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Pharmacologic Studies on Synthetic Analogues of Heparin with Selective Affinity to Endogenous Serine Protease Inhibitors

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PHARMACOLOGIC STUDIES ON SYNTHETIC ANALOGUES OF HEPARIN WITH SELECTIVE AFFINITY TO ENDOGENOUS SERINE PROTEASE INHIBITORS

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ABSTRACT

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Heparin is a mixture of glycosaminoglycan chains which is used to prevent thrombosis in a number of clinical indications. Heparin is known to promote the inhibition of blood coagulation via the plasmatic cofactors antithrombin III (ATIII) and heparin cofactor II (HCII).

The objective of this dissertation was to examine the importance of heparin cofactors in the mediation of the anticoagulant and antithrombotic effects of heparin. To achieve this goal, synthetic heparin analogues exhibiting a more selective interaction with heparin cofactors were studied using a number of *in vitro* systems and *in vivo* models.

Three analogues were selected for this study. The pentasaccharide representing the minimal ATIII binding sequence of heparin was observed to inhibit factor Xa but not thrombin via activation of ATIII. Aprosulate inhibited thrombin via HCII but did not exhibit activate ATIII. GL-522-Y-1 also promoted the inhibition of thrombin via HCII but did not interact with ATIII.

The *in vitro* actions of each agent were investigated in plasma based clotting assays, Plasmatic and non-plasmatic amidolytic protease generation assays and in endothelial cell cultures. The *in vivo* pharmacologic actions were studied in terms of

antithrombotic and hemorrhagic activities in a rabbit stasis thrombosis model, a rat jugular vein clamping model of thrombosis and a rabbit ear bleeding model. The impact of the administration of synthetic heparin analogues on plasma TFPI levels was assessed in several species. TFPI antigen levels were also measured in primates and as part of two phase I human trials with aprosulate.

From these studies, it was observed that ATIII plays a primary role in mediating heparin's anticoagulant and antithrombotic actions. In both models of thrombosis, agents capable of interacting with ATIII proved more potent than those which solely inhibited thrombin via HCII. Interaction with plasma based SERPINs was not predictive of hemorrhagic potential as both pentasaccharide and aprosulate failed to produce hemorrhage. These results also indicate a relative unimportance of HCII mediated thrombin inhibition in the mediation of anticoagulant and antithrombotic activities. While aprosulate and GL-522-Y-1 were observed to inhibit thrombin via HCII with similar potencies the *in vitro* and *in vivo* activities of these agents were widely divergent suggesting the importance of mechanisms other than protease inhibition by ATIII and HCII for the mediation of the biologic effects of these agents.

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LIST OF STANDARD ABBREVIATIONS

ADP	Adenosine diphosphate
ALA	Alanine
ANOVA	Analysis of variance
ARG	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
C	Carbon
^{13}C	Carbon 13
$CaCL_2$	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic Guanosine monophosphate
cm	Centimeter
$-COO^-$	Carboxylate
CPM	Counts per minute
Da	Dalton
dL	Deciliter
DNA	Deoxyribonucleic acid
E. coli	*Escherechia coli*

^{19}F	Fluorine 19
g	Gram
GLY	Glycine
H	Hydrogen
^{1}H	Proton
HCl	Hydrochloride
HIV	Human Immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IgG	IgG antibody
IL-1	Interleukin I
IL-6	Interleukin 6
i.m.	Intramuscularly
i.p.	Intraperitoneally
IV	Intravenous
K	Degree Kelvin
k_d	Dissociation rate constant
kDa	Kilodalton
kg	kilogram
K_m	Michaelis constant
LDL	Low density lipoprotein
LEU	Leucine
M	Molar

mg	Milligram
MHz	Megahertz
mL	Milliliter
mM	Millimolar
mOsmol	Milliosmole
N	Nitrogen
NaCl	Sodium chloride
Na_2SO_4	Sodium Sulfate
nm	Nanometer
nM	Nanomolar
NO	Nitric Oxide
$-NHSO_3^-$	Amino sulfate
-OH	Hydroxyl
$-OSO_3^-$	Sulfate
nMol	nanomole
^{31}P	Phosphorus 31
pM	Picomolar
ppm	part per million
RNA	Ribonucleic acid
rpm	Revolution per minute
^{35}S	Sulfur 35
SC	Subcutaneous

SEM	Standard Error of the mean
SER	Serine
TNF	Tumor necrosis factor
U	Unit
UDP	Uridine diphosphate
USP	United States Pharmacopeia
UV	Ultraviolet
vs.	Versus
5-HT	5-hydroxy tryptamine; serotonin
μg	Microgram
μM	Micromolar
μmol	Micromole
@	at
°C	Degree Celsius
"	minutes
®	Registered trademark

LIST OF NON-STANDARD ABBREVIATIONS

ACT	Activated clotting time
Anti-IIa	Antithrombin
Anti-Xa	Anti-factor Xa
APTT	Activated partial thromboplastin time
ATIII / AT-III	Antithrombin III
b.i.d.	Twice daily
$Conc_{100s}$	Concentration which elevates clotting time to 100 seconds
C-terminal (terminus)	Carboxy terminal (terminus)
DDAVP	Desamino-D-arginine vasopressin
DIC	Disseminated intravascular coagulation
EDRF	Endothelium derived relaxation factor
EDTA	Ethylenediaminetetraacetic acid
ED_{50}	Dose which produces 50 % maximal activity
ELISA	Enzyme linked immunosorbant assay
EPI	Extrinsic pathway inhibitor
FEIBA	Factor eight inhibitor bypass activator
FIX	Coagulation factor IX
FVIII	Coagulation factor VIII

FVIII:C	Factor VIII coagulant activity
FX	Coagulation factor X
FXa	Activated coagulation factor X
GP Ia/IIa	Glycoprotein Ia/IIa
GP Ib	Glycoprotein Ib
GP IIb/IIIa	Glycoprotein IIb/IIIa
HCII	Heparin cofactor II
HIT	Heparin induced thrombocytopenia
HMC	Heparin Mass Calibrator
IC_{25}	Concentration producing 25 % maximal inhibition
IC_{50}	Concentration producing 50 % maximal inhibition
II	Prothrombin
IIa	Thrombin
ISI	International sensitivity index
LALLS	Low angle laser light scatter
LACI	Lipoprotein associated coagulation inhibitor
LMWH	Low molecular weight heparin
mau	Milliabsorbance unit
MPS	Mucopolysaccharide
M.W.	Molecular weight
nkat	Nanokatal

NMR	Nuclear magnetic resonance spectroscopy
NRP	Normal Rabbit Plasma
N-terminus (terminal)	Amino-terminus (terminal)
O.D.	Optical density
PDA	Diaminopropane
PDGF	Platelet Derived Growth Factor
PGI_2	Prostaglandin I_2; prostacyclin
PF4	Platelet factor 4
pNA	Para-nitroanaline
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
RI	Refractive index
RT	Retention time
TEG	Thrombelastograph
TFPI	Tissue factor pathway inhibitor
TFPI:Ag	Tissue factor pathway inhibitor antigen
tPA	Tissue plasminogen activator
Tris	Tris(hydroxymethyl)aminomethane
TXa_2	Thromboxane A_2
VLA	Very late antigen
Xa	Coagulation factor Xa

5U TT	5 unit thrombin time

CHAPTER I

LITERATURE REVIEW

A.Overview of Blood Coagulation

1. Introduction

Hemostasis as defined by Virchow in the last century is a fine balance between blood flow, humoral factors, and cellular elements of the vascular system. Today, biotechnology has advanced our understanding of the thrombotic process and its regulation. Whereas in the past, heparin and warfarin have been the sole antithrombotic agents available, specific sites in the thrombotic network can now be targeted. Antibodies against specific platelet receptors as well as specific antithrombin and anti-Xa agents are being developed. Mutations of endogenous inhibitors have been identified as causes of congenital thrombophilias. The use of heparin has also advanced. Heparin is no longer solely a surgical anticoagulant, but is used to treat a variety of conditions including venous thrombosis, unstable angina and myocardial infarction and is used in procedures such as angioplasty and stent implantation. The mechanism of heparin's action has become more complex with the discovery of tissue factor pathway inhibitor.

Blood normally is maintained in the fluid state so that nutrients can be delivered to the various tissues of the body. When the integrity of the vascular system has been compromised, it becomes necessary for the blood to clot. The initial response to a break in the continuity of the vasculature is the formation of the platelet plug. Platelets in the

flowing blood rapidly adhere to the exposed subendothelial vessel wall matrix and become activated. During this activation process, components of the platelet α and ß granules (ATP, ADP, factor V, 5-HT) are released causing further platelet aggregation. Also during these morphologic changes, activated platelets express protein and cell receptors and procoagulant phospholipids are expressed upon their surface.

The negatively charged phospholipid phosphatidylserine is asymmetrically distributed in mammalian cell membranes, primarily on the inner leaflet. Upon exposure to collagen or thrombin, the distribution of phospholipids changes with increasing phosphatidylserine in the external membrane leaf (Bevers et al., 1985). The increased expression of phosphatidylserine on the outer leaflet of the membrane creates a procoagulant surface on which several steps of the coagulation cascade take place.

The platelet plug initially arrests the loss of blood. This, however, is not a permanent blockade. The formation of a fibrin based clot acts to stabilize the initial platelet plug. The coagulation system is a complex network of zymogens which must be activated to ultimately form the fibrin strands of the blood clot. Upon activation, most of these coagulation proteins are converted into active serine proteases which are similar to trypsin and chymotrypsin. Traditionally, coagulation has been viewed as having two distinct branches (Davie et al., 1964; MacFarlane et al., 1964), the intrinsic and the extrinsic pathways. Today it has been established that the two pathways are linked prior to the generation of factor Xa (Osterud et al., 1977). A schematic of the coagulation cascade is depicted in Figure 1.

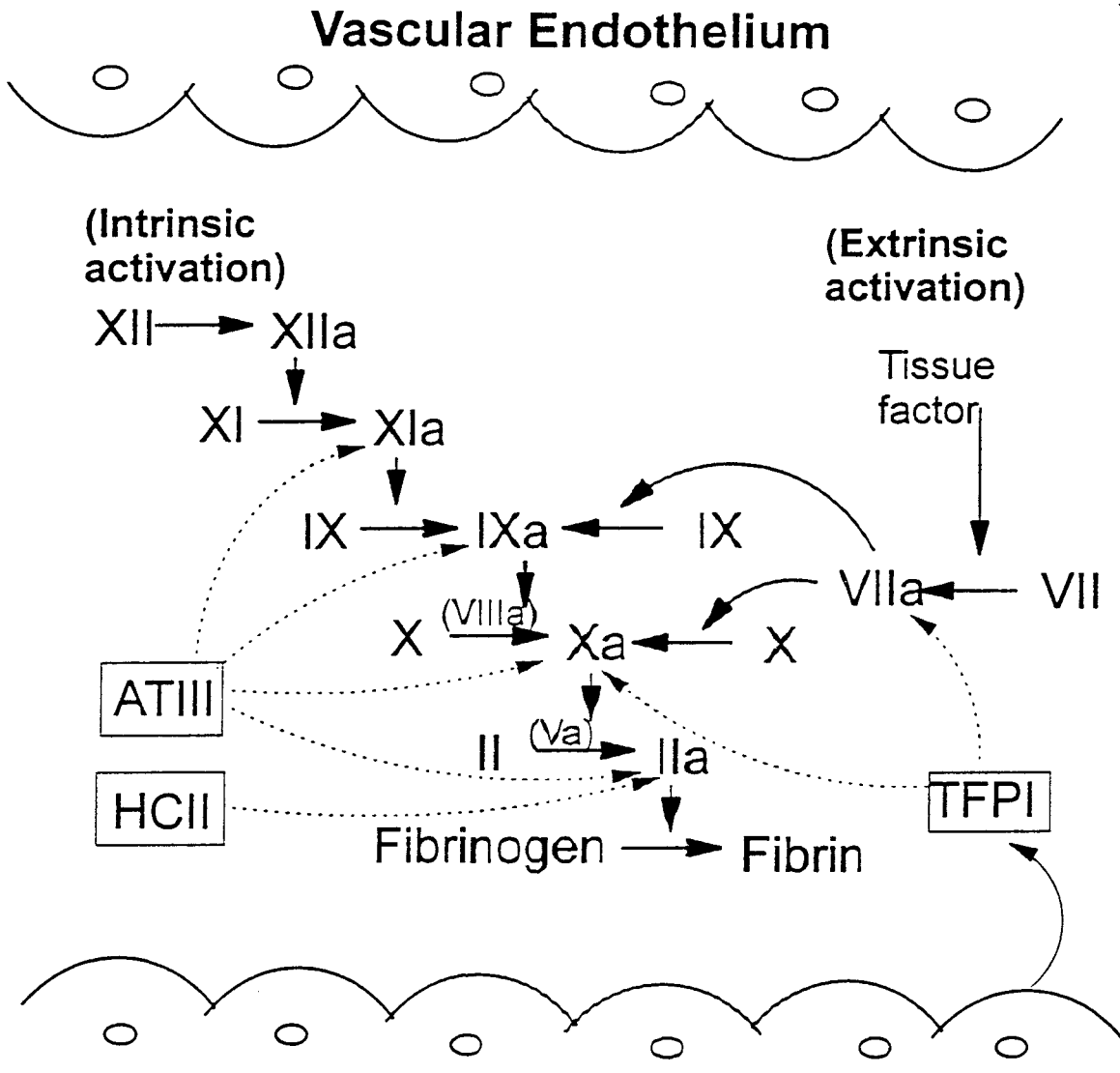


Figure 1. Schematic of the coagulation cascade. The formation of the fibrin clot can be initiated in one of two ways. Activation of the contact system results in the sequential activation of factors XII, XI, IX, X and II. Exposure of tissue factor leads to activation of factors IX and X via a complex with factor VIIa. Solid arrows indicate enzymatic conversion. Broken arrows indicate inhibition. Factors in parenthesis are act as cofactors. ATIII primarily inhibits in the intrinsic pathway. TFPI regulates the extrinsic pathway. HCII is limited to inhibiting thrombin.

2.Intrinsic Pathway of Coagulation

In the intrinsic pathway, factor XII becomes activated in the contact phase of coagulation. This occurs when factor XII, factor XI, prekallikrein, and high molecular weight kininogen come together on a negatively charged surface. While this reaction can take place in the laboratory on a negatively charged surface such as glass or kaolin, the physiologic surface is unknown. It has been proposed that this could be a tissue rich in collagen or sulphatides (Scully et al., 1992). By binding to the negatively charged surface, factor XII is converted to its active form through an unknown mechanism. The formation of factor XIIa is amplified by a positive feedback loop. Factor XIIa is capable of converting prekallikrein to kallikrein. Likewise, kallikrein converts factor XII to its active form. Factor XIIa also converts factor XI to factor XIa which in turn activates factor IX. Factor IX along with its cofactor factor VIII, calcium ions, and phospholipid membranes form the "tenase" complex which converts factor X to factor Xa thereby initiating the common pathway of coagulation. The phospholipid membrane in these complexes serves to lower the Km of the reaction. The phospholipid allows the enzyme to become saturated more easily and serves to localize the coagulation response to where it is most needed. The cofactor, factor V, increases the catalytic efficiency of the enzyme (Hemker et al., 1991). Factor Xa joins with its cofactor factor V, calcium ions and phospholipid membranes to form the prothrombinase complex. The prothrombinase complex then acts to convert prothrombin into the active enzyme thrombin. Factors V and VIII are activated through proteolytic cleavage by factor Xa or thrombin. They are not, however, active proteases. Factor V is believed to have two rate enhancing effects

on the prothrombinase complex. In the prothrombinase complex, factor Xa and factor V are present in stoichiometric amounts resulting in an unknown alteration in the active site of factor Xa which increases its catalytic efficiency (Mann et al., 1988). Factor V also binds to prothrombin thus sequestering it at the site of assembly of the prothrombinase complex. Overall, these two actions of factor V result in a 300,000-fold increase in the rate of prothrombin conversion.

Thrombin serves many functions in coagulation. First, thrombin cleaves the soluble protein fibrinogen to generate the insoluble fibrin monomer. Fibrinogen circulates as a disulfide-linked dimer containing two A-α chains, two B-ß chains and two gamma chains. Cleavage of fibrinogen by thrombin results in the release of fibrinopeptides A and B and the exposure of charged domains at opposite ends of the molecule (Roberts et al., 1992). Exposure of these charged domains leads to polymerization of the monomers. The release of fibrinopeptides A and B occur at different rates with fibrinopeptide A preferentially removed in mammalian systems (Blomback et al., 1958; Shainoff et al., 1960). Removal of fibrinopeptide A leads to end-to-end fibrin polymerization whereas loss of fibrinopeptide B allows side-to-side polymerization of the end-to-end linked monomers (Laurent et al., 1958). It is these monomers which are cross-linked by the transaminase factor XIIIa to form the meshwork of the thrombus. Thrombin also acts to augment its own generation by being a part of several positive feedback loops in the coagulation cascade. In these loops, thrombin activates factors XII, XI, VIII, and V. By activating the precursors to its own generation, thrombin greatly amplifies its own generation. Thrombin also activates platelets (Coughlin et al., 1992), activates the

inhibitor Protein C through binding with thrombomodulin (Esmon et al., 1989), and stimulates activated endothelial cells to release tissue plasminogen activator (Olsen et al., 1992).

3. Extrinsic Pathway of Coagulation

The extrinsic pathway of coagulation is activated when circulating factor VII encounters tissue factor. Tissue factor is a transmembrane glycoprotein which is normally expressed by subendothelial fibroblast-like cells which surround the blood vessel. An intact endothelium normally shields the circulating blood from exposure to tissue factor. The tissue factor molecule consists of a 219 amino acid hydrophilic extracellular domain, a 23 amino acid hydrophobic region which spans the membrane, and a 21 amino acid cytoplasmic tail which anchors the molecule to the cell membrane (Bach et al., 1988; McVey et al., 1994). Other sites of tissue factor expression include activated monocytes, activated endothelial cells, and atherosclerotic plaques.

Factor VII exhibits a weak procoagulant activity on its own, typically accounting for about 1-2% of the total factor VII/VIIa activity (Morrisey et al., 1993). Upon binding to tissue factor, a 10,000,000 fold increase in factor VIIa enzymatic activity is observed (Edgington et al., 1991). Both factor VII and factor VIIa bind to tissue factor with equal affinity (Nemerson et al., 1988). How factor VII is initially activated is not known, though it is hypothesized that factor Xa can activate factor VII in a back activation reaction. The factor VIIa - tissue factor complex can then activate factor X leading to the generation of thrombin and ultimately to the formation of fibrin strands.

