.2±0.2	0.0±0.0	0.6±0.4
30	1.0±0.5	2.6±0.3	-	2.2±0.6
60	2.8±0.2	3.3±0.4	1.8±0.3	3.2±0.4
90	3.0±0.4	-	3.2±0.4	-

D-MePhe-Pro-Arg-H was injected at 1.94 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 0.10 μmol/kg, hirudin at 0.04 μmol/kg and heparin at 0.02 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

Table 20a -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after S.C. injection, 45 min prior to r-TF injection and after 10 and 20 min stasis.

D-Me-Phe-Pro-Arg-H			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.00	3.2±0.2	3.6±0.3
100	0.19	3.6±0.3	3.8±0.2
250	0.49	2.8±0.2	3.6±0.3
500	0.97	2.4±0.3	2.8±0.2
1000	1.94	1.2±0.4	2.3±0.5
2500	4.85	0.0±0.0	1.2±0.4

* Each value represents the mean ± SEM of 6 independent determinations.

Table 20b -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after S.C. injection, 45 min prior to r-TF injection and after 10 and 20 min stasis.

Ac-D-Phe-Pro-boroArg-OH			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.00	3.2±0.2	3.6±0.3
25	0.05	3.1±0.1	3.4±0.2
50	0.10	2.4±0.3	3.2±0.4
100	0.20	1.7±0.4	2.5±0.4
250	0.50	0.0±0.0	0.8±0.4

* Each value represents the mean ± SEM of 6 independent determinations.

Table 20c -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after S.C. injection, 45 min prior to r-TF injection and after 10 and 20 min stasis.

Hirudin			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.000	3.2±0.2	3.6±0.3
25	0.004	4.0±0.0	3.8±0.2
50	0.007	2.8±0.2	3.2±0.2
100	0.014	2.5±0.2	2.7±0.2
250	0.036	2.0±0.3	2.3±0.5
500	0.072	1.3±0.5	2.0±0.4
1000	0.144	0.4±0.3	1.0±0.6

* Each value represents the mean ± SEM of 6 independent determinations.

Table 20d -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rabbit model of jugular vein stasis thrombosis, after S.C. injection, 45 min prior to r-TF injection and after 10 and 20 min stasis.

Heparin			
Dose μg/kg	Dose μmol/kg	Clot Score After 10 min Stasis*	Clot Score After 20 min Stasis*
0	0.000	3.2±0.2	3.6±0.3
500	0.046	3.6±0.2	3.8±0.2
1000	0.091	3.3±0.2	3.1±0.2
2750	0.250	1.3±0.5	2.0±0.6
5000	0.455	0.7±0.2	0.8±0.3

* Each value represents the mean ± SEM of 6 independent determinations.

Table 20e -- Time dependance of antithrombotic effects of thrombin inhibitors in the rabbit model of jugular vein stasis thrombosis, after S.C. injection and 10 minutes stasis.

	Clot Scores After 10 min Stasis*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
15	0.0±0.0	0.0±0.0	0.4±0.3	1.2±0.2
45	0.0±0.0	0.3±0.4	0.4±0.3	0.7±0.2
90	0.6±0.4	0.2±0.2	0.7±0.5	0.3±0.2

D-MePhe-Pro-Arg-H was injected at 4.86 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 0.50 μmol/kg, hirudin at 0.14 μmol/kg and heparin at 0.47 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

Table 20f -- Time dependance of antithrombotic effects of thrombin inhibitors in the rabbit model of jugular vein stasis thrombosis, after S.C. injection and 20 minutes stasis.

	Clot Scores After 20 min Stasis*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
15	0.2±0.2	1.4±0.3	0.8±0.4	1.8±0.3
45	1.2±0.4	1.2±0.5	1.0±0.6	0.8±0.3
90	1.7±0.5	1.7±0.6	0.9±0.4	1.2±0.3

D-MePhe-Pro-Arg-H was injected at 4.86 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 0.50 μmol/kg, hirudin at 0.14 μmol/kg and heparin at 0.47 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

Table 21a -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rat model of laser induced thrombosis, after I.V. injection, 5 min prior to initiation of the laser injuries.