It has been shown in 1977 and more recently appreciated that the tissue factor - factor VIIa complex also activates factor IX to factor IXa, thus interacting with "intrinsic" pathway enzymes (Osterud et al., 1977). This is believed to be important for maintaining the clotting process. Direct activation of factor X by factor VIIa - tissue factor can rapidly initiate coagulation, but both of these enzymes are quickly inhibited by the endogenous inhibitor tissue factor pathway inhibitor. By activating factor IX, the tissue factor - VIIa complex initiates two pathways for thrombin generation. The small amounts of factor Xa generated prior to TFPI inhibition are sufficient to cleave prothrombin and generate a small amount of thrombin. This thrombin is then capable of back-activating factors V, VIII and possibly XI, thereby sustaining clot formation through generation of thrombin via the intrinsic pathway. It has been observed that the activation of factor X by the factor IXa-VIII complex in the presence of calcium and phospholipids is 50 times greater than by the tissue factor-VIIa complex (Mann et al., 1990). Factor XI activation has been shown to occur in the presence of thrombin and a polyanion cofactor (Naito et al., 1991; Gailani et al., 1991). Activation with the cofactor has been observed to be poor. A physiologic cofactor has not been elucidated. It has been reasoned that if the direct activation of factor X by VIIa - tissue factor is the sole source of thrombin generation, there would be no manifestation of hemophilia, a genetic deficiency of either factor IX or factor VIII.

4. Role of Platelets

Platelets are disc-shaped, anuclear cells which circulate in a non-adhesive state

in the undamaged circulation (Packham, 1994). These cells contain a contractile system and a number of storage granules. The α storage granules contain platelet factor 4 (PF4), ß-thromboglobulin, platelet derived growth factor (PDGF), fibrinogen, factor V, and von Willebrand factor (Kaplan et al., 1981). The dense or ß-granules contain ATP, ADP, and serotonin (Holmsen et al., 1987; Niewiarowski et al., 1987).

The first step toward platelet aggregation is platelet adhesion. Normally, platelets do not adhere to the vessel walls due to the non-thrombogenic properties of the endothelium. Endothelial cells produce heparan sulfate (to activate antithrombin III), thrombomodulin (for activation of protein C), plasminogen activators (to induce fibrin degradation) and TFPI (to inhibit tissue factor activity). In addition, these cells also produce prostacyclin (PGI_2) which inhibits platelet activation by raising platelet cAMP levels and endothelial derived relaxing factor (EDRF; NO) which inhibits platelet activation through a cGMP dependent mechanism. When this antithrombotic continuum of cells is interrupted by vascular injury, platelets adhere to the exposed subendothelial tissues.

Following adhesion, platelets become activated. In this activation process, there is a morphologic shape change in the platelet, with pseudopod formation observed. This brings about a change in the conformation of the glycoprotein IIb/IIIa receptor on the platelet surface which allows for fibrinogen binding (Packham, 1994). Fibrinogen binding serves as a bridge which links individual platelets into larger aggregates. An increase in cytosolic calcium levels leads to activation of internal platelet enzymes with the subsequent release of platelet granule contents. The formation of these platelet aggregates

is the process of primary hemostasis, the first step to arrest blood loss.

The release of platelet granule contents leads to further platelet activation and aggregation and an activation of coagulation. Most of the known aggregating agents cause release of the platelet storage granule contents. These agonists include thrombin, ADP, collagen, TXA_2, platelet activating factor, serotonin, epinephrine, immune complexes, and fibrinogen (Packham, 1994). Thrombin is the most potent aggregating agent, capable of causing platelet aggregation without any contribution from thromboxane A_2 or ADP (Packham, 1994). Serotonin and epinephrine do not induce aggregation on their own, but synergistically promote aggregation induced by other agents (Coller et al., 1992; Hourani et al., 1991; Siess et al., 1989).

Platelet membranes contain a variety or receptors for the various agonists including the thrombin receptor, the TXA_2 receptor, 5-HT_2 receptors, and α_2-adrenergic receptors. In addition, a number of glycoproteins present on the membrane serve as receptors for collagen (GP Ia/IIa), fibrinogen (GP IIb/IIIa), von Willebrand factor (GP Ib) and fibronectin (GP IIb/IIIa) (Coller et al., 1992; Hourani et al., 1991; Siess et al., 1989). A high molecular weight chondroitin sulfate proteoglycan has been shown to be released from the surface of the platelet during the aggregation process (Nader, 1991). This proteoglycan contains homopolymers of 4-O chondroitin sulfate which inhibit ADP induced aggregation of platelets.

Activated platelets also provide a procoagulant surface on which several reactions of the coagulation cascade take place. Unstimulated platelets provide only a minimally effective surface on which the "tenase" and prothrombinase complexes can

assemble (Zwaal et al., 1989; Wiedman et al., 1986; Esmon, 1993). This is due to the bilayer partitioning of various phospholipids. In unstimulated platelets, the outer leaflet of the membrane consists of mostly phosphatidylcholine while the inner leaf contains most of the phosphatidylserine. Two mechanisms have been proposed for maintaining this distribution (Sandberg, et al., 1985; Tilly et al., 1990). When platelets are stimulated to release their granular contents, the procoagulant phospholipids are brought to the surface as the granules fuse to the membranes (Zwaal et al., 1989). This expression of phosphatidylserine on the outer leaflet along with factor V release from the α-granule greatly accelerates the formation of thrombin (Tracy et al., 1992; Miletich et al., 1977; Ittyerah et al., 1981).

Platelet activation leads to the formation of platelet derived microparticles derived from the platelet surface. These microvesicles typically account for 25 to 30 % of platelet procoagulant activity and factor V binding sites (Sandberg et al., 1985; Sims et al., 1989).

5. Role of Platelet Integrins

A number of the glycoproteins on the surface of the platelet belong to the superfamily of adhesive protein receptors known as integrins. Integrins are α/ß heterodimer protein complexes which are present on the surface of adherent cells of most species (Bogaert et al., 1987; DeSimone et al., 1988; Marcantonio et al., 1988). These integrins mediate cell-cell and cell-matrix interactions involved in a diverse number of biologic functions (Hynes et al., 1987; Takada et al., 1987). Integrins are divided into

subfamilies based on the identity of the ß-subunit. The first two subfamilies of integrins, the VLA complexes and the Leu-Cams, are found on white cells and mediate various leukocyte aggregation responses (Hemler et al., 1983; Anderson et al., 1987). Platelets contain two members of the third subfamily of integrins, glycoprotein IIb/IIIa or P-selectin and the vitronectin receptor (Cheresh et al., 1987; Lam et al., 1989; Bray et al., 1987; Zimrin et al., 1988).

Integrins function by interacting with a number of extracellular glycoprotein ligands such as fibronectin, laminin, collagen, vitronectin, fibrinogen, and von Willebrand's factor (Bennett et al., 1991). Integrins are capable of binding several ligands and the nature of the ligand specificity is not known.

Platelet membranes contain five integrin-like receptors which are involved in the formation of the primary hemostatic plug. These include VLA-2, VLA-5, VLA-6, glycoprotein IIb/IIIa and the vitronectin receptor. Of these, GP IIb/IIIa is the most abundant (Phillips et al., 1991). VLA-2 (GPIa/IIa) is the binding site for collagen on the platelet surface (Staatz et al., 1989). VLA-5 and VLA-6 are responsible for the binding to vitronectin and laminin, respectively (Hemler et al., 1988; Pischel et al., 1988). The extent to which these receptors contribute to platelet adhesion *in vivo* is not known. The physiologic function of the vitronectin receptor is not known.

Platelet aggregation requires that platelets become activated by at least one platelet agonist, the presence of functional GPIIb/IIIa molecules and the presence of at least one GPIIb/IIIa ligand (Shattil et al., 1981). Lack of GPIIb/IIIa complexes leads to the congenital bleeding disorder known as Glanzmann's thrombasthenia (Bennett et al.,

1979). In nonactivated platelets, GPIIb/IIIa is capable of binding only immobilized fibrinogen. Platelet activation allows plasma-borne adhesive proteins to bind to GPIIb/IIIa complexes (Jackson et al., 1993). The activation of the IIb/IIIa complex occurs by an unknown mechanism though the number of receptors on the membrane is not altered by activation (Phillips et al., 1991). Fibrin polymers bind to the activated GPIIb/IIIa complexes and anchor the platelet plug in place.

Recent studies have shown that the binding of ligands to GPIIb/IIIa also activates a number of cellular processes important for platelet stimulation (Phillips et al., 1991) including the synthesis of 3-phosphorylated phosphatidylinositols, the release of arachidonic acid, and the increase in plasma calcium levels. Stimulation of these processes allows for bidirectional signalling between the intracellular and extracellular compartments.

6. Role of Leukocytes

Leukocytes typically express minimal amounts of procoagulant activity in the unstimulated state (Drake et al., 1989). Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) can elicit the expression of tissue factor on endothelial and mononuclear cells (Carlsen et al., 1988). Monocyte procoagulant activity is also induced by endotoxin, the complement system, phorbol esters, prostaglandins, and a number of other agonists (Edwards et al., 1992). Procoagulant activity associated with leukocytes is not limited to the expression of tissue factor. Several monocyte/macrophage derived procoagulant activities have been characterized. These include tissue factor (Gregory et

al., 1989; McGee et al., 1990, Schwartz et al., 1981), factor VII (McGee et al., 1989; Chapman et al., 1985), and factor XIII (Weisberg et al., 1987). In addition, some monocytes and macrophages have been shown to express functional factor V/Va (Rothberger et al., 1984) and to possess binding sites for factor X (Altieri et al., 1988). The factor Xa binding site on leukocytes has been shown to be the integrin CD11b/CD18 (Altieri et al., 1988). Not only does this integrin bind factor X, but it also proteolytically activates factor X to Xa, allowing for initiation of coagulation on the surface of the monocytes and neutrophils (Altieri et al., 1988b). Monocytes have also been shown to contain a receptor for the factor IXa/VIII complex which allows the reactions of the intrinsic pathway of coagulation to take place on the surface of the monocyte (McGee et al., 1991).

Prothrombin has been shown to be efficiently activated on the cell surface of monocytes and lymphocytes (Tracy et al., 1983; Tracy et al., 1985). As with platelets, the prothrombinase activity on monocytes is increased with activated monocytes as compared to the non-activated cells (Robinson et al., 1992).

It has been stated that when coagulation takes place on the surface of leukocytes, it "... assumes the aspects of a broad inflammatory mechanism, directly influencing cellular motility and adhesion, phagocytosis, cell-cell communication, and normal or deregulated cellular growth" (Altieri et al., 1993). Fibrin formation not only forms the basis for a blood clot, but can also serve to limit the inflammatory response. In addition, products of the coagulation process such as thrombin, fibrinopeptides, and fibrin degradation products have chemotactic and mitogenic properties (Perdue et al., 1981;

Senior et al., 1986; Shavit et al., 1983).

Studies have indicated that leukocytes play a critical role in the activation of coagulation in patients with septicemia and in animal models of acute lung injury (Okajima et al., 1991; Car et al., 1991). One study has presented direct evidence indicating the role of tissue factor expression on activated endothelial cells on *in vivo* thrombogenesis (Nawroth et al., 1986).

7. Role of Cytokines

Cytokines produced during inflammatory responses as in sepsis lead to several alterations of the hemostatic system. Primarily affected are the endothelium and leukocytes. Following an injection of endotoxin, it has been shown that there is an increase in tissue factor expression on monocytes leading to the formation of the VIIa-tissue factor complex. (Edgington et al., 1992; ten Cate et al., 1994). Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) are known to alter the antithrombotic properties of the endothelium. Among these changes is an increased expression of adhesion molecules for neutrophils and lymphocytes on the endothelium (Savage et al., 1993; Redl et al., 1994; Waage et al., 1993). Both cell types are capable of directly damaging the endothelium (Savage et al., 1993). Damage to the endothelium leads to both an activation of coagulation (Levi et al., 1993; Maruyama, 1994) and to delayed alterations in the balance of fibrinolytic activators and inhibitors (Gertler et al., 1992), both of which can promote thrombus formation.

B. Endogenous Inhibitors of Coagulation

1. Antithrombin III

Antithrombin III is a single chain glycoprotein with a molecular weight of approximately 58,000 Da (Mourey et al., 1990). The primary structure of this serine protease inhibitor (SERPIN) has been determined by protein and cDNA sequencing of clones from several species (Beck et al., 1982; Chandra et al., 1983; Petersen et al., 1979; Prowchownil et al., 1983; Sheffield et al., 1992; Stackhouse et al., 1983). Normal plasma levels of antithrombin III are approximately 2 to 3 μM (Conrad et al., 1983).

In the beginning of the century, it was suspected that a natural inhibitor of thrombin was present in the plasma (Howell, 1918). The first hints of antithrombin III's existence were detected shortly after the discovery of heparin when it was discovered that heparin required a cofactor to exhibit its anticoagulant activity (Howell, 1925; Brinkhous et al., 1939). At this point, the molecule was termed heparin cofactor (Brinkhous et al., 1939). It was not until the late 1960's that Abildgaard demonstrated that the proteins antithrombin and heparin cofactor were one in the same (Abildgaard et al., 1968).

Antithrombin III is a member of the SERPIN superfamily of proteins which includes the inhibitors α_2-antiplasmin, α_1-antichymotrypsin, and α_1-proteinase inhibitor (Pizzo et al., 1994). Antithrombin III is considered to be the primary inhibitor of coagulation (Pratt et al., 1991) and targets most coagulation proteases as well as the enzymes trypsin, plasmin, and kallikrein (Bjork et al., 1986; Travis et al., 1983). Inhibition takes place when a stoichiometric complex between the active site serine of the protease and the ARG393-SER294 bond of antithrombin III forms (Rosenberg et al.,

1973; Jornvall et al., 1979; Damus et al., 1973; Owen et al., 1975).

The tertiary structure of antithrombin III resembles α_1-antitrypsin in that it is folded into N-terminal domain helices and ß-sheets. This tertiary structure is maintained by the formation of three disulfide bonds (Mourey et al., 1990). Four glycosylation sites exist on human antithrombin III, two of which are suspected to actually contain carbohydrate chains. The glycosylation of these sites appears to effect heparin binding to the inhibitor (Brennan et al., 1987; Brennan et al., 1988).

The efficient inhibition of proteases by antithrombin III requires heparin as a cofactor. Without heparin, the inhibition rate constants for thrombin and factor Xa have been estimated to be 1×10^3 and 3×10^3 L/mol sec^{-1}, respectively. In the presence of heparin, these rates of inhibition are accelerated to 3×10^7 and 4×10^6 L/mol sec^{-1}, respectively, for thrombin and factor Xa (Jordan et al., 1980). The binding site for heparin is located on the N-terminal domain of the molecule.

Two mechanisms have been proposed to account for heparin's ability to catalyze the antiprotease actions of antithrombin III. The first suggests that heparin binds to antithrombin III and causes a conformational change at the active site (Rosenberg et al., 1973). The second model, the ternary complex or template model, proposes that heparin acts catalytically by binding both antithrombin III and the serine protease, thereby bringing them in close proximity (Bjork et al., 1986). Both models may be operative depending upon the serine protease being inhibited. Conformational changes of ATIII upon heparin binding have been observed spectroscopically (Villaneuva et al., 1979; Shore et al., 1989; Rosenberg et al, 1973). Furthermore, the ability of a pentasaccharide

region of heparin to promote the antithrombin III mediated inhibition of factor Xa supports this model. The inhibition of thrombin appears to be better explained by the template model. Conformational changes induced by heparin binding do not alter the reactivity of antithrombin III towards thrombin (Peterson et al., 1987). In addition, heparin pentasaccharides do not promote thrombin inhibition. Rather, chains of greater than 18 saccharide units are needed for this inhibition. Kinetic studies indicate that heparin must bind both thrombin and antithrombin III (Nesheim et al., 1986; Hoylaerts et al., 1984). It is not clear if one binding must precede the other for optimal inhibition to occur (Griffith et al., 1982; Pletcher et al., 1983).

Deficiency of antithrombin III predisposes the patient to thrombotic complications. Antithrombin III deficiencies can be the result of low protein levels or due to functionally abnormal molecules. Low protein levels can be brought about by reduced synthesis or an increased turnover of the molecule. Functional deficiencies can be brought about by mutations in either the reactive site or heparin binding sites. A number of such mutations have been documented (Erdjument et al., 1988; Lane et al., 1987; Stephens et al., 1987; Bock et al., 1985; Koide et al., 1984; Owen et al., 1987; Borg et al., 1988; Chang et al., 1986)

2. Heparin Cofactor II

Heparin cofactor II is a second plasma SERPIN which has resemblance to antithrombin III in that it is activatable by glycosaminoglycan binding. This protein has also been called antithrombin BM (Wunderwald et al., 1982), dermatan sulfate cofactor

(Abildgaard et al., 1984) and human leuserpin 2 (Ragg et al., 1986). The existence of this second inhibitor and heparin cofactor was first shown by Briginshaw in 1974 (Briginshaw et al., 1974a,b). Whereas antithrombin III is observed to have progressive antithrombin activity and to also inhibit factor Xa, the second cofactor exhibits only weak progressive activity and does not inhibit factor Xa. Tollefsen observed two different thrombin inhibitor complexes, one of which could not be identified with antisera to known protease inhibitors (Tollefsen et al., 1981). Several clinical studies observed a discrepancy between heparin cofactor activity levels and plasma antithrombin III antigen levels (Friberger et al., 1982; Griffith et al., 1983). The existence of the inhibitor was confirmed when the protein was isolated from human plasma (Tollefsen et al., 1982) and from Cohn fraction IV (Wunderwald et al., 1982). The heparin cofactor II protein has a molecular weight of 62,000 to 72,000 Da depending upon the methodology used (Tollefsen et al., 1982; Tran et al., 1986).

Like antithrombin III, heparin cofactor II inhibits proteases by forming a 1:1 stoichiometric complex with the enzyme. The protease attacks the reactive site of heparin cofactor II located on the C-terminus, resulting in the formation of a covalent bond. Heparin cofactor II has a higher protease specificity than antithrombin III. Of the coagulation enzymes, heparin cofactor II is known only to inhibit thrombin (Travis et al., 1983). Additionally, heparin cofactor II has been shown to inhibit chymotrypsin (Church et al., 1985) and leukocyte cathepsin G (Parker et al., 1985). This protease specificity appears to be due to the active site bond present in heparin cofactor II. Whereas antithrombin III contains an Arg-Ser bond as its active site, heparin cofactor II is unique

in containing a Leu-Ser bond. This suggests than another portion of the heparin cofactor II molecular may be required for protease binding.

As in the case of antithrombin III, the inhibition of protease activity by heparin cofactor II is promoted by glycosaminoglycan binding. Whereas the activation of antithrombin III is dependent upon the presence of a specific sequence in the heparin chain, heparin cofactor II can be activated by a wide variety of agents. Heparins, heparans, and dermatan sulfate all promote thrombin inhibition via heparin cofactor II. Agents with relatively little sulfation such as chondroitin 4-O- or 6-O-sulfate, keratan sulfate or hyaluronic acid do not activate heparin cofactor II. Heparan sulfate containing 0.97 sulfates per disaccharide has been shown to be a better activator of heparin cofactor II than heparan sulfate containing 0.67 sulfates per disaccharide (Tollefsen et al., 1989). In addition, sulfated, synthetic agents are able to activate heparin cofactor II. Both pentosan polysulfate (Scully et al., 1984; Scully et al., 1986) and dextran sulfate (Yamagishi et al., 1984) have been shown to activate heparin cofactor II.

Both dermatan sulfate and heparin have been fractionated in order to study their heparin cofactor II binding characteristics. In studies performed by Tollefsen et al. dermatan sulfate octasaccharides with higher negative charge have been shown to bind to heparin cofactor II better than those with a lower charge (Tollefsen et al., 1986). Although these octasaccharides bind to heparin cofactor II, dermatan sulfate chains of 12 to 14 saccharides are required to promote thrombin inhibition. This is consistent with the template model of inhibition. Heparin has been fractionated by charge density and subsequently on an ATIII-Sepharose column into high and low affinity fractions (Hurst

et al., 1983). It has been observed that for a given charge density, antithrombin III affinity is unrelated to the ability of the fraction to activate heparin cofactor II. High and low affinity fractions equally activated heparin cofactor II when charge density is constant. To date, definitive data supporting the existence of a minimally required sequence to activate heparin cofactor II has not been reported.

Rogers et al. have probed the role of thrombin exosites in the heparin cofactor II mediated inhibition of this enzyme (Rogers et al., 1992). In the absence of glycosaminoglycan, thrombin variants recognize antithrombin III and heparin cofactor II to a similar degree, indicating that neither the autolysis loop nor the ß-loop of thrombin is required for SERPIN/protease interaction. Upon addition of heparin, the interaction of antithrombin III with the thrombin variants is not altered suggesting the importance of the anion binding exosite II for the heparin bridge between thrombin and antithrombin III. These same studies indicate the importance of anion binding exosite I for the inhibition of thrombin by heparin cofactor II as gamma thrombin, lacking this site, is not inhibited. Based on these results, a complex double bridge mechanism for heparin cofactor II mediated thrombin inhibition has been postulated. In this mechanism, heparin or dermatan sulfate binds to the glycosaminoglycan binding site on heparin cofactor II and anion binding site I on thrombin. Upon heparin binding to heparin cofactor II, the acidic domain is displaced and is free to interact with the ß-loop region of the anion binding exosite of thrombin, facilitating its rapid inhibition.

The normal plasma level of heparin cofactor II is approximately $1.2 \pm 0.2\ \mu M$ (Tollefsen et al., 1985). Two patients to date have been described as having heparin

cofactor II deficiency related to thrombosis (Sie et al., 1985; Tran et al., 1985).

3. Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is one of the coagulation protease inhibitors found endogenously within the vasculature. TFPI has alternately been known as lipoprotein associated coagulation inhibitor (LACI) or extrinsic pathway inhibitor (EPI). This 42 kDa inhibitor has been shown to contain three Kunitz domains tandemly linked between a negatively charged amino terminus and a positively charged carboxy terminus (Girard et al., 1989). The active site of the first Kunitz domain binds to the active site of the VIIa - tissue factor complex while the active site of the second Kunitz domain binds to the active site of factor Xa. Mutation of the active site of the third Kunitz domain has no effect on the inhibition of either factor VIIa or factor Xa. Modification of the second Kunitz domain has also been shown to result in a loss of inhibition of tissue factor-VIIa activity. In experiments where the third Kunitz domain has been truncated, TFPI still inhibits factor VIIa tissue factor complexes on cell surfaces in culture (Hamamoto et al., 1993). The carboxy terminus of TFPI is required for the optimal inhibition of factor Xa (Wesselschmidt et al., 1992; Nordfang et al., 1991), perhaps effecting the rate at which TFPI can bind to Factor Xa. No difference is observed between the inhibition of factor VIIa - tissue factor by full-length TFPI or by a truncated form of TFPI (Wesselschmidt et al., 1992). Two studies have examined the kinetics of TFPI inhibition of factor Xa (Lindhout et al., 1993; Huang et al., 1993). Both studies have indicated that more than just the second Kunitz domain is required for factor

Xa binding as the association rate constants for full-length TFPI are higher than for carboxy-terminus or Kunitz 3 truncated TFPI. The third Kunitz domain has recently been shown to contain a second heparin binding site (Enjyoji et al., 1995).

TFPI has been cloned from a number of species including humans, rabbits, rats, and monkeys (Sprecher et al., 1994; Warn-Cramer et al., 1992; Kamei et al., 1994; Wun et al., 1988; Enjyoji et al., 1992). Monkey TFPI was observed to be similar in structure and function to human TFPI. Rabbit TFPI was shown to have a weaker antiprotease activity compared to human TFPI and not to associate with lipoproteins (Warn-Cramer et al., 1992).

In normal tissues of the vasculature, TFPI is produced by megakaryocytes and the endothelium (Werling et al., 1993). Once produced, this TFPI is stored in three intravascular pools. These pools are located in the plasma, in platelets, and bound to the endothelium (Lindahl et al., 1992). The smallest pool of TFPI is found in the platelets, accounting for less than 2.5 % of the intravascular total. This small pool of TFPI is released upon platelet activation (Novotny et al., 1988). 10 to 50 % of the intravascular TFPI is in the plasma. Most plasma based TFPI is bound to plasma lipoproteins (Novotny et al., 1991; Broze et al., 1987). Approximately 5 % of the plasma pool of TFPI circulates in the free form (Novotny et al., 1989; Lindahl et al., 1991). The lipoprotein bound TFPI is reported to be of relatively low inhibitory activity (Lindahl et al., 1991). The largest pool of TFPI is found bound to the endothelial surface (Novotny et al., 1991; Sandset et al., 1988; Lindahl et al., 1990). This pool can account for 50 to 90% of the total intravascular TFPI.

The TFPI pool bound to the endothelium has been shown to be heparin releasable in a number of studies (Ariens et al., 1994; Warn-Cramer et al., 1993; Bara et al., 1993; Holst et al., 1993; Novotny et al., 1991d). Venous occlusion (Sandset et al., 1988) and agents such as DDAVP which induce exocytosis of endothelial granular proteins (Warr et al., 1989) do not cause the release of TFPI. Repeated heparin administration is observed to release similar amounts of TFPI (Ariens et al., 1994) with no tachyphylaxis. It is believed that the endothelial pool of TFPI is bound to glycosaminoglycans on the surface of the endothelium. Heparin injection, then, is thought to displace TFPI from the endogenous glycosaminoglycans. The amount of TFPI in the plasma following heparin administration is determined by the heparin concentration. TFPI levels 2 to 10 fold baseline have been reported following heparin and low molecular weight heparin administration. The chemical nature of the low molecular weight heparin also effects the degree of TFPI release. It has been shown that when different LMWHs are administered at the same anti-Xa unit dosage, plasma TFPI levels vary by as much as 30 % (Vogel, 1995). Neutralization of heparin by protamine sulfate or protamine chloride results in a dramatic decrease in plasma TFPI levels (Harenberg et al., 1993; Hoppensteadt et al., 1995).

TFPI acts *in vitro* as an anticoagulant when measured by a number of assays. Both the thromboplastin induced clotting time and the activated partial thromboplastin time are prolonged by TFPI (Lindahl et al., 1991b; Lindahl et al., 1991c). Factor Xa based assays such as the Heptest® and the amidolytic anti-Xa assay are also affected by recombinant TFPI (Kristensen et al., 1992). Higher amounts of TFPI are required in the

prothrombin time and APTT for prolongation of the clotting time than are needed in the Heptest®. The prothrombin time is a more sensitive assay for the anticoagulant effects of TFPI than is the APTT, suggesting that the main *in vitro* inhibitory effect of TFPI is the inhibition of factor VIIa (Lindahl et al., 1991d). Co-supplementation of heparin and rTFPI to plasma *in vitro* has differing effects depending upon the assay used. Kristensen observed that heparin and rTFPI additively prolong the Heptest® clotting time. It has been shown that the prolongation of the APTT and PT assays by heparin and TFPI is synergistic (Valentin et al., 1991; Wun et al., 1992). A study by Nordfang et al., however, suggest that the increased effect of TFPI in the presence of heparin is due to heparin antithrombin III complexes as addition of heparin exhibited no effect in antithrombin III deficient plasma (Nordfang et al., 1993). The rate of Xa inhibition by rTFPI was observed to increase 2.5 fold upon the addition of heparin (Broze et al., 1988), though not with full-length TFPI (Wesselschmidt et al., 1992).

TFPI, when administered to rabbits, has been shown to have an antithrombotic effect when thromboplastin was used as a thrombogenic challenge (Day et al., 1990). TFPI was also shown to be an effective inhibitor when thrombosis was induced in rabbit jugular veins by endothelial destruction and restricted blood flow (Holst et al., 1994). The antithrombotic and antiprotease actions of TFPI have been tested in several other animal models. Warn-Cramer et al. investigated the effect of immunodepletion of TFPI in factor VIIa and Xa induced coagulation in rabbits (Warn-Cramer et al., 1993b). These rabbits were observed to be sensitized to the procoagulant effects of factor VIIa, but not factor Xa in the absence of factor VIIa. Two studies have indicated that TFPI

administration reduces the lethal effects of *E. coli* administration in a septic shock model in baboons (Carr et al., 1995; Creasey et al., 1993). These studies also indicated that TFPI may have an anti-inflammatory effect as an attenuation of the IL-6 response was also observed. Administration of TFPI has been observed to prevent reocclusion of arteries in dogs following clot lysis with t-PA (Haskel et al., 1991). Topical administration of TFPI has been shown to prevent thrombosis in a rabbit model of vascular trauma (Khouri et al., 1993).

4. Protein C

The protein C pathway is one of the natural anticoagulant systems which keeps blood in the fluid state. When thrombin is formed, it stimulates coagulation and its own formation by activating factors V and VIII through proteolytic cleavage (Jenny et al., 1994; Kane et al., 1988). Factors VIIIa and Va bind to negatively charged phospholipids on activated platelets and act as binding sites for factors IXa and Xa, respectively, allowing for formation of the "tenase" and prothrombinase complexes (Mann et al., 1990).

Thrombin can also act to limit its own procoagulant activity. When thrombin is in circulation, it binds a high affinity receptor on the endothelium known as thrombomodulin (Esmon et al., 1981). The k_d for this binding is 0.2 to 0.5 x 10^{-9} M (Owen et al., 1981). Thrombomodulin is a membrane spanning protein containing multiple functional domains and a molecular weight of approximately 60,000 Da (Dahlback et al., 1995). When thrombin binds to thrombomodulin, a change in substrate

specificity is noted. While this complex is a potent activator of protein C, the bound thrombin no longer cleaves fibrinogen, is not able to activate other coagulation proteases such as factors V and VIII and does not activate platelets (Esmon et al., 1982; Esmon et al., 1983). The thrombin-thrombomodulin complex is a 20,000 fold better activator of protein C than is free thrombin (Esmon et al., 1981; Owen et al., 1981). Thrombomodulin is present on the endothelium in most arteries, veins, and capillaries (Maruyama et al., 1985; DeBault et al., 1986).

Protein C is a vitamin K-dependent zymogen identified by Stenflo (Stenflo et al., 1976) which has been shown to be identical to autoprothrombin IIa (Seegers et al., 1976). Upon activation, protein C exhibits anticoagulant properties (Kisiel et al., 1977; Kisiel et al., 1979). Alterations of thrombin's substrate specificity upon binding to thrombomodulin are thought to be due both to steric hinderance of thrombin's active site and to conformational changes in the active site (Musci et al., 1988; Ye et al., 1991; Holtin et al., 1991). Protein C is made up of disulfide linked heavy and light chains and has a molecular weight of approximately 62,000 Da (Beckman et al., 1982; Foster et al., 1984). Protein C derives its anticoagulant properties from its ability to cleave and inactivate membrane bound forms of factors Va and VIIIa (Esmon et al., 1992; Esmon et al., 1993; Walker et al., 1992). Factors V and VIII as well as non-membrane bound forms of factors Va and VIIIa are not cleaved by protein C.

Protein C requires two cofactors in order to express its anticoagulant activity, protein S and factor V. Protein S is another vitamin K dependent plasma protein whose free form expresses protein C cofactor activity for the degradation of factors Va and

VIIIa (Dahlback et al., 1994). Protein S is a single chain, 70,000 Da glycoprotein (Dahlback et al., 1995), which has the highest affinity for negatively charged phospholipids among vitamin K dependent proteins (Nelsestuen et al., 1978). Protein S forms a 1:1 complex with protein C on the lipid membrane which may account for its ability to increase the affinity of activated protein C for such membranes (Walker et al., 1981; Walker et al., 1984). Though the mechanism of action of protein S is not completely understood, it may be related to its ability to make factors Va and VIIIa available for proteolytic cleavage by activated protein C (Regan et al., 1994; Solymoss et al., 1988). Less is known about factor V's role as an activated protein C cofactor, though it is hypothesized that factor V and protein S may synergistically act to localize protein C activity to the surface of membranes (Shen et al., 1994; Dahlback et al., 1994b).

As low levels of protein C activation peptide are found in healthy individuals, it is suggested that protein C is constantly activated to a small degree (Bauer et al., 1984). Protein C administration has been shown to inhibit both arterial and venous thrombosis in animal models (Gruber et al., 1990; Arnljots et al., 1994; Wakefield et al., 1993). Heterozygous protein C deficiency or activated protein C resistance due to factor V mutation are thought to explain 60 to 70 % of the cases of familial thrombophilia (Dahlback et al., 1995).

5. Protease Nexins

Protease nexins 1 and 2 are endogenous serine protease inhibitors which have

molecular weights of 43 and approximately 100 kDa, respectively (Preissner et al., 1988; van Nostrand et al., 1992). Both protease nexin 1 and protease nexin 2 have effects on the coagulation system. Based on cell culture studies, protease nexin 1 appears to be produced by fibroblasts, smooth muscle cells, and epithelial cells (Baker et al., 1980; Knauer et al., 1983; Eaton et al., 1983). Protease nexin 1 has a 30 percent sequence homology with antithrombin III and like ATIII, has a high affinity heparin binding site. Heparin binding to protease nexin 1 accelerates protease inhibition (Baker et al., 1987; Kruithof et al., 1988). Protease nexin 1 appears to be limited to the extravascular compartment as human plasma contains only small amounts of this inhibitor (20 pM) (Preissner et al., 1988). Protease nexin 1 inhibits several serine proteases including thrombin, urokinase, plasminogen activator, and activated protein C (Hermans et al., 1993; Scott et al., 1983; Bergman et al., 1984). Upon formation of a stable complex with the target protease, the complex binds back to the cells where it is internalized and degraded (Cunningham et al., 1992). The physiologic role of protease nexin 1 appears to be related to protection of the extracellular matrix from degradation by urokinase and plasminogen activator (Rao et al., 1989). This is supported by the fact that protease nexin 1 binds tightly to the extracellular matrix, thereby localizing its activity (Farrell et al., 1988).

Protease nexin 2 is identical to the secreted form of the amyloid precursor protein containing the Kunitz-type serine protease inhibitor domain (van Nostrand et al., 1989; Oltersdorf et al., 1989). Protease nexin 2 circulates in blood stored as a platelet α-granule protein which is secreted upon platelet activation (van Nostrand et al., 1990a).

Protease nexin 2 inhibits trypsin- and chymotrypsin-like serine proteases and is also a potent inhibitor of factor XIa (Smith et al., 1990; van Nostrand et al., 1990a, 1991a, 1991b; Sinha et al., 1990). Its location in platelets and its ability to inhibit factor XIa suggests a role in regulating blood coagulation for protease nexin 2.

6. Other Inhibitors

A number of other serine protease inhibitors are known to play a role in modulating physiologic functions. Plasminogen activator inhibitors serve to limit the normal activation of the fibrinolytic process. High levels of PAI-1 are associated with an increased risk of thromboembolic disease (Reilly et al., 1994). PAI-1 has also been shown to regulate the degradation of extracellular matrix which may be important in modulating cancer invasion. α_2-Antiplasmin rapidly inhibits the fibrinolytic activity of plasmin (Edelberg et al., 1994). α_2-Macroglobulin has been described as a "panproteinase inhibitor" in light of evidence that it interacts with nearly any proteinase (Borth, 1992). In addition, α_2-macroglobulin may play a role in inflammation and immune reactions through its ability to regulate the distribution and activity of numerous cytokines including transforming growth factor ß, tumor necrosis factor α, platelet derived growth factor, and several interleukins (LaMarre et al., 1991; Chaudhuri et al., 1993; Chu et al., 1994; Borth, 1994; Bonner et al., 1995). The complement and contact systems are regulated by c_1-esterase inhibitor through the inhibition of complement components C1r and C1s (Hack et al., 1994; Zahedi et al., 1993). Deficiency of c_1-esterase inhibitor is associated with angioedema (Carreer, 1992). Histidine-rich glycoprotein has been shown

to bind to plasminogen an interfere with its interaction with fibrin (Lijnen et al., 1980; Munkvad, 1993). Additionally, histidine-rich glycoprotein is known to bind to heparin and related glycosaminoglycans (Lijnen et al., 1983). High levels of this protein have not been definitively linked to thrombosis (Engesser et al., 1987).

C. Heparin

1. Discovery of Heparin

Heparin was first discovered in 1916 by Jay McLean while he was studying the procoagulant actions of phospholipids (McLean, 1916). In these early years, heparin was initially thought to be a phospholipid as it was isolated using procedures designed to separate phospholipids. Today we know that heparin is a glycosaminoglycan structurally related to the dermatans and chondroitins. More specifically, heparin has been defined as "... a family of polysaccharide species, whose chains are made up of alternating, 1-4 linked and variously sulfated residues of uronic acid and D-glucosamine" (Casu, 1989). The uronic acid residues are either L-iduronic acid or D-glucuronic acid. The glucosamine residues are either N-sulfated or N-acetylated. Typically, the iduronic acid moieties are 2-O sulfated whereas the glucosamine residues contain 6-O sulfate groups and a small proportion are 3-O sulfated (Brenkowski et al., 1985).