D-Me-Phe-Pro-Arg-H		
Dose μg/kg	Dose μmol/kg	# Laser Exposures*
500	0.97	4.3±0.8
1000	1.94	6.3±1.5
1750	3.40	6.0±1.1
2500	4.85	4.5±1.2
0	0.00	2.9±0.1

* Each value represents the mean ± SEM of 6 independent determinations.

Table 21b -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rat model of laser induced thrombosis, after I.V. injection, 5 min prior to initiation of the laser injuries.

Ac-D-Phe-Pro-boroArg-OH		
Dose μg/kg	Dose μmol/kg	# Laser Exposures*
50	0.10	3.5±0.2
75	0.15	5.7±0.6
100	0.20	10.2±0.4
0	0.00	2.9±0.1

* Each value represents the mean ± SEM of 6 independent determinations.

Table 21c -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rat model of laser induced thrombosis, after I.V. injection, 5 min prior to initiation of the laser injuries.

Hirudin		
Dose μg/kg	Dose μmol/kg	# Laser Exposures*
50	0.007	3.8±0.5
100	0.014	5.3±0.5
500	0.072	8.2±0.7
0	0.000	2.9±0.1

* Each value represents the mean ± SEM of 6 independent determinations.

Table 21d -- Comparative dose-dependent antithrombotic effects of thrombin inhibitors as studied in the rat model of laser induced thrombosis, after I.V. injection, 5 min prior to initiation of the laser injuries.

Heparin		
Dose μg/kg	Dose μmol/kg	# Laser Exposures*
500	0.047	4.5±0.5
1000	0.093	6.5±0.5
5000	0.467	8.0±1.0
0	0.000	2.9±0.1

* Each value represents the mean ± SEM of 6 independent determinations.

Table 22a -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after I.V. injection, 5 and 20 min prior to initiation of the bleeding procedure.

D-Me-Phe-Pro-Arg-H			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 5 min*	RBC (10^9/L) After 20 min*
0	0.00	0.100±0.075	0.100±0.075
500	0.97	0.155±0.055	0.165±0.110
1000	1.94	0.990±0.525	0.445±0.300
2500	4.85	4.445±0.730	1.715±0.475

* Each value represents the mean ± SEM of 6 independent determinations.

Table 22b -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after I.V. injection, 5 and 20 min prior to initiation of the bleeding procedure.

Ac-D-Phe-Pro-boroArg-OH			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 5 min*	RBC (10^9/L) After 20 min*
0	0.00	0.100±0.075	0.100±0.075
50	0.10	0.215±0.075	0.065±0.035
125	0.25	2.24±1.065	0.515±0.220
250	0.50	1.520±0.54	1.395±0.465
500	1.00	3.245±1.080	11.790±7.295

* Each value represents the mean ± SEM of 6 independent determinations.

Table 22c -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after I.V. injection, 5 and 20 min prior to initiation of the bleeding procedure.

Hirudin			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 5 min*	RBC (10^9/L) After 20 min*
0	0.000	0.100±0.075	0.100±0.075
500	0.046	0.470±0.245	0.100±0.060
1000	0.144	3.880±1.075	0.140±0.050
2500	0.359	4.910±1.485	3.025±1.015

* Each value represents the mean ± SEM of 6 independent determinations.

Table 22d -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after I.V. injection, 5 and 20 min prior to initiation of the bleeding procedure.

Heparin			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 5 min*	RBC(10^9/L) After 20 min*
0	0.000	0.100±0.075	0.100±0.075
500	0.046	0.975±0.370	0.420±0.275
1000	0.091	0.870±0.530	0.465±0.250
2500	0.227	0.745±0.270	0.920±0.480
5000	0.455	1.115±0.630	0.370±0.130

* Each value represents the mean ± SEM of 6 independent determinations.

Table 22e -- Time dependance of hemorrhagic effects of thrombin inhibitors in the rabbit ear bleeding model, after I.V. injection.