2. Chemistry of Heparin

Heparin is synthesized by a number of tissues and mast cells as part of a high molecular weight proteoglycan (molecular weight $\approx$ 750 to 1000 kDa). This

proteoglycan consists of a peptide core which is composed of 20 to 25 residues each of glycine and serine (Robinson et al., 1978). Attached to this peptide are 15 polysaccharide chains with molecular weights ranging from 60 to 100 kDa. The polysaccharide chains are attached to the peptide core via a galactosyl-galactosyl-xylosyl trisaccharide sequence (Lindahl et al., 1972).

The polysaccharide chains are formed by stepwise transfer of D-glucuronic acid and N-acetyl-D-glucosamine from their UDP sugar nucleotide forms to the non-reducing end of the polysaccharide chain (Helting et al., 1972; Helting et al., 1973; Forsee et al., 1981). Presumably these sugar moieties are polymerized directly to the linkage region of the protein core. The alternating sequence of glucuronic acid and hexosamine is due to the substrate specificity of the glycosyl transferases (Lindahl et al., 1989). Following polysaccharide chain elongation, the polymer undergoes a series of modification reactions.

Heparin is structurally heterogeneous due to incomplete structural modifications. Four enzymatic modifications of the polysaccharide backbone occur following its synthesis. The majority of the N-acetyl groups on the glucosamine residues are removed (Hook et al., 1975). The N-deacetylated glucosamines are subsequently sulfated. In the next step, D-glucuronic acid residues are epimerized to L-iduronic acid units by uronosyl C-5 epimerase (Lindahl et al., 1976; Malmstrom et al., 1980). During the epimerization process, most iduronic acids are 2-O sulfated. Finally, 3-O and 6-O sulfate groups are added onto the glucosamine units. Previous N-sulfation allows more efficient O-sulfation to occur (Lindahl et al., 1989). Several more recent studies have indicated that chain

elonation and modification may occur simultaneously during heparin synthesis (Lidholt et al., 1989; Linholt et al., 1992)

Nuclear resonance spectroscopy involves the measurement of radiofrequency radiation absorption by a given sample when it is placed in a strong magnetic field. The nuclei of many atoms act as magnetic dipoles in that they can be aligned either with or against the magnetic field. These nuclei include ^{1}H, ^{13}C, ^{19}F, and ^{31}P. ^{1}H and ^{13}C are the most commonly studied nuclei due to their abundance in organic materials. The ground state of a nuclei is the energy level when the dipole is aligned along the magnetic field. The excited state occurs when the nuclei are aligned against the magnetic field. Transfer of nuclei from the ground to the excited state occurs when nuclei absorb radiofrequency radiation. The amount of power which is absorbed is dependent not only on the molecular properties of the given sample, but also upon the surrounding magnetic field. The surrounding magnetic field includes the field produced by the instrument as well as any field produced by adjacent nuclei. Adjacent nuclei can either shield (decrease) of deshield (increase) the magnetic field produced by the instrument. Resonance frequencies of nuclei vary depending upon the chemical environment of the nuclei. This is expressed in terms of a chemical shift (δ). Chemical shift is usually given in ppm and is independent of the applied magnetic field. As there is no way of measuring the strength of the magnetic field at a given nucleus without shielding, chemical shifts are determined relative to an internal standard. The chemical shifts of nuclei are dependent upon the nearby chemical structures. Structural characteristics of a molecule can be elucidated using this technique as the various protons (^{1}H) and ^{13}C nuclei are present in different

environments with respect to the local charge density and, therefore, are excited to different extents by the introduction of the radiofrequency pulse.

Monodimemsional spectroscopy of polysaccharides such as heparin is often of limited value due to the intrinsically broad signals and the large number of overlapping signals. Bidimensional spectroscopy is a more useful technique as it allows for the correlation of two similar (^1H - ^1H) or different (^1H - ^{13}C) nuclei in a manner such that the signals relating to intramolecular interactions can be identified. These are termed homonuclear and heteronuclear correlations.

3. Biologic Effects of Heparin - Non-anticoagulant

Heparin is a strongly anionic polyelectrolyte which at physiologic pHs contains three acidic functional groups which are fully dissociated; $-OSO_3^-$, $-NHSO_3^-$, and $-COO^-$ (Nieduszynski, 1989). Owing to this fact, heparin has a large number of pharmacologic properties. Among these are its antilipemic and antihemolytic actions (Bradshaw et al., 1975; Levy et al., 1958). Heparin is also known to inhibit a variety of enzymes including myosin ATPase, RNA dependent DNA polymerase, elastase, and renin (Cruz et al., 1967; Neuhoff et al., 1970; Sealey et al., 1967). Heparin inhibits tumor growth (Lippman et al., 1965; Folkman et al., 1985). Additionally, heparin exhibits antibacterial and antiviral properties (Corrigan et al., 1977; Vaheri et al., 1964).

4. Pharmacokinetics of Heparin

Heparin is administered either by intravenous infusion or by subcutaneous

injection. Upon entering the blood stream, heparin binds to a variety of plasma proteins, thereby lowering its bioavailability and producing a variable anticoagulant response (Hirsh et al., 1976). These proteins include histidine rich glycoprotein, platelet factor 4, vitronectin, and von Willebrand factor (Lane, et al., 1986; Lijnen et al., 1983; Peterson et al., 1987; Holt et al., 1985; Preissner et al, 1987; Dawes et al., 1991; Sobel et al., 1991). Heparin exhibits a complex pharmacokinetics and is cleared by two mechanisms. The rapid, saturable phase of elimination is thought to be due to receptor mediated internalization of heparin by endothelial cells and macrophages (Glimelius et al., 1978; Mahadoo et al., 1978; Friedman et al., 1974). A slower, nonsaturable renal mechanism also clears heparin from the plasma (de Swart et al., 1982; Olsson et al., 1963; Bjornsson et al., 1982). The anticoagulant effect of heparin is therefore not linearly related to dose when in the therapeutic range (Hirsh et al., 1994). The biologic half-life of heparin increases from 30 minutes following an IV bolus dose of 25 U/kg to 150 minutes following a dose of 400 U/kg (de Swart et al., 1982; Olsson et al., 1963; Bjornsson et al., 1982).

5. Clinical Use of Heparin

Heparin is used in the therapy of several cardiovascular disorders including prevention and treatment of venous thromboembolism, treatment of unstable angina, acute myocardial infarction, cardiac and vascular surgery, coronary angioplasty, stent implantation, and as an adjunctive agent during thrombolysis. Heparin is also the anticoagulant of choice during pregnancy.

Studies have demonstrated that there is a reduction in mortality in patients receiving heparin for the treatment of pulmonary embolism (Barritt et al., 1960; Brandjes et al., 1992). In addition, recurrent thrombosis was not common during the heparinization period, but increased significantly when heparin was stopped and no other anticoagulant therapy was utilized (Hull et al., 1979; Lagerstedt et al., 1985). Heparin is effective in treating venous thrombosis. This effectiveness has been shown to be dependent upon the anticoagulant effect achieved (Hull et al., 1986; Turpie et al., 1989). Heparin is also effective prophylactically, reducing the risk of venous thrombosis and pulmonary embolism 60 to 70 percent (Clagett et al., 1988; Collins et al., 1988). Heparin is effective short-term in preventing acute myocardial infarction and recurrent refractory angina in patients with unstable angina (Theroux et al., 1990; Theroux et al., 1992; Neri Serneri et al., 1990). This beneficial effect is lost upon cessation of heparin therapy. In patients with previous myocardial infarction, heparin administration has been shown to significantly reduce reinfarction and death when compared to untreated controls (Neri Serneri et al., 1987). Heparin has been tested as an adjunct in thrombolytic therapy where it appears to increase patency during the initial stages of recanalization by preventing rethrombosis (Bleich et al., 1990; de Bono et al., 1992). Heparin is the anticoagulant of choice in pregnancy as it does not cross the placental barrier and is not known to cause unwanted effects on the fetus (Hirsh et al., 1994; Hyers et al., 1992).

6. Effect of Heparin on Platelets

The effect of heparin on platelets is controversial. Studies by Ellison and

Thomson have shown that heparin decreases the threshold for ADP and epinephrine induced aggregation and enhances the platelet release reactions by these agonists (Ellison et al., 1978; Thomson et al., 1973). Treatment with heparin was also observed to increase platelet retention on cellophane membranes. Other studies have indicated the opposite effects on platelets. Besterman showed that irreversible aggregation induced by collagen and epinephrine was reduced in patients treated with 2500 to 5000 U of unfractionated heparin (Besterman et al., 1973). In these same patients, no effect to a slight increase in aggregation was observed with ADP (Zucker et al., 1977). Heiden demonstrated a loss of [^{14}C]-5-HT release in PRP of individuals treated with 100 U/kg heparin in response to collagen, epinephrine, and ADP (Heiden et al., 1977). An indirect mechanism was suggested to account for this observation based on the finding that *in vitro* addition of heparin caused no effect on aggregation (Eika et al., 1972). Salzman et al. have shown that concentrations of heparin as low as 10 μg/mL induces aggregation in platelet rich plasma, but not in washed platelets (Salzman et al., 1980).

Heparin administration is also known to cause an adverse effect on platelets known as heparin induced thrombocytopenia (HIT). Type I HIT occurs early in heparin treatment and causes a transient reduction in platelet count. Patients usually remain asymptomatic. Type II HIT is a more severe thrombocytopenia of delayed onset. This form of HIT often results in thrombosis and is associated with a high degree of mortality. While the exact mechanism is unknown, it appears that an IgG antibody is generated whose F-ab portion binds to the heparin/PF4 complex. Optimal platelet activation occurs when heparin and platelet factor 4 are in near equimolar concentrations. The Fc portion

of the antibody binds to the FcIIa receptor on the platelet surface and activates the platelets. The differences in response to these heparin/PF4 antibodies may be due to differences in the genotype of the FcIIa receptor. Studies have indicated that the IgG antibody:heparin:PF4 complex forms in solution an then binds to the platelet FcIIa receptor on platelets.

7. Chemically Modified Heparins

a. Hypersulfated heparins

The anticoagulant and antithrombotic actions of heparins containing higher than normal degrees of sulfation have been examined in several studies. In a laser model of thrombosis, a supersulfated low molecular weight heparin was observed to require a 10 fold lower dose than native heparin or low molecular weight heparin to achieve a comparable antithrombotic effect (Krupinski et al., 1990). In another study, oversulfation of low molecular weight heparin was observed to reduce the *ex vivo* anticoagulant activity relative to LMWH which has not been oversulfated. Addition of sulfate groups, however, did not effect the antithrombotic activity in a rat venous stasis-thrombosis model and did not significantly increase the bleeding time (Naggi et al,, 1987). The release of lipoprotein lipase by the supersulfated low molecular weight heparin was twice that of heparin. In a pure biochemical system, the inhibition of thrombin via heparin cofactor II by supersulfated low molecular weight heparin was approximately 100 fold stronger than for low molecular weight heparin (Jeske et al., 1995).

b. Desulfated heparins

N- and O- desulfated heparins have to be examined for a number of pharmacologic properties. In general, a reduction in the sulfation of heparin results in a decrease in the given biologic activity. N- and 6-O-desulfation significantly decreased the antiviral activity of heparin with respect to herpes simplex I binding (Herold et al., 1995). Heparin potentiates the binding of vascular endothelial growth factor (VEGF 165) to its cellular receptors. O- and N- desulfated heparins potentiated this binding to a lesser extent (Soker et al., 1994). Rajtar demonstrated that N- desulfated heparins were less effective at inhibiting platelet function than native heparin (Rajtar et al., 1993). Both fully desulfated heparin and N-desulfated heparin lack the ability to bind heparin binding growth factor (Belford et al., 1992).

The anticoagulant and antithrombotic effects of desulfated heparins have also been examined. A partially N-desulfated heparin has been shown to have no measurable anticoagulant or antiprotease activity, but to dose-dependently impair thrombogenesis *in vivo* (Sache et al., 1989). A completely N-desulfated heparin derivative lacked both *in vitro* and *in vivo* activity (Inoue et al., 1976). Other investigators have shown that N-desulfated heparins have minimal anticoagulant activity (Bjornsson et al., 1988; Danishefsky et al., 1977). N-desulfated heparin has been shown to be cleared approximately 6 fold faster than native heparin (Bjornsson et al., 1988). The weak anticoagulant activity is attributable to the lack of interaction with antithrombin III (Danishefsky et al., 1977).

8. Low Molecular Weight Heparin

The depolymerization of heparin either chemically by nitrous acid degradation, benzylation - alkaline hydrolysis, or peroxidative cleavage, or enzymatically using heparinase results in the production of another clinically useful drug known as low molecular weight heparin. Low molecular weight heparins exhibit several distinct properties which differentiate them from standard unfractionated heparin. Through the depolymerization process, the molecular weight is reduced to approximately one third that of the parent material (Fareed, 1995). This is important for two reasons. First, the largest heparin chains are not well absorbed following subcutaneous administration. The bioavailability of heparin is only 20 to 30 percent (Fareed, 1995). The bioavailability of LMWH is nearly 100 percent when measured using an amidolytic anti-Xa assay. The smaller molecular size of the LMWHs also has an effect on the biologic activity of these agents. The LMWHs have a lower anticoagulant potency than unfractionated heparin. This is a reflection of the lower antithrombin activity of these agents. Heparin exhibits a 1:1 ratio of antithrombin to anti-Xa activity whereas for LMWHs the ratio ranges from 1:2 to 1:4 depending upon the molecular weight composition of the given LMWH (Hirsh et al., 1994).

LMWHs are the agents of choice in European countries for the prophylaxis of deep venous thrombosis due to their efficacy and safety. Owing to their high bioavailability, LMWHs exhibit a sustained pharmacologic effect such that once daily dosing is sufficient to keep the patient in an antithrombotic state. Heparin, in contrast, requires 2 or 3 daily injections to achieve a similar effect (Fareed, 1995). Due to their

near complete availability and limited plasma protein binding (Sobel et al., 1991; Lane et al., 1986; Lijnen et al., 1983; Dawes et al., 1991), LMWHs exhibit predictable clinical effects and do not require daily monitoring of plasma levels (Handeland et al., 1990). Heparin levels are monitored frequently using the APTT assay. There has also been the suggestion that LMWHs may cause less hemorrhagic side-effects, less osteoporosis, and decreased cytopenia relative to standard heparin (Fareed, 1995).

Low molecular weight heparins have been examined for their efficacy in the treatment and prevention of venous thromboembolism. In general surgical patients, randomized trials have indicated that LMWH is both effective and safe. Statistically significant reductions in thromboembolic mortality was observed without a significant increase in major hemorrhage. Most clinical trials indicate a bleeding effect which is equal to or less than that caused by heparin (Ockelford et al., 1989; Pezzuoli et al., 1989; Kakkar et al., 1985; Bergqvist, 1988). In orthopedic patients, LMWHs have exhibited equal or superior efficacy compared to low dose heparin (Planes et al., 1988; Eriksson et al., 1991), adjusted dose heparin (Leyvraz et al., 1991; Dechavanne et al., 1989), warfarin (Heit et al., 1991), and dextran (Bergqvist et al., 1991). In medical patients, LMWHs have been shown to reduce the risk of thromboembolism relative to placebo (Prins et al., 1987; Turpie et al., 1987) and heparin (Green et al., 1990; Turpie et al., 1992). LMWHs have been shown to be effective in treating established thrombosis, both preventing further extension of the thrombus and enhancing its regression (Hull et al., 1992; Prandoni et al., 1992).

D. Non-heparin Glycosaminoglycan Agents

1. Heparan Sulfate

Heparan sulfate has a backbone which is structurally similar to heparin. Heparan sulfate generally contains more than 20 % N-acetylated glucosamine and nearly equal amounts of N- and O-sulfation. In contrast, the ratio of O- to N-sulfation in heparin is almost 4 to 1 (Gallagher et al., 1985; Lindahl et al., 1991). Like heparin, heparan sulfate primarily inhibits proteases via activation of antithrombin III and has been shown to catalyze the formation of thrombin-antithrombin complexes (Hatton et al., 1978) and also to exhibit anti-factor Xa activity (Thomas et al., 1979). As it does not completely inhibit prothrombin activation, it is much less effective than heparin. The antithrombotic dosage of heparan sulfate has been shown to be approximately 500 to 600 μg/kg compared with 60 to 70 μg/kg for heparin (Ofosu, 1989)..

2. Dermatan Sulfate

Dermatan sulfate is a glycosaminoglycan polymer of iduronic acid and N-acetylated galactosamine. Due to a difference in the molecular backbone, dermatan sulfate is unable to interact with antithrombin III (Ofosu et al., 1985), but rather complexes with heparin cofactor II to mediate thrombin inhibition (Tollefsen et al., 1983). The anticoagulant potency of dermatan sulfate is less than that of heparan sulfate (Teien et al., 1976; Ofosu et al., 1984). Anticoagulant activity as measured by the APTT and thrombin time is nearly undetectable (Sie et al., 1991). Dermatan sulfate inhibits thrombin as it is formed rather than preventing its generation (Ofosu et al., 1989). It has

been shown that thrombin generation inhibition by dermatan sulfate is much less than for an equigravimetric amount of heparin (Merton et al., 1987). At a dosage of 150 μg/kg, dermatan sulfate caused a 28 % inhibition of thrombin generation compared to an 83 % inhibition by heparin. While dermatan octasaccharides bind heparin cofactor II, 12 to 14 residues are required for thrombin inhibition. Dermatan sulfate chains with a higher charge density appear to bind to heparin cofactor II better than those with a lower charge.

Dermatan sulfate is active *in vivo* as an antithrombotic agent in the rabbit stasis thrombosis model, but to a lesser extent than heparin (Merton et al., 1987). Though both agents inhibited thrombus formation to the same extent at an equigravimetric dosage of 150 μg/kg after 10 minutes of stasis, dermatan sulfate was ineffective at inhibiting thrombus formation following 20 minutes stasis time at a dose 8 times higher than that of heparin. The advantage dermatan sulfate has over heparin as an antithrombotic agent is a lower risk of bleeding complications (Fernandez et al., 1986); Desnoyers et al., 1989). Dermatan sulfate has been shown not to significantly increase bleeding versus a saline control at doses which are antithrombotically effective.

It is thought that the decreased bleeding seen with dermatan sulfate is a result of its minor effects on platelets. Sie et al. have shown that while thrombin induced platelet aggregation is inhibited by dermatan sulfate in the presence of heparin cofactor II, arachidonic acid, ADP and collagen induced aggregations were not effected (Sie et al., 1982). In addition, it has been shown that dermatan sulfate has a smaller effect on heparin induced thrombocytopenic serum induced platelet aggregation than did heparin

(Hoppensteadt et al., 1991).

3. Chondroitin Sulfate

Chondroitin sulfate is a glycosaminoglycan which consists of alternating D-glucuronic acid and N-acetylated D-galactosamine residues. The galactosamine residues are typically 4-O or 6-O sulfated (Casu et al., 1991). Chondroitin sulfate has a lower degree of sulfation than the heparin or heparan-type glycosaminoglycans, with a SO^{3-}/COO- ratio equal to 1. Due to a low degree of sulfation, and the lack of iduronic acid moieties, chondroitin sulfate does not have strong interactions with the endogenous coagulation inhibitors antithrombin III or heparin cofactor II or with lipoprotein lipase or low density lipoproteins as heparin does (Casu et al., 1991). Chondroitin sulfates are typically found in cartilage. Chondroitin sulfate has been used in the treatment of osteoarthritis as it inhibits elastase and hyaluronidase in the synovial fluid which can damage joint cartilage (Pepitone et al., 1991). The anticoagulant activity of chondroitin sulfate is minimal, with potency designated as less than 5 USP IU/mg and 5 anti-factor Xa U/mg by the Yin and Wessler test (Bianchini et al., 1985).

The antithrombotic activity of chondroitin sulfate and its oversulfated derivatives was examined in a rat model of thrombosis and was observed to be minimal (Pescador et al., 1991). Oversulfation of the molecules also did not enhance the negligible anticoagulant activity of the native chondroitin sulfate. The chondroitin sulfate present in Org 10172 was not observed to enhance the antithrombotic effect of the high affinity material present in the preparation (Zammit et al., 1994).

Chondroitin sulfate has been shown to have several biologic functions. Evidence has been presented indicating that chondroitin sulfate proteoglycans interact with the herpes simplex virus (Banfield et al., 1995). Chondroitin sulfate offers neuroprotective effects against glutamate induced neuronal cell death (Okamoto et al., 1994) and may play a role in neuronal patterning (Brittis et al., 1992). Chondroitin sulfate has been shown *in vitro* to inhibit the activation of the complement system (Biffoni et al., 1991). This potency was related to the sulfate content of the chondroitin. Chondroitin sulfate exhibits *in vitro* anti-HIV-1 activity (Jurkiewicz et al., 1982). When administered to cholesterol fed rabbits, chondroitin sulfate suppressed cholesterol deposition in the aorta due to a decrease in plasma LDL cholesterol and to a change in arterial metabolism (Matsushima et al., 1987). Chondroitin sulfate E was shown to be a weak activator of heparin cofactor II, accelerating the inhibition of thrombin approximately 200 fold. In addition, chondroitin sulfate may exhibit some anticoagulant activity due to a interference in the thrombin-fibrinogen interaction (Scully et al., 1986).

E. Homologues of Heparin

1. K-5 Derived Agents

Heparin-like agents can be derived from bacterial polysaccharides. The K5 capsular polysaccharide from *E. coli* is a polymer of glucuronic acid and N-acetylated glucosamine. This is the same chemical structure as N-acetyl heparosan, a precursor in mammalian heparin formation (Casu et al., 1992). To make a heparin-like substance, this precursor is first deacetylated with hydrazine (Jann et al., 1992) and then N-sulfated. The

resultant sulfamino heparosan undergoes C5 epimerization to produce iduronic acid moieties (Kusche et al., 1991) and finally O-sulfation produces a substance resembling mammalian heparin. NMR analysis of the resultant polysaccharides indicate strong signals characteristic of N-sulfated groups, glucosamine and glucuronic acid residues (Jann et al., 1992). These modified polysaccharides have also been shown to contain the 3-O sulfate group on glucosamine which is required for high affinity heparin binding to ATIII (Casu et al, 1994). These agents have been shown to produce similar *in vitro* anticoagulant and *in vivo* antithrombotic effects as low molecular weight heparin (Fareed et al., 1995).

2. Pentosan Polysulfate

Pentosan polysulfate is a linear polymer of sulfated 1-4 linked ß-xylopyranose units which is derived from the bark of the beech tree (Aspinsall, 1959). All available hydroxyl groups on pentosan are sulfated. The molecular weight of pentosan polysulfate preparations is in the range of that of the low molecular weight heparins (4 to 6.5 kDa).

Like unfractionated heparin, pentosan polysulfate has multiple pharmacologic actions. It has been widely utilized as an antilipemic agent (Barrowcliffe et al., 1986), shown to selectively inhibit HIV-1 replication (Baba et al., 1988) and to have anti-tumor effects in animals (Wellstein et al., 1991).

A number of investigators have examined the anticoagulant actions of pentosan polysulfate. Unlike heparin, pentosan polysulfate prevents coagulation independently of AT-III (Fischer et al., 1982; Scully et al., 1983). Pentosan polysulfate has been shown

to inhibit coagulation by two mechanisms. Firstly, pentosan polysulfate prevents thrombin generation through its ability to inhibit the thrombin dependent activation of factor V (Ofosu et al., 1987). Thereby, pentosan polysulfate inhibits the formation of the prothrombinase complex and the generation of thrombin. Secondly, pentosan polysulfate promotes the inhibition of preformed thrombin via HC-II (Scully et al., 1984). Consequently, pentosan polysulfate has been shown to have low anticoagulant potency *in vitro* (Scully et al., 1983; Soria et al., 1980). The potency of pentosan polysulfate has been reported as 12 anti-IIa U/mg and 8 anti-Xa U/mg.

Despite its low anticoagulant activity, a number of investigators have demonstrated the antithrombotic efficacy of this agent. In a rabbit model of stasis-thrombosis, pentosan polysulfate was shown to inhibit factor Xa, thrombin, and thromboplastin induced thrombogenesis to varying degrees. Thromboplastin was most potently inhibited (van Ryn-McKenna et al., 1989). Similarly in rats, pentosan polysulfate was observed to dose-dependently increase the number of laser injuries required to induce thrombogenesis, though at much higher doses than were required for heparin or low molecular weight heparin (Krupinski et al., 1990).

The major side effects of pentosan polysulfate are reported to be similar to those of heparin. A recent report has indicated that pentosan polysulfate induced thrombocytopenia and thrombosis in patients receiving repeated administrations of the agent (Tardy-Poncet et al., 1994). Positive cross-reactivity with heparin and low molecular weight heparin was observed in a large number of these cases.

3. Sulfated Mucopolysaccharides

Sulfated mucopolysaccharides are mixtures of glucosaminoglycans and galactosaminoglycans extracted from cartilage. These substances are frequently used in rheumatology for the treatment of joint degeneration. Several of these agents have also been tested for their effects on the hemostatic system. Gorog et al. demonstrated that a high concentration of a galactosaminoglycan caused a concentration dependent inhibition of platelet reactivity. In addition, doses from 1 to 10 mg/kg were seen to inhibit thrombus formation in a laser model of thrombosis (Gorog et al., 1987). Bauer et al. examined the anticoagulant properties of three mucopolysaccharides used clinically to treat degenerative joint diseases (Bauer et al., 1983). Arteparon was observed to have an *in vitro* anticoagulant potency one quarter that of heparin as measured by the APTT. *In vivo* administration of this agent confirmed its anticoagulant properties.

F. Synthetic Heparin Analogues

1. Pentasaccharide

By measuring the antithrombin III affinity of a series of heparin fragments, it was determined that the minimal antithrombin III binding sequence resides in an irregular region of heparin and is a pentasaccharide (Rosenberg et al., 1979; Lindahl et al., 1979; Choay et al., 1980). In the irregular region of heparin, 25 to 30 percent of the glucosamine residues contain a unique 3-O sulfate group. The importance of this sulfate group to both the *in vitro* anticoagulant and the *in vivo* antithrombotic activities has been demonstrated (Walenga et al., 1988). Subsequent to its discovery, this pentasaccharide

was chemically synthesized (Petitou et al., 1986). Higher yields of the methyl α-glycoside derivative of the pentasaccharide have been achieved (Petitou et al., 1987). The chemical structure of this pentasaccharide is depicted in Figure 2. This derivative exhibits the same biologic properties as the native agent.

By allometry, it has been estimated that the half-life of pentasaccharide in humans is approximately 14 hours (Herault et al., 1995a). The first clinical study performed in man with pentasaccharide has confirmed the long duration of action of this agent following subcutaneous administration (Boneu et al., 1995). Half-lives of 13.1 to 13.9 hours were observed in healthy volunteers. The peak plasma concentrations were linearly related to dose and plasma clearance was observed to be three fold higher than that of typical low molecular weight heparins. This agent has been reported to exhibit a very good tolerance with no prolongation of the bleeding time, APTT, or PT. Van Amsterdam has shown that there is a rapid clearance of unbound pentasaccharide from the plasma, but that antithrombin III bound pentasaccharide is cleared similarly to endogenous antithrombin III (van Amsterdam et al., 1993). This was also observed following administration of the highest dose in the study of Boneu.

The inhibition of thrombin generation was studied following subcutaneous administration to normal volunteers (Lormeau et al., 1995). In this study, thrombin generation was observed to be inhibited for periods of up to 18 hours. TFPI release was not observed with subcutaneously administered pentasaccharide doses as high as 12,000 anti-Xa units. It was concluded from this study that thrombin generation by pentasaccharide is mediated exclusively through selective ATIII mediated inhibition of

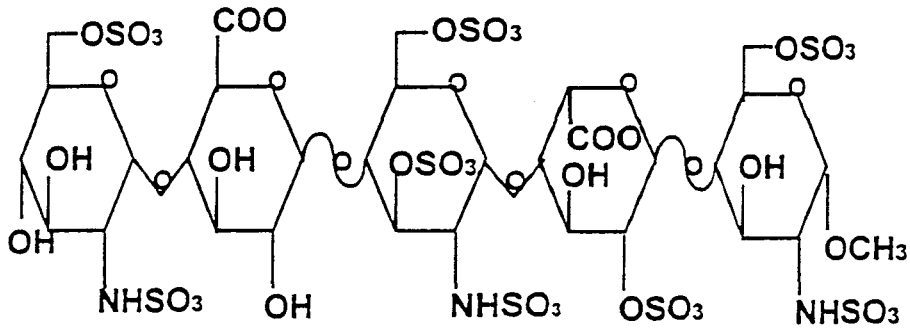


Figure 2. depicts the chemical structure of a methyl α-glycoside derivative of the high ATIII affinity binding pentasaccharide of heparin. The pentasaccharide contains the unique 3-0 sulfate group required for binding to antithrombin III. The molecular weight of this agent is 1728 Da. This structure was adapted from Petitou et al., 1991.

factor Xa.

Studies have shown that the pentasaccharide has no measurable activity in the prothrombin time or in the APTT assays. In addition, pentasaccharide was seen to be ineffective in both coagulant and amidolytic antithrombin assays (Walenga et al., 1985). This pentasaccharide does exhibit a very high anti-factor Xa potency (Choay et al., 1983). Pentasaccharide has been shown to concentration dependently inhibit thrombin generation in normal human plasma (Lormeau et al., 1993). Thrombin generation following extrinsic pathway activation was more potently inhibited than following intrinsic pathway activation. This weaker inhibition of the intrinsic pathway is likely due to the pentasaccharide's inability to inhibit factor V and factor VIII formation (Ofosu et al., 1991). It has been shown both *in vitro* and *in vivo* that thrombin generation is not completely inhibited by pentasaccharide. At doses of pentasaccharide which completely prevented thrombus formation in a modified Wessler model, thrombin generation was only inhibited sixty percent (Walenga et al., 1988). Clots formed in this model contain predominantly red cells and fibrin, but few platelets. The thrombi formed in the arterio-venous shunt model contain red cell, fibrin strands, and a large amount of platelets. Pentasaccharide was able to inhibit thrombus formation in both models. Hobbelen showed that pentasaccharide dose-dependently inhibited thrombosis caused by various triggers in a rat thrombosis model (Hobbelen et al., 1990). Additionally, this study had shown that pentasaccharide causes a smaller increase in blood loss than heparin at antithrombotically active doses. Impaired thrombin generation rather than anti-Xa activity was observed to correlate with prevention of stasis induced thrombus formation (Thomas et al., 1989).

The antithrombotic effect of this agent was also tested in baboons using a modified arterio-venous shunt in which both arterial-type and venous-type thrombi can form (Cadroy et al., 1993). While both types of thrombi were inhibited by pentasaccharide, higher doses of the agent were needed to achieve a comparable level of inhibition on the arterial side. The pentasaccharide was also observed to have no effect on platelet function as measured by the template bleeding time. The pentasaccharide has been observed to reduce thrombus formation following an electrical stimulation injury in rabbit carotid arteries. This agent was also observed to enhance clot lysis induced by tissue plasminogen activator.

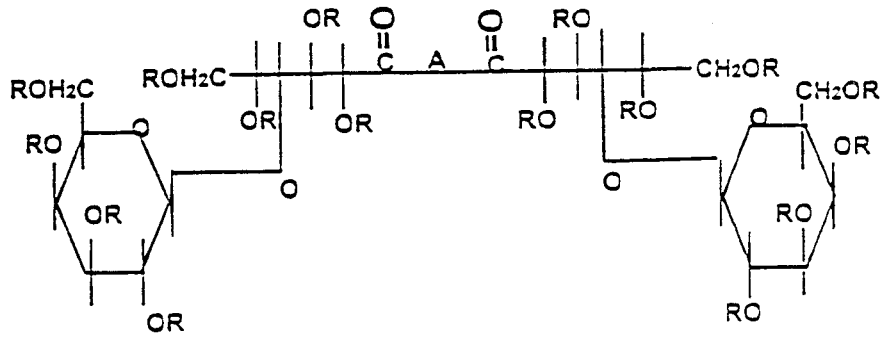
A series of structural analogues of the native pentasaccharide sequence have been synthesized (Meulemen et al., 1991). The analogues contain an additional 3-O sulfate group on glucosamine residue H. Additionally, some of the analogues also contained a 3-O or 4-O sulfate group on glucosamine residue D. All of the analogues with additional 3-O sulfate groups were observed to exhibit a higher anti-Xa potency than the native pentasaccharide (1230 anti Xa U/mg vs. 700 anti-Xa U/mg). The higher specific activity is proposed to be due to a tighter two site binding to antithrombin III. As expected, all of the analogues were active in a rat stasis thrombosis model. The duration of the antithrombotic effect was four to five times longer than the natural pentasaccharide. The half-life of the anti-Xa activity of the modified analogues was approximately twice that of the natural pentasaccharide. Other pentasaccharide analogues have been reported (Herault et al., 1995b). Conversion of the N-sulfate groups to O-sulfates has been shown to increase antithrombin III affinity 2 fold. O-methylation leads

to an 8 fold increase in affinity.

2. Aprosulate

Sulfated bis-aldonic acid amides are a new class of synthetic low molecular weight polyanions whose anticoagulant and antithrombotic activity is dependent on their chemical structure. These agents are synthesized by conversion of aldonic acids to their corresponding cyclic ester, or lactone. Two such lactone molecules are linked via amide bonds with an alkylene diamine. The intermediate is then fully sulfated using a pyridine-SO_3 complex resulting in a homogeneous product (Klauser et al., 1991). Lactobionic, D-gluconic, L-mannonic, D-galactonic, milibionic, and maltobionic acids were used as starting materials and were linked by alkylene diamine bridges ranging from 2 to 12 units in length. The compound which has shown the best anticoagulant and antithrombotic profile is one which is produced by linking two lactobionic acid moieties with a trimethylene diamine (Klauser et al., 1991). This compound is known as aprosulate. The chemical structure of aprosulate is depicted in Figure 3. The series of compounds is characterized by a defined structure and has been shown to have limited dispersity (Klauser et al., 1991). The molecular weights of these compounds are lower than those of the low molecular weight heparins with the molecular weight of aprosulate being 2388 Da.

The anticoagulant properties of several aldonic acid amides has been reported (Raake et al., 1991; Jeske et al., 1993; Sugidachi et al., 1994). These agents exhibited activity in the APTT and Heptest® assays indicating that they inhibit the intrinsic



$A = NH\text{-}(CH_2)_3\text{-}NH$

$R = -SO_3Na$

Figure 3 depicts the chemical structure of aprosulate. Its structure is characterized by two fully sulfated disaccharides linked via amide bonds by a propylene chain. One saccharide of each disaccharide is in open chain conformation. The molecular weight of aprosulate is 2388 Da. This structure was adapted from Raake et al., 1991.

coagulation pathway enzymes. This activity was low when compared to heparin. Activity in a chromogenic anti-Xa was 200 times less than that of heparin (Raake et al., 1991).

Fareed et al. examined the pharmacodynamics of aprosulate with the purpose of determining the usefulness of various assay methods for determining the amount of aprosulate in plasma samples. In this study, Rhesus monkeys (*Macaca mulatta*) were given aprosulate by both intravenous and subcutaneous injection, with blood samples drawn over a period of six hours (Fareed et al., 1991). Both the APTT, 5U thrombin time and the Heptest® were dose-dependently prolonged by aprosulate. The amidolytic activity of thrombin was inhibited only up to 30 minutes post-injection. This data indicates that aprosulate is absorbed following subcutaneous administration. Based on this data, the biologic half-life of aprosulate was estimated to be 45 minutes.

Protamine sulfate is a heterogeneous mixture of polybasic proteins derived from various types of fish (Meischer et al., 1874) which is used clinically to neutralize the anticoagulant effects of heparin. *In vitro, ex vivo,* and *in vivo* neutralizations of aprosulate have been examined. With the *in vitro* samples, complete neutralization in the Heptest® and thrombin time assays was observed when a two to four fold gravimetric excess of protamine was used. Despite this high level of protamine, the APTT values remained elevated (Hoppensteadt et al., 1991). Aprosulate's effects on the amidolytic anti-IIa and anti-Xa assays were completely neutralized by equigravimetric amounts of protamine. Protamine effectively neutralized aprosulate when an intravenous injection of aprosulate was followed by an equigravimetric intravenous injection of protamine as measured using the Heptest® or thrombin time (Hoppensteadt et al., 1991; Kijowski et

al., 1994). It has also been demonstrated that protamine dose-dependently neutralizes the antithrombotic activity of aprosulate as measured in a rat jugular vein clamping model of thrombosis. A 2.5 fold gravimetric excess of protamine was required to completely neutralize the antithrombotic activity (Raake et al., 1993). The anticoagulant activity of aprosulate was not effected by high concentrations of the native heparin inhibitor, platelet factor 4 (Klauser et al., 1991).

The mechanism of action of aprosulate has been examined in several studies. Aprosulate was seen to inhibit approximately 50 % of thrombin activity in the presence of plasma when 5 to 20 μg/mL of the agent was used. If purified antithrombin III was substituted for the plasma, aprosulate was completely ineffective at inhibiting thrombin. This is in contrast to heparin which was observed to be more potent in the presence of purified antithrombin III. To further examine aprosulate's interaction with antithrombin III, the APTT was measured with and without the presence of rabbit anti-antithrombin III antibodies. Addition of the antibodies had no effect on the prolongation of the APTT by aprosulate whereas heparin's ability to prolong the APTT was almost completely neutralized in the presence of the antibodies (Klauser et al., 1991). Aprosulate is able to dose-dependently inhibit thrombin when incubated with purified heparin cofactor II (Hoppensteadt et al., 1988; Jeske et al., 1993b). This was seen to be a slow reaction, with 15 minutes of incubation needed to achieve full inhibition. Ofosu et al. demonstrated that aprosulate was able to inhibit intrinsic prothrombin activation by a mechanism similar to that of dermatan sulfate, via heparin cofactor II. Even at high concentrations, aprosulate was unable to prevent the onset of tissue factor dependent factor X and factor

V activation. The lag time for intrinsic prothrombin activation was, however, increased by aprosulate. Though several bis-aldonic acid amides were shown to release tissue plasminogen activator in an isolated pig ear perfusion model, aprosulate did not (Klocking et al., 1991).

Aprosulate is an effective antithrombotic agent *in vivo* in several animal models. In the Harbauer model (Harbauer et al., 1984) of venous thrombosis in rabbits, 1 mg/kg intravenously administered aprosulate was observed to almost completely prevent thrombosis. The same effect was observed two hours post-subcutaneous administration of the same dose. This effect was stronger than that seen with an equigravimetric dose of heparin (Raake et al., 1989). A significant inhibition of thrombus formation in this model was seen to last at least six hours following subcutaneous administration. In a rat jugular vein clamping model (Raake et al., 1989), aprosulate was observed to be an effective antithrombotic agent (Raake et al., 1991). A dose of 1 mg/kg was effective in preventing thrombosis. With increasing doses, an increased number of clampings was required to elicit thrombus formation. Aprosulate was less potent than heparin in preventing thrombosis in this model. Aprosulate has been shown to be effective in an arterio-venous shunt model of thrombosis (Sugidachi et al., 1993). The hemorrhagic effect of aprosulate has been examined in a rat tail transection model. Antithrombotically effective doses of aprosulate were not associated with significant bleeding risks.

Aprosulate has been administered to humans in two phase I clinical trials. In the first trial, ascending doses from 0.25 to 2.0 mg/kg were administered to healthy individuals on alternate days. Both the APTT and the Heptest® were dose-dependently

elevated. No effect was observed on the thrombin time. There was no indication of a thrombocytopenic response during this study (Papoulias et al., 1993). In the second trial, three doses of aprosulate were studied in a repeated administration protocol for a period of one week. In this study, an increase in plasma TFPI antigen levels was observed following each administration of aprosulate. The TFPI antigen level was observed to correlate with the plasma aprosulate levels (Jeske et al., 1995).

3. GL-522-Y-1

GL-522-Y-1 is a synthetic sulfonated calix[8]arene which exhibits weak antithrombotic properties. This compound does not have a saccharidic backbone like heparin, yet maintains a degree of antithrombotic activity. The chemical structure of GL-522-Y-1 is depicted in Figure 4. Unlike heparin, GL-522-Y-1 exhibits its anticoagulant activity in global clotting assays only at very high concentrations. Additionally, negligible activity is observed in chromogenic antithrombin and anti-factor Xa assays at these concentrations (Jeske et al., 1992). GL-522-Y-1 exhibits no affinity to antithrombin III and only a weak affinity for heparin cofactor II. This compound does inhibit factor Xa and thrombin generation as measured in amidolytic, fibrinogen deficient plasma systems. GL-522-Y-1 is a protease generation inhibitor following both intrinsic and extrinsic pathway activation. This inhibition was observed to be more potent than that of either pentasaccharide or aprosulate. Additionally, this inhibition was observed at lower concentrations than were required to activate heparin cofactor II. The mechanism by which this agent achieves this inhibition is not known.

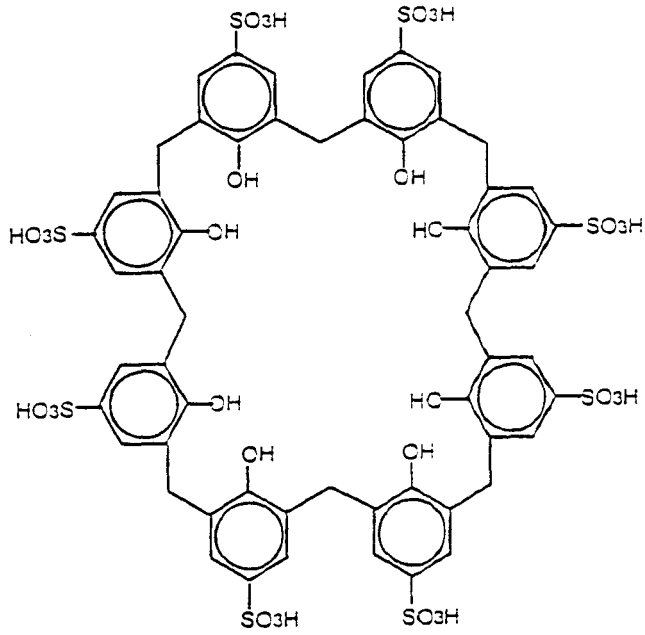


Figure 4 depicts the chemical structure of GL-522-Y-1. This agent is not saccharidic in nature but rather is a cyclic polymer of phenol residues alternately separated by a methyl group. The charge density of this agent is derived from sulfone groups rather than sulfate groups as found in heparin. The molecular weight of this agent is 1488 Da.

GL-522-Y-1 has been shown to be effective as an antithrombotic agent in the rabbit stasis thrombosis model (Jeske et al., 1993a). This agent was effective following both intravenous and subcutaneous administration (Lee et al., 1993). GL-522-Y-1 was also observed to be effective in a laser injury model of thrombosis following intravenous and oral administration (Giedrojc et al., 1993). The antithrombotic activity following oral administration was observed to have a duration of six hours. Administration of GL-522-Y-1 to primates (*Macaca fascicularis*) resulted in an increase in plasma functional TFPI levels (Jeske et al., 1993b).

G. Factor Xa Inhibitors

1. DX-9065a

The synthetic factor Xa inhibitor DX-9065a is currently being investigated. This amidinonaphthalene derivative exhibits high specificity for factor Xa over thrombin and other coagulation proteases. The k_i for factor Xa was determined to be 41 nM versus 2.3 μM for plasma kallikrein, 21 μM for t-PA, 23 μM for plasmin, and $>$ 2000 μM for thrombin (Hara et al., 1994). Trypsin is also inhibited by DX-9065a. Both free factor Xa as well as factor Xa found in the prothrombinase complex on platelets was inhibited by DX-9065a. Moreover, this inhibition has been shown to be independent of antithrombin III. DX-9065a demonstrates several advantages over other heparin-like antithrombotics. First, this agent is reported to be absorbed orally. It has been shown that the PT, APTT, and anti-Xa activity in blood samples drawn 30 to 240 minutes post-oral administration to rats are dose-dependently increased. Second, as DX-9065a does not directly inhibit

thrombin and has minimal effects on platelet response to known aggregants, almost no effect was observed on the bleeding time in rats (Tanabe et al., 1993). This compound has also been examined for its ability to abrogate disseminated intravascular coagulation (DIC) induced by endotoxin or thromboplastin administration to rats (Yamazaki et al., 1994). In both models, oral administration of DX-9065a inhibited the reduction of platelet counts and fibrinogen levels characteristic of DIC.

2. Antistasin

Antistasin is a 119 amino acid protein which has been isolated from the saliva of the leech *Haementaria officinalis* (Nutt et al., 1988). This protein is a slow, tight-binding inhibitor of factor Xa with an estimated dissociation rate constant of 0.3 nM (Dunwiddie et al., 1989). Antistasin is a selective inhibitor of factor Xa, with no observed inhibition of thrombin at concentrations 1000 fold higher than that of thrombin (Vlasuk et al., 1991; Ohta et al., 1994). Antistasin inhibits factor Xa in a manner similar to other serine protease inhibitors in which a rigid, substrate-like reactive site is presented to the enzyme (Hoffman et al., 1992). Studies have shown that antistasin has a specific and saturable binding site for heparin (Manley et al., 1992; Brankamp et al., 1992). In contrast to LMWHs, antistasin is capable of dose-dependently prolonging the APTT, suggestive of an ability to inhibit prothrombinase bound factor Xa (Vlasuk et al., 1991).

Antistasin has been studied in several animal models for its *in vivo* antithrombotic effects. In a rabbit model, antistasin was observed to reduce angiographic

restenosis following balloon angioplasty (Ragosta et al., 1994). In a dog model, co-administration of recombinant antistasin with tPA dramatically reduced the time to reperfusion (Mellott et al., 1992). In a monkey model of mild DIC, 1 mg/kg recombinant antistasin was shown to be comparably effective to 1000 U/kg unfractionated heparin in reducing fibrinopeptide A generation. This effect was observed to have a duration of at least 5 hours (Dunwiddie et al., 1992). Platelet and fibrinogen deposition onto a Dacron graft in a femoral arteriovenous shunt can be completely inhibited by antistasin infusion (Schaffer et al., 1992). In models of thrombosis, recombinant antistasin was observed to be as effective as conventional heparin therapy in preventing venous thrombosis (Vlasuk et al., 1991). Additionally, antistasin is effective in arterial models of thrombosis where heparin has limited effects (Hauptmann et al., 1993). While template bleeding times are not increased by antistasin administration (Schaffer et al., 1992), its clinical utility may be limited by the generation of neutralizing antibodies following repeated administration (Dunwiddie et al., 1992).

CHAPTER II

STATEMENT OF PURPOSE

It is the purpose of this dissertation to test the hypothesis that antithrombin III and heparin cofactor II are not the sole mediators of the anticoagulant and antithrombotic actions of heparin.

Heparin has long been used clinically for the prevention and treatment of thrombosis. In more recent times, the use of heparin has expanded to include treatment of angina and myocardial infarction and is used extensively in cardiovascular surgery and invasive cardiologic procedures such as coronary angioplasty. In addition, depolymerized forms of heparin known as low molecular weight heparins have been developed for a variety of thrombotic indications. It has been initially established that the plasma serine protease inhibitor antithrombin III is required for the mediation of the anticoagulant activity of heparin (Abildgaard, 1968). The activities of other plasma serine protease inhibitors are also known to be modulated by heparin.

To study the role of endogenous serine protease inhibitors in the mediation of heparin's anticoagulant and antithrombotic activities, a systematic approach using synthetic heparin analogues is utilized. These analogues offer the advantage of being chemically homogeneous substances. As these agents exhibit varying degrees of

interaction with antithrombin III and heparin cofactor II, they produce a more specific and targeted range of functional properties inherent to heparin.

For this dissertation, three low molecular weight analogues of heparin have been selected for both the biochemical and pharmacologic studies. These include a heparin pentasaccharide, a sulfated bis-lactobionic acid amide (aprosulate), and a cyclic aromatic polysulfonate derivative (GL-522-Y-1). Their activities in biochemical and biologic assays are compared with those of unfractionated heparin.

Pentasaccharide, which mimics the minimal sequence in heparin that binds with high affinity to AT-III, has been selected for study in this dissertation as it selectively interacts with ATIII. This molecule has been shown to exhibit relatively little interaction with HCII. This agent, produced synthetically through a collaboration between Sanofi Recherche, France and Organon, Oss, the Netherlands, is a potent catalyst for the AT-III mediated inhibition of factor Xa. Unlike the larger heparin chains of which it is normally a part, this molecule is too small to catalyze thrombin inhibition. Despite its different mechanism of action, pentasaccharide exhibits potent antithrombotic actions in animal models.

Aprosulate is a synthetic polyanion developed by Luitpold-Pharma (Munich, Germany) whose backbone is saccharidic in nature though structurally dissimilar to that of heparin and pentasaccharide. This agent has been selected for study as it has been shown to lack the sulfate conformation required to activate AT-III, but to potently catalyzes the HCII mediated inhibition of thrombin. Aprosulate has also been shown to be an effective antithrombotic agent in animal models.

GL-522-Y-1 has been developed by Genelabs, Inc., Redwood City, CA, as an antithrombotic agent. GL-522-Y-1 has been selected for study as it is structurally distinct from the other polyanions chosen. GL-522-Y-1 is not saccharidic in nature and derives its polyanionic character from sulfonate rather than sulfate groups. Despite these chemical differences, GL-522-Y-1 has been shown to potentiate thrombin inhibition via HCII. Like aprosulate, this agent has been shown to be unable to catalyze the AT-III mediated inhibition of either factor Xa or thrombin.

Physical characterization of these agents has been performed using gel permeation chromatography (GPC), nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS). GPC analysis provides data concerning molecular mass and molecular weight distribution of each agent. This is particularly important for unfractionated heparin as it is a mixture of various size glycosaminoglycan chains. NMR and mass spectral analysis of these compounds has provided additional information on the structural and molecular characteristics of these agents.

The *in vitro* biochemistry of the synthetic heparin analogues has been defined using a variety of assays. These included a battery of clotting and amidolytic antiprotease assays designed to examine various steps of the coagulation cascade. The effects on the intrinsic pathway are investigated using the activated partial thromboplastin time (APTT). Effects on the extrinsic pathway are investigated using the prothrombin time (PT). The common pathway of coagulation is iinvestigated using the thrombin time, Heptest®, and amidolytic anti-factor IIa and anti-factor Xa assays.

Interactions of the various analogues with the endogenous SERPINs is examined

using amidolytic assays in which the ability of each agent to mediate inhibition of factor Xa or thrombin via antithrombin III or heparin cofactor II activation is examined in a concentration dependent manner.

Protease generation inhibition assays provide a useful set of data to explain the mechanism of action of the synthetic analogues as well as to determine the relative importance of heparin cofactor II and antithrombin III activation for the inhibition of these processes. Plasmatic and defined non-plasmatic amidolytic assay systems are utilized for these purposes. Non-plasmatic systems provide the opportunity to study the effect of each agent on protease generation in the absense of ATIII and HCII.

As platelets play an important role in the hemostatic process (forming the primary hemostatic plug, catalyzing several reactions of the coagulation cascade, releasing procoagulant or heparin neutralizing compounds), the effects of the synthetic analogues on various platelet functions are also investigated. Agonist specific platelet aggregation inhibition is assessed by determining the individual modulatory profile of these agents in platelet rich plasma. The potential for each of these analogues to produce platelet activation in a heparin induced thrombocytopenia screening assay is also investigated.

The *in vivo* pharmacology of the heparin analogues is examined using valid models of thrombosis and bleeding. The activity of each agent is determined in a dose dependent manner in order to compare the potency of each agent. Two models of thrombosis are utilized. In the rabbit model of stasis thrombosis, thrombus formation is induced by the administration of activated coagulation factors and a stasis of blood flow in a segment of jugular vein. In the rat model of jugular vein clamping, thrombus

formation is triggered by vascular damage. The rat model offers the advantage of maintaining blood flow through the damaged area over the experimental course. The rabbit model offers the advantage of allowing the drawing of several blood samples over the course of the experiment for *ex vivo* analysis of coagulation parameters. By measuring various SERPIN mediated events (anti-IIa activity, anti-Xa activity, APTT, PT) *ex vivo* in blood samples taken during the thrombotic models, the relative importance of these actions to producing the antithrombotic state can be more clearly elucidated.

In the rabbit ear bleeding model, the number of red blood cells lost in a given period of time is quantitated and used as a measure of hemorrhagic potential in terms of a bleeding index.

The effects measured in these *in vivo* models are compared to heparin cofactor II and antithrombin III mediated activities, activity in *in vitro* systems, and *ex vivo* clotting activities. Since these analogues are structurally defined, their actions are presented in terms of molar dosage and a comparison of the activities can be readily achieved.

Tissue factor pathway inhibitor (TFPI) has been shown to be an important mediator of the pharmacologic actions of heparin. The effect of the heparin analogues on plasma TFPI levels has been assessed in several animal models. An assay to measure functional TFPI levels in rabbit samples obtained during the stasis-thrombosis experiments has been optimized. Additionally the effect of intravenous administration of pentasaccharide and GL-522-Y-1 on TFPI levels in non-human primates is also assessed. The effect of aprosulate on plasma TFPI levels has been assessed in human volunteers

as part of two phase I clinical studies.

By characterizing the actions of the selected heparin analogues in parallel with unfractionated heparin, the importance of SERPIN activation for the antithrombotic and bleeding effects of these agents is determined. The data obtained from the current research provids a defined biochemical and pharmacologic basis for understanding the molecular mechanisms by which heparin mediates its antithrombotic actions and bleeding effects.

CHAPTER III

MATERIALS AND METHODS

A. Materials

1. Unfractionated Heparin

Unfractionated porcine mucosal heparin (lot H410) was obtained from Sanofi Pharma, Paris, France. The molecular weight of this heparin was approximately 10.5 kDa. The potency of this preparation was 160 IU/mg when cross referenced to International Standard #3. The heparin was provided as a white powder and was stored in a desiccator at room temperature. Stock solutions were made in physiologic saline as needed. Unused solutions were stored at 1 °C and were discarded after two weeks. The molecular profile of this heparin is depicted in Appendix 1. Product specifications are listed in Appendix 2.

2. Pentasaccharide

The pentasaccharide representing the minimal antithrombin III binding sequence (O-(2-deoxy-2-sulfamido-6-O-sulfo-α-D-glucopyranosyl)-(1-4)-O-(ß-D-glucopyranosyluronic acid)-(1-4)-O-(2-deoxy-2-sulfamido-3,6-di-O-sulfo-α-D-glucopyranosyl)-(1-4)-O-(2-O-sulfo-α-L-idopyranosyluronic acid)-)1-4)-2-deoxy-2-

sulfamido-6-O-sulfo-D-glucopyranose decasodium salt (SR90107A lot JMS07046) was obtained from Sanofi Pharma, Paris, France. The molecular weight of this pentasaccharide is 1.724 kDa. The potency of this compound was designated at 650 aXa units/mg in human plasma. Stock solutions were made in physiologic saline as needed. Unused portions of the solutions were frozen at -70 °C for future use. The molecular profile of this heparin is depicted in Appendix 1. Product specifications are listed in Appendix 2.

3. Aprosulate

Aprosulate, lot 90416/7/72, is a sulfated bis-lactobionic acid amide (Hexadecasodium trimethylenebis-[4-O-ß-D-galactopyranosyl-D-gluconoylamino octasulfate]) obtained from Luitpold-Pharma, Munich, Germany. Its molecular weight is 2.388 kDa. The potency of aprosulate was determined to be 4.6 USP units/mg using the USP XXII method. Purity of this substance was determined to be greater than 99.7 %. It was provided as a colorless powder whose solubility in water was determined to be up to 60 %. This agent is not soluble in methanol, ethanol, or acetone. Stock solutions were made in physiologic saline as needed. Unused solutions were stored at 1 °C and were discarded after two weeks. The molecular profile of this heparin is depicted in Appendix 1. Product specifications are listed in Appendix 2.

4. GL-522-Y-1

GL-522-Y-1, a sulfonated calix[8]arene, (Lot Sabinsa #4) was obtained from

Genelabs Inc. (Redwood City, CA). This compound has a molecular weight of 1.488 kDa. Purity was determined to be greater than 99 % by reverse-phase ion pairing HPLC following two recrystallizations. The solubility of GL-522-Y-1 in water is greater than 1000 mg/mL. The material was provided as a white powder which was reconstituted in physiologic saline as needed. Unused solutions were stored at 1 °C and were discarded after two weeks. The molecular profile of this heparin is depicted in Appendix 1. Product specifications are listed in Appendix 2.

5. Reagents

a. Enzymes

Human thrombin (Fibrindex®) was purchased from Ortho Diagnostics (Raritan, NJ). The lyophilized material was reconstituted in physiologic saline (Baxter Healthcare Corp., Deerfield, IL) prior to use with the remaining material frozen in aliquots at -70 °C. Bovine factor Xa was obtained from Enzyme Research Laboratories (South Bend, IN) and was stored in aliquots at -70 °C. This material was reconstituted in Tris buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA; pH = 8.4 at 25 °C) immediately prior to use.

b. Substrates

Chromogenic substrates, Spectrozyme TH (HDCT-Ala-Arg-pNA) and Spectrozyme FXa (CH_3OCDHG-Gly-Arg-pNA), were obtained from American Diagnostica (Greenwich, CT). They were reconstituted in deionized water to

concentrations of 1.0 and 2.5 mM, respectively, for use in the amidolytic antiprotease assays.

c. Clotting Assays

Reagents for the APTT, 0.025 M $CaCl_2$ and a micronized silica based APTT reagent, were purchased from Organon Teknika (Parsippany, NJ). The APTT reagent was reconstituted by the addition of 5 mLs of sterile, deionized water. 0.025 M $CaCl_2$ was used at 37 °C. Once reconstituted, the APTT reagents were refrigerated (4 °C) for periods of up to one week.

Thromboplastin C®, used in the PT and protease generation assays, was obtained from Baxter Healthcare, Dade Division (Miami, FL). ISI = 2.69. Each bottle was reconstituted with 20 mLs of sterile, distilled water, gently shaken, and allowed to sit for 20 minutes prior to use. Once reconstituted, the thromboplastin reagent was refrigerated (4 °C) for periods of up to one week.

Reagents for the Heptest®, bovine FXa and Recalmix®, were purchased from Haemochem (St. Louis, MO). Both were obtained in lyophilized form and each vial was reconstituted with 2 mLs of deionized water prior to use. Recalmix®, an optimized mixture of $CaCl_2$ and brain cephalin in a bovine plasma fraction was used at 37 °C. Bovine factor Xa was used at room temperature. Once reconstituted, the Heptest® reagents were refrigerated (4 °C) for periods of up to one week.

Purified human AT-III was obtained from Kabi Vitrum (Stockholm, Sweden). It was stored in aliquots of 500 U/mL at -70 °C and was diluted with physiologic saline

to obtain desirable concentrations immediately prior to use.

Purified HC-II (lot 1180) was obtained from Diagnostica Stago (Gennevieres, France) in lyophilized form. Each vial contained 100 μg of HC-II purified from human plasma and was reconstituted with 200 μL of physiologic saline to make a 500 μg/mL solution. Biologic activity was designated as 1.6 U/vial.

Tris (hydroxymethyl)aminomethane (Trizma base and Trizma HCl) was purchased from Sigma (St. Louis, MO). Buffers of various pHs and osmolality were prepared as described in Methods.

Fibrinogen deficient plasma was purchased from George King Biomedical (Overland Park, KS). Fibrinogen levels were less than 25 mg/dL.

Actin®, activated cephaloplastin reagent, was purchased from Baxter Healthcare, Dade Division (Miami, FL). Once reconstituted, the Actin® reagent was refrigerated (4 °C) for periods of up to one week.

Konyne® brand of prothrombin complex concentrate was purchased from Cutter Laboratories (Berkeley, CA). This reagent contains purified human coagulation factors II, VII, IX, and X in a stabilized matrix.

FEIBA® brand of activated prothrombin complex concentrate was purchased from Immuno, AG (Vienna, Austria) in lyophilized form. Each bottle was reconstituted with 20 mLs of sterile water prior to use with any remaining portions frozen in aliquots of 25 U/mL at -70 °C. This complex concentrate contains purified human coagulation factors II, VIIa, IX, and X in a stabilized matrix.

d. Anesthetics

Ketaset®, ketamine hydrochloride, was purchased from Aveco Co. Inc. (Fort Dodge, IA) as a 100 mg/mL injectable solution. Rompun®, xylazine, was purchased from Mobay Corporation (Shawnee, KS) as a 100 mg/mL injectable solution.

Beuthanasia®-D, a solution of sodium pentobarbital and phenytoin sodium, was obtained from Schering-Plough Animal Health, Kenilworth, NJ.

e. Other agents

Physiologic saline, obtained from Baxter Healthcare (Deerfield, IL), contained 308 mOsmol/L NaCl at a pH of 5.5. Sterile irrigation water prepared by distillation was obtained from Baxter Healthcare Corp., Deerfield, IL.

Celite coagulation tubes were purchased from International Technidyne (Edison, NJ). Each evacuated tube contained 12 mg of diatomaceous earth and was designed to activate 2 mLs of blood.

Mineral oil was obtained from Sigma (St. Louis, MO).

Albumin was obtained from Sigma (St. Louis, MO).

Sodium sulfate was purchased from Mallinkrodt (Paris, KY).

6. Major Instrumentation

Beckman DU®-7 spectrophotometer (Beckman Instruments Inc., Fullerton CA); ACL 300 Plus fast kinetics coagulation analyzer (Coulter, Hialeah, FL); fibrometers® (Becton Dickinson and Co, Rutherford, NJ); Multistat III centrifugal analyzer (Instrumentation Laboratory, Lexington, MA); Thrombelastograph CTEG model #3000

(Hellige GmbH, Freiburg, Germany); ACT machine (International Technidyne, Edison, NJ), model 801; Light microscope (model BH-2, Olympus); HPLC (Waters, Lexington, MA), consisting of a VAX 3100 computer, a LAC/E interface module, two model 510 HPLC pumps, a 712 WISP autoinjector, a model R401 differential refractometer, and a model 484 tunable absorbance detector; platelet aggregometer (BioData Corporation, Hatboro, PA) and an IBM compatible personal computer linked to a Hewlett Packard (San Diego, CA) Laserjet IIIsi printer for wordprocessing, statistical analysis, and graphics were used in these studies.

7. Animals

Male, White New Zealand rabbits (2.5 to 3.5 kg, 10 to 12 weeks of age) obtained from LSR Industries Inc. (Union Grove, WI) were housed in individual cages in the Animal Research Facility of Loyola University Medical Center (AAALAC accredited effective 3/9/93; PHS Animal Welfare Assurance ID # A 3117-01). The rabbits were fed a diet of standard rabbit chow and had free access to water. The ambient temperature was kept at 65 °F with approximately 45 % humidity. The animals were kept on a 12 hour light/dark cycle with the light period beginning at 7:00 a.m. All experiments were performed during the animals light cycle. Rabbits were anesthetized during all procedures with ketamine (50 mg/kg intramuscularly) and xylazine (25 mg/kg intramuscularly). Beuthanasia®-D, Schering-Plough Animal Health, Kenilworth, NJ was administered at a dose of 0.1 mL/kg for euthanasia.

Male Sprague-Dawley rats (300 to 400 g, 73 to 121 days in age) were obtained

from Harlan Industries, Indianapolis, IN and were housed two per cage in the Animal Research Facility of Loyola University Medical Center (AAALAC accredited effective 3/9/93; PHS Animal Welfare Assurance ID # A 3117-01). The rats were fed a diet of standard rat chow and had free access to water. The animals were kept on a 12 hour light/dark cycle with the light period beginning at 7:00 a.m. All experiments were performed during the animals light cycle. The rats were anesthetized with an intraperitoneal injection of urethane (15 % in physiologic saline) for all procedures. Rats were euthanized with a 0.1 mL/kg intravenous injection of Beuthanasia®-D.

B. Physical Characterization of Agents

1. Molecular Weight Profile by Gel Permeation Chromatography (GPC)

Calibration curves made using three different types of calibrators were prepared by running the appropriate compounds on a Waters 845 GPC-HPLC system (Millipore-Waters, Lexington, MA, U.S.A.) equipped with Expert Ease® software designed for polymer analysis. Each calibrator was dissolved in 0.5 M Na_2SO_4 to give a final concentration of 10 mg/mL. The HPLC system consisted of a VAX 3100 computer (Digital Corp.), a LAC/E interface module, two Waters model 510 HPLC pumps, a Waters model 712 WISP autoinjector, a model 401 differential refractometer (RI), and a Waters 484 tunable absorbance detector. The UV and the RI detector were linked in series, with the outlet end of the columns attached to the UV detector.

20 μL of each sample was injected onto a tandem column system (TSK 2000 /

TSK 3000, Tosoh Haas, Tokyo, Japan) for analysis. The mobile phase, 0.5 M Na_2SO_4, was pumped through the columns at a flow rate of 0.5 mL/minute. All analyses were made at room temperature. The run time for each sample was set at 65 minutes. UV determinations were made at 234 nm. The system was equilibrated each morning using freshly degassed mobile phase until a stable baseline was obtained.

Nineteen narrow range calibrators of known molecular weight were supplied by Choay Laboratories, Roen, France. They were prepared by fractionation of heparin on Ultragel AcA44 agarose acrylamide (LKB-Produkteur AB) gel permeation chromatographic columns. The narrow range fractions were further analyzed for their molecular weights by running them through TSK G2000 SW and TSK 3000 SW analytical gel permeation columns which had been calibrated against other well defined reference standards. The weights of these fractions were found to be: 22,500; 17,300; 14,800; 12,900; 11,500; 10,100; 8,650; 7,540; 6,670; 6,150; 5,730; 5,360; 5,000; 4,530; 4,000; 3,410; 2,440; 1,880 and 1,320 Da. These molecular weights were confirmed by viscometry, LALLS, or sedimentation analysis.

The log molecular weight of each calibrator was plotted against its retention time. Using the Expert Ease® software, a third order polynomial regression equation was fitted to the data points to give an expression in the form :

$$\log \text{M.W.} = D_0 + D_1(RT) + D_2(RT)^2 + D_3(RT)^3$$

where M.W. = calculated molecular weight and

RT = sample retention time

D_0, D_1, D_2, and D_3 = coefficients calculated in the curve fitting process

The molecular weight profile of each agent was determined based on this calibration curve. The molecular weight profile consisted of the parameters weight average molecular weight, number average molecular weight, peak molecular weight, and dispersity.

2. ^1H and ^{13}C Nuclear Magmetic Resonance (NMR) Spectra

Proton (^1H) NMR spectra of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were detected with an AC300 or AMX 500 NMR spectrometer (Bruker, Germany) at either 300 or 500 MHz in collaboration with Professor B. Casu (Ronzoni Institute, Milan, Italy). Carbon 13 (^{13}C) spectra were detected using an AC300 NMR spectrometer at 75 MHz. For this analysis, the samples were dissolved in 99.9% deuterium oxide at concentrations varying from 1 to 10 % depending upon the sample and the magnetic field. For aprosulate and heparin, spectra were obtained at a temperature of 313 K. The spectra of GL-522-Y-1 were obtained at 300 K while those of pentasaccharide were obtained at 298 K. Proton spectra were made at 300 MHz and ^{13}C spectra were made at 75 MHz. All chemical shifts were given in ppm downfield from at internal sodium-3-(trimethylsilyl)-propionate standard.

3. Mass Spectral Analysis

Mass spectra of aprosulate and GL-522-Y-1 were obtained using a Finnigan

MAT900 2-sector mass spectrometer equipped with an Antek cesium ion gun. For the analysis, an aliquot of the sample was dissolved in 1 μL of solvent and added to 1 μL of matrix on a probe tip. The matrix for these analyses was triethanolamine. The probe tip was subsequently inserted into the ion source of the mass spectrometer. The sample was ionized by liquid secondary ion mass spectrometry. All data was collected in the negative ion mode.

C. *In vitro* Biochemical and Pharmacologic Profile

The comparative *in vitro* biochemical and pharmacologic analysis of heparin and its synthetic analogues was carried out utilizing biochemically defined, normal human pooled plasma and normal rabbit pooled plasma based assays. In addition, protease generation assays were carried out utilizing fibrinogen deficient plasma and activated and non-activated prothrombin complex concentrate supplemented systems. The modulatory actions of each of these agents were investigated utilizing endothelial cell cultures and platelet rich plasma harvested from citrated whole blood obtained from human volunteers.

1. SERPIN Activity Assays

a. Antithrombin III (AT-III)

The ability of an agent to mediate antiprotease activity via AT-III was measured utilizing an amidolytic substrate assay run on the ACL-300+ fast kinetics analyzer. Test agents were diluted in saline and placed in sample cups such that the final assay

concentrations ranged from 0 to 40 μM. 12.5 μL of a 1.25 U/mL human AT-III solution was added to the sample cups such that the final concentration of AT-III in the assay was 0.0625 U/mL. The first reagent consisted of 1.25 U/mL thrombin or 0.625 nkat/mL factor Xa in Tris buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH = 8.4 @ 25 °C). The second reagent consisted of 0.5 mM Spectrozyme TH or 1.25 mM Spectrozyme FXa. The instrument was programmed such that 100 μL of sample and 100 μL of reagent 1 were placed into individual compartments in a reaction rotor. These reagents were incubated at 37 °C for 1 minute and were then mixed together. 50 μL of reagent 2 was then added to the rotor and the reaction rotor was spun at 1200 r.p.m. Optical density readings at 405 nm were made continuously for all cells for 60 seconds. The data was downloaded for analysis to an IBM PC containing the Instrument Laboratories research program. A printout of the change in optical density with time was obtained. Percent inhibition and maximal reaction rates were determined.

Potency evaluations were made by comparing IC_{50} values for each agent in both systems. IC_{50} values were calculated in the following manner: Data from each individual run was plotted as percent inhibition relative to saline versus concentration (μM). Regression analysis was performed on the linear portion of each curve. The agent concentration resulting in 50 % maximal inhibition was extrapolated from the best fit line. The mean and standard deviation of the extrapolated IC_{50}'s was determined. Statistical differences between the respective IC_{50}'s were determined by one way ANOVA followed by the Newman-Keuls test.

b. Heparin Cofactor II (HC-II)

The ability of an agent to mediate antiprotease activity via HC-II was measured utilizing an amidolytic substrate assay run on the ACL-300+ fast kinetics analyzer. Agents were diluted in saline and placed in sample cups such that the final assay concentrations ranged from 0 to 40 μM. 4.2 μL of a 500 μg/mL human HC-II solution was added to the sample cups such that the final concentration of HC-II in the assay was 8.4 μg/mL. The first reagent consisted of human thrombin diluted to 2.08 U/mL with Tris buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH = 8.4 @ 25 °C). The second reagent consisted of 0.625 mM Spectrozyme TH. The instrument was programmed such that 100 μL of sample and 50 μL of reagent 1 were placed into individual compartments in a reaction rotor. These reagents were incubated at 37 °C for 1 minute and were them mixed together. 100 μL of reagent 2 was then added to the rotor and the reaction rotor was spun at 1200 r.p.m. Optical density readings at 405 nm were made continuously for all cells for 60 seconds. The data was downloaded for analysis to an IBM PC containing the Instrument Laboratories research program. A printout of the change in optical density with time was obtained. Percent inhibition and maximal reaction rates were determined.

Potency evaluations were made by comparing IC_{50} values for each agent. IC_{50} values were calculated in the following manner: Data from each individual run was plotted as percent inhibition relative to saline versus concentration (μM). Regression analysis was performed on the linear portion of each curve. The agent concentration resulting in 50 % maximal inhibition was extrapolated from the best fit line. The mean

and standard deviation of the extrapolated IC_{50}'s was determined. Statistical differences between the respective IC_{50}'s were determined by one way ANOVA followed by the Newman-Keuls test.

2. Anticoagulant Profile

a. Activated Partial Thromboplastin Time (APTT)

The APTT is a global clotting assay which is used to measure inhibition of coagulation factors in the intrinsic pathway and is commonly used to monitor heparin therapy. The assay was performed in the following manner. 100 μL of APTT reagent was added to 100 μL of test plasma and was incubated for 5 minutes at 37 °C. Clotting time was measured using a Fibrometer® (BBL, Cockeysville, MD) following the addition of 100 μL of prewarmed 0.025 M $CaCl_2$. Measurement of clotting time was stopped at 300 seconds as clotting times beyond 300 seconds were outside of the linear range of the instrument.

b. Heptest®

The Heptest® (Haemochem, St. Louis, MO) is an assay which is used to measure clotting times after the addition of purified bovine factor Xa. The assay was performed in the following manner. 100 μL of plasma was added to 100 μL of bovine factor Xa and was incubated for 2 minutes at 37 °C. Clotting time was measured with a Fibrometer® (BBL, Cockeysville, MD) after the addition of 100 μL of prewarmed Recalmix®. Measurement of clotting time was stopped at 300 seconds as clotting times

beyond 300 seconds were outside of the linear range of the instrument.

c. 5 U Thrombin Time (5 U TT)

The 5 U thrombin time measures the time needed to convert fibrinogen to fibrin by preformed thrombin. The assay was performed in the following manner. 200 μL of plasma was equilibrated to 37 °C for 3 minutes. The clotting time was measured with a Fibrometer® (BBL, Cockeysville, MD) following the addition of 100 μL of human thrombin. This thrombin had previously been calibrated to 5 U/mL by adjusting its concentration such that the clotting time of pooled normal human plasma was 18 to 20 seconds. Measurement of clotting time was stopped at 300 seconds as clotting times beyond 300 seconds were outside of the linear range of the instrument.

d. Prothrombin Time (PT)

The PT is a global clotting test which is used to measure inhibition of coagulation factors in the extrinsic pathway (FVIIa and FXa). This assay is commonly used to monitor oral anticoagulant therapy. The assay was performed in the following manner. 100 μL of plasma was incubated at 37 °C for 3 minutes. Clotting time was measured using a Fibrometer® (BBL, Cockeysville, MD) following the addition of 200 μL of prewarmed Thromboplastin C® (Dade, FL). Measurement of clotting time was stopped at 300 seconds as clotting times beyond 300 seconds were outside of the linear range of the instrument.

3. Amidolytic Antiprotease Assays

a. Anti-thrombin assay (Anti-IIa)

Residual thrombin activity was measured using a amidolytic substrate based assay on a Beckman spectrophotometer. Anticoagulant present in the plasma sample would inhibit a given portion of the available added thrombin. Any remaining thrombin would cleave the amidolytic substrate and release p-nitro aniline. The reaction was followed by monitoring the change in the amount of free chromophore (pNA) at an optical density of 405 nm. The assay was performed in the following manner. 400 μL of buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH = 8.4 @ 25 °C) and 25 μL of the test plasma was prewarmed for 1 minute at 37 °C in a quartz cuvette. 25 μL of 10 U/mL thrombin was then added. Thrombin for this assay was calibrated to give an optical density change of 0.65 to 0.75 mau/min in a saline control. Following an incubation time of exactly 1 minute, 50 μL of a 1 mM solution of the amidolytic substrate Spectrozyme TH was added and the change in optical density was measured for 1 minute. Percent inhibition was determined in relation to unsupplemented baseline plasma using the following formula:

% I = {(delta O.D. baseline - delta O.D. sample) / delta O.D. baseline} * 100 %

b. Anti-factor Xa assay (Anti-Xa)

Residual factor Xa activity was measured using a chromogenic substrate assay developed by Hoppensteadt et al. (Hoppensteadt et al., 1985). Anticoagulant present in

plasma may inhibit a given fraction of added factor Xa. Any residual factor Xa was able to cleave an amidolytic substrate to release p-nitro aniline. The reaction was monitored by measuring the change in the amount of free chromophore (pNA) by measuring the change in optical density at 405 nm. The assay was performed in the following manner. 375 μL of buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH = 8.4 @ 25 °C) was incubated with 25 μL of the plasma sample for 2 minutes at 37 °C. 50 μL of bovine FXa was added. Prior to running the assay, an aliquot of factor Xa was reconstituted in reaction buffer such that in a saline control a change in absorbance of 0.65 to 0.75 mau/min was observed. Following a 2 minute incubation at 37 °C, 50 μL of Spectrozyme FXa (2.5 mM) was added and the change in optical density at 405 nm was measured for 1 minute. Percent inhibition was determined in relation to unsupplemented baseline plasma using the following formula:

% I = {(delta O.D. baseline - delta O.D. sample) / delta O.D. baseline} * 100 %

4. FVIII:C assay

Factor VIII coagulant (FVIII:C) activity was determined using an amidolytic assay (Coatest Factor VIII, USA Helena Laboratories, Beaumont, TX) in which a purified FVIII solution (4 U/mL) was supplemented with the agent being studied over a concentration range of 0 to 100 μM. 300 U of lyophilized FVIII (Armour, Kankakee, IL) was reconstituted with 15 mLs of physiologic saline and was stored in 0.5 mL aliquots at -70 °C. Immediately prior to the experiment, 0.2 mLs of the 20 U/mL aliquot was

diluted with 0.8 mL of Owren's Veronal Buffer (pH = 7.35 ± 0.1; American Dade, Aguada, Puerto Rico). 40 μL of each sample was incubated with 420 μL of buffer (0.05 M Tris + 0.2 % bovine albumin; pH = 7.3) and 200 μL of a solution containing factor IXa, factor X, and phosphatidylserine for 5 minutes. 100 μL of $CaCl_2$ (0.025 M) was added and the solution was incubated for another 5 minutes. 200 μL of the factor Xa substrate, Spectrozyme Xa (2.7 mM) was added and the change in absorbance at 405 nm was measured for 60 seconds. Percent inhibition was determined in relation to a saline control using the following formula:

% I = {(delta O.D. baseline - delta O.D. sample) / delta O.D. baseline} * 100 %

5. Protease Generation Assays

a. Fibrinogen Deficient Plasma

Inhibition of thrombin and factor Xa generation was measured utilizing an amidolytic substrate method (Kaiser et al., 1992) run on the ACL-300+ fast kinetics analyzer. Agents were diluted in saline to the appropriate concentrations and placed in the sample cup carousel. Reagent position 1 contained fibrinogen deficient plasma diluted 1:8 in Tris buffer (pH = 8.5, 100 mM Tris base adjusted with HCl). Reagent position 2 contained Actin (Dade,, Miami, FL) diluted 1:1 with Spectrozyme TH or Spectrozyme FXa (1 mM made in 0.025M $CaCl_2$) for measuring intrinsic IIa and Xa generation or Thromboplastin C® diluted 1:6 with the chromogenic substrates for measuring extrinsic thrombin and FXa generation. 60 μL of sample and 60 μL of reagent 1 were pipetted

into the rotor and were incubated at 37 °C for 5 minutes. 60 μL of reagent 2 was pipetted into the rotor and the rotor was spun at 1200 r.p.m. 1040 optical density measurements were made over a period of 999 seconds for each cuvette. This data was downloaded to an IBM PC for analysis by the IL research program. Kinetics curves were generated. Percent inhibition was determined according to the following formula:

% I = {(delta O.D. baseline - delta O.D. sample) / delta O.D. baseline} * 100 %

b. Prothrombin Complex Concentrate Based (Konyne) System

Thrombin and FXa generation inhibition were measured in a non-plasma system in which a prothrombin complex concentrate provides the necessary coagulation factors for the reaction in the absence of plasma. This system used Konyne® brand of prothrombin complex concentrate (Cutter Laboratories, Berkeley, CA), containing factors II, VII, IX, and X. This amidolytic assay was run on the Multi-stat III centrifugal analyzer. The reagents were added to the rotor in the following order. 25 μL of the drug dilution (0 to 100 μM in saline) and 140 μL of buffer (Thromboquant aPTT buffer, Boehringer Mannheim, GmbH, Mannheim, Germany, lot 1313205) was added to the outer well of the reaction rotor. 10 μL of the same buffer and 25 μL of Konyne® (10 U/mL) were added to the inner well of each cuvette. 25 μL of Thromboplastin C® (Dade, FL) diluted 1:10 with 0.025 $CaCl_2$ was added to the outer well. Immediately before the rotor was placed in the instrument, 25 μL of Spectrozyme TH or FXa made at 2.5 mM in deionized water was added to the outer well. The rotor was incubated for 30 seconds

at 37 °C before optical density readings at 405 nm were made. The rotor was spun at 600 r.p.m. Optical density was read once every 60 seconds for a period of twelve minutes. Data printouts contained optical density readings at each time point as well as the overall optical density change. The data was plotted as kinetic curves (Δ O.D. vs. time) and as % inhibition relative to saline control vs. concentration (μM). Percent inhibition is calculated as above.

c. Activated Prothrombin Complex Concentrate (FEIBA®) System

Thrombin and FXa generation inhibition were measured in a non-plasmatic system in which an activated prothrombin complex concentrate provided the necessary coagulation factors for the reaction in the absence of plasma. This system used FEIBA® brand of prothrombin complex concentrate containing factors II, VIIa, IX, and X. This was an amidolytic assay run on the Multi-stat III centrifugal analyzer. The reagents were added in the following order. 25 μL of the drug dilution (0 to 100 μM in saline) and 140 μL of buffer (Thromboquant aPTT buffer, Boehringer Mannheim, GmbH, Mannheim, Germany, lot 1313205) was added to the outer well of the reaction rotor. 10 μL of the same buffer and 25 μL of FEIBA® (10 U/mL) were added to the inner well of each cuvette. 25 μL of Thromboplastin C® (Dade, FL) diluted 1 : 10 with 0.025 $CaCl_2$ was added to the outer well. Immediately before the rotor was placed in the instrument, 25 μL of Spectrozyme TH or FXa made at 2.5 mM in deionized water was added to the outer well. The rotor was incubated for 30 seconds at 37 °C before optical density readings (405 nm) were made. The rotor was spun at 600 r.p.m. Optical density was

read once every 60 seconds for a period of twelve minutes. Data printouts contained optical density readings at each time point as well as the overall optical density change. The data was plotted as kinetic curves (Δ O.D. vs. time) and as % inhibition relative to saline control vs. concentration in μM.

6. Endothelial Cell Culture System

Rabbit aortic endothelial cells were grown to confluence in F12 culture medium supplemented with fetal calf serum. Confluence was achieved after approximately 2 weeks. Immediately prior to the experiment, serum factors were washed out of the cultures with fresh F12 medium. The experimental media consisted of 900 μL of F12 medium + ^{35}S (made by adding 125 μL ^{35}S to 20 mLs of F12 medium) and 100 μL of the stock polyanion solution. Stock solutions of 0.001 to 1.0 mg/mL for the various polyanions were utilized. Controls were treated with 100 μL of F12 medium in place of the polyanion solution. The experimental media remained with the cells for a period of 20 hours at which time the media was removed and the cells were washed. Both the cells and the culture media were frozen for future analysis.

To analyze the newly synthesized glycosaminoglycans in the media and the cells, cells were removed from the freezer and 250 μL of a Superase solution was added to degrade the cellular proteins. The cells were stirred and scraped into a tube containing 250 μL of Tris HCl buffer and 10 μL of a non-labelled chondroitin sulfate + heparan sulfate + dermatan sulfate standard. In separate tubes, 100 μL of the media + 10 μL Superase + 10 μL of the standard solution were added. These solutions were incubated

overnight. Heparan sulfate, chondroitin sulfate and dermatan sulfate were separated by agarose gel electrophoresis in PDA buffer and visualized by toluidine blue staining. Each gel was run for 30 minutes. Newly synthesized heparan and chondroitin sulfate in each sample was quantitated using scintillation counting of the respective bands.

7. *In Vitro* Supplementation to Freshly Drawn Human Blood

Each heparin analogue was supplemented to freshly drawn human blood at a final concentration of 0.25 μM. Stock solutions of each agent were prepared at 2.5 μM in physiologic saline. 500 μLs of each stock solution was placed in individual polypropylene syringes. Blood was drawn from normal human volunteers via the antecubital vein using a double syringe technique. After discarding the initial 2 mLs of blood, blood was drawn to the 5 mL mark in the syringes containing the test agent. These syringes were gently inverted to mix the blood and test agent. Celite and saline activated clotting times were performed to determine the anticoagulant activity of each agent. TEG analysis was also performed to assess the ability of each agent to effect clot formation.

8. Effect of Heparin Analogues on Platelet Function

a. Agonist Induced Platelet Aggregation

The effects of the synthetic heparin analogues on platelet function were assessed by platelet aggregometry. In the first set of experiments, blood was drawn from 10 volunteers (5 males and 5 females) using a double syringe technique to avoid

contamination by tissue factors released upon venipuncture. Standard venipuncture was performed using a 21 gauge Butterfly® (3/4 x 12" tubing) infusion set (Abbott Hosp. Inc., North Chicago, IL). 9 mL of blood was drawn into a 10 mL polyethylene syringe. The blood was immediately transferred to plastic test tubes containing 1 mL of 3.8% sodium citrate. The blood was mixed by gentle inversion. Platelet rich plasma (PRP) was prepared by spinning the citrated blood at 800 r.p.m. for 15 minutes at room temperature. PRP was carefully removed and kept in capped plastic tubes. The remaining blood was spun further at 1200 x g for 15 minutes to obtain platelet poor plasma (PPP). PPP was used to blank the aggregometer (BioData Corporation, Hatboro PA). Aggregations were performed in the following manner. 450 μL of platelet rich plasma was pipeted into the aggregometer tubes and allowed to incubate at 37 °C for 2 to 4 minutes to assure that the platelets do not self aggregate. 50 μL of agonist was pipetted into each tube. The agonists which were tested included ADP (1.15 and 0.58 μg/mL), collagen (0.8 μg/mL), epinephrine (10 μg/mL), arachidonic acid (300 μg/mL), and human thrombin (1 U/mL). All agonist concentrations are final assay concentrations. Platelet rich plasma was supplemented with each analogue at a concentration 10 μM. The aggregation profile was analyzed according to two parameters: maximum percent aggregation and slope. Saline supplementation was used as a control.

b. Heparin Induced Thrombocytopenia Screening

The synthetic analogues were also analyzed for their ability to promote heparin-induced thrombocytopenia (HIT) according to the method of Brace (Brace et al., 1990).

290 μL of PRP and 160 μL of heat inactivated HIT serum were warmed in a cuvette for 1 to 2 minutes at 37 °C. 5, 10, or 20 μL of a 1 mg/mL solution of the analogue being tested was then added. The aggregation response was monitored for 30 minutes. The inactivated HIT serum was made by allowing the blood of known heparin-induced thrombocytopenic individuals to clot. The clotted blood was then centrifuged and the serum drawn off and aliquoted for use in this screening.

D. *In vivo* Pharmacologic studies:

The *in vivo* pharmacologic studies were carried out in established models of rabbit stasis thrombosis and rabbit ear bleeding, and a rat jugular vein clamping model of thrombosis. Both intravenous and subcutaneous dosing protocols were utilized. To measure the pharmacodynamics of parenterally administered heparin and its analogues, whole blood investigations employing ACT and TEG were performed. In addition, *ex vivo* analysis of blood samples included global anticoagulant profiling and the measurement of functional TFPI.

1. Rabbit Stasis Thrombosis Model

The modified stasis thrombosis model of Fareed (Fareed et al., 1985) was used to study the *in vivo* antithrombotic effects of the synthetic heparin analogues. Male white New Zealand rabbits (2.5 to 3.5 kg) were administered Ketaset® (ketamine hydrochloride) at a dose of 50 mg/kg and Rompun® (xylazine) at a dose of 25 mg/kg intramuscularly to induce anesthesia. When the rabbits appeared too lightly anesthetized

during the procedure, an additional injection of Ketaset® (25 mg/kg) was given. A scalpel, forceps, and cautery were used to isolate the jugular veins from the facia while causing minimal trauma to the vessels. A 2 cm segment of each jugular vein, including the bifurcation, was isolated. The right carotid artery was cannulated for the purpose of obtaining blood samples. When long circulation times were employed, baseline samples were drawn via the medial ear artery. Heparin analogues were administered either subcutaneously in the abdominal region or intravenously via the marginal ear vein. 7.5 U/kg FEIBA® was administered via the marginal ear vein as a thrombogenic challenge. The FEIBA® was allowed to circulate for exactly 20 seconds before the jugular vein segments were ligated to induce stasis. After 10 minutes of stasis time, the left vein segment was excised, opened, and the clot graded according to the following scale. +0 is scored when the blood in the segment is completely unclotted. +1 is scored when a small number of microscopic clots are present in largely unclotted blood. +2 is scored when a larger number of small clots are present. +3 is scored when a single large clot is present with few unclotted blood cells. +4 is scored when a solid clot with no unclotted blood is present. The right jugular segment was removed after 20 minutes of stasis time and the clots were graded using the above scale. One blue top tube (4.5 mLs blood in 0.5 mLs of 3.8 % sodium citrate) and one red top tube (3 mLs whole blood) were drawn at baseline, post-drug, and 6 minutes post-FEIBA®. The blue top tube was spun at 1200 x g for 20 minutes to obtain platelet poor plasma. This plasma was aliquoted and frozen at -70 °C for future analysis using the PT, APTT, TT, Heptest®, anti-IIa, anti-Xa, and functional TFPI assays. The blood saved in a red top tube was

analyzed immediately for activated clotting time and by thrombelastography. 5 rabbits per drug per dose were used. Controls were run using saline. Following each experiment, the rabbit was euthanized with an intravenous injection of 0.1 mL/kg Beuthanasia®-D.

2. Rabbit Ear Bleeding Model

The rabbit ear bleeding model (Cade et al., 1984) was used to evaluate the hemorrhagic potential of each agent. A white New Zealand male rabbit was anesthetized with 25 mg/kg Rompun® (xylazine) and 50 mg/kg Ketaset® (ketamine hydrochloride) intramuscularly. The rabbit's ear was immersed in a physiological saline bath kept at 37 °C. Using transillumination, an area was selected which was free of major blood vessels. A #20 Bard-Parker scalpel blade (Becton Dickinson AcuteCare, Franklin Lakes, NJ) was used to make 5 uniform, full thickness incisions through the ear. The ear was immediately re-immersed in the saline bath for 10 minutes. After 10 minutes, the saline bath was collected and bottled. The red blood cells in each sample were counted using a Hycel red cell counter. The rabbit ear was bandaged as necessary and the rabbit was allowed to recover in its cage for minimally one week prior to use in the rabbit stasis thrombosis model.

3. Rat Jugular Vein Clamping Model of Thrombosis

Male Sprague-Dawley rats were anesthetized using an i.p. injection of 1 mL/100 g body weight of a 15 % solution of urethane (Raake et al., 1989). The skin on the neck

was shaved and an incision was made centrally above the trachea. The right jugular vein was isolated and covered with ultrasound transmission gel. A bi-directional Doppler probe was used to measure blood flow through the vessel. The jugular vein was clamped using a mosquito forceps for 1 minute and then released in order to measure flow. Clamping of the blood vessel caused endothelial damage and initiated clot formation. Blood flow was measured for five minutes between clampings. This process was repeated until no flow was measured at five minutes post-clamping. The effectiveness of the antithrombotic agent was determined by the number of clampings required to cause vascular occlusion.

4. *Ex Vivo* Analysis of Anticoagulant Activity

a. Activated Clotting Time (ACT)

2 mLs of blood freshly drawn via carotid catheter was added to blood coagulation tubes containing 12 mg celite to activate clotting. The tube was agitated vigorously for a few seconds and immediately inserted into a Hemochron® test well. The time taken for the blood to clot was displayed digitally on the face of the instrument.

b. Thrombelastography (TEG)

The TEG automatically recorded viscoelastic changes in a sample of whole blood as the sample clotted. The resultant profile was a measure of the kinetics of clot