	RBC (10^9/L)*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
5	8.655±2.865	9.830±5.830	4.910±2.130	1.115±0.630
20	1.865±0.425	4.680±2.380	3.025±1.590	0.370±0.130
30	1.090±0.580	6.650±4.455	0.590±0.275	1.835±0.540
45	0.210±0.080	1.245±0.425	0.130±0.035	1.180±0.415
60	0.135±0.025	0.440±0.265	-	0.140±0.050
90	0.150±0.155	0.065±0.045	-	0.165±0.090

D-MePhe-Pro-Arg-H was injected at 4.86 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 0.50 μmol/kg, hirudin at 0.36 μmol/kg and heparin at 0.47 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

Table 23a -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after S.C. injection, 45 and 60 min prior to initiation of the bleeding procedure.

D-Me-Phe-Pro-Arg-H			
Dose μg/ml	Dose μmol/kg	RBC (10^9/L) After 45 min*	RBC (10^9/L) After 60 min*
0	0.00	0.100±0.075	0.100±0.075
1000	1.94	0.125±0.080	0.080±0.055
2500	4.85	0.855±0.280	0.225±0.105
5000	9.71	0.680±0.190	0.375±0.280

* Each value represents the mean ± SEM of 6 independent determinations.

Table 23b -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after S.C. injection, 45 and 60 min prior to initiation of the bleeding procedure.

Ac-D-Phe-Pro-boroArg-OH			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 45 min*	RBC (10^9/L) After 60 min*
0	0.00	0.100±0.075	0.100±0.075
100	0.20	0.235±0.075	0.030±0.020
250	0.50	0.730±0.265	0.465±0.180
500	1.00	1.405±0.945	1.150±0.934
1000	2.01	7.190±3.151	2.645±0.655

* Each value represents the mean ± SEM of 6 independent determinations.

Table 23c -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after S.C. injection, 45 and 60 min prior to initiation of the bleeding procedure.

Hirudin			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 45 min*	RBC (10^9/L) After 60 min*
0	0.000	0.100±0.075	0.100±0.075
500	0.046	0.135±0.070	0.040±0.035
1000	0.144	0.315±0.285	0.215±0.170
2500	0.359	0.225±0.140	0.140±0.075
5000	0.718	1.865±0.715	0.350±0.140

* Each value represents the mean ± SEM of 6 independent determinations.

Table 23d -- Comparative dose-dependent hemorrhagic effects of thrombin inhibitors as studied in the rabbit ear bleeding model, after S.C. injection, 45 and 60 min prior to initiation of the bleeding procedure.

Heparin			
Dose μg/kg	Dose μmol/kg	RBC (10^9/L) After 45 min*	RBC (10^9/L) After 60 min*
0	0.000	0.100±0.075	0.100±0.075
5000	0.455	0.095±0.060	0.100±0.055
10,000	0.909	0.395±0.215	0.160±0.065

* Each value represents the mean ± SEM of 6 independent determinations.

Table 23e -- Time dependance of hemorrhagic effects of thrombin inhibitors in the rabbit ear bleeding model after S.C. injection.

	RBC (10^9/L)*			
Time (min)	D-Me-Phe-Pro-Arg-H	Ac-D-Phe-Pro-boroArg-OH	Hirudin	Heparin
15	1.445±0.620	2.445±0.700	0.160±0.150	0.100
30	0.520±0.325	1.245±0.529	0.020±0.010	0.150
45	0.680±0.190	7.190±3.151	1.865±0.715	0.395±0.215
60	0.375±0.280	2.646±0.732	0.350±0.140	0.160±0.065
90	0.140±0.110	1.438±0.412	0.045±0.025	0.035±0.025
120	0.755±0.290	1.663±0.443	0.220±0.125	0.055±0.020

D-MePhe-Pro-Arg-H was injected at 9.72 μmol/kg, Ac-(D)Phe-Pro-Arg-OH at 2.01 μmol/kg, hirudin at 0.72 μmol/kg and heparin at 0.93 μmol/kg. * Each value represents the mean ± SEM of 6 independent determinations.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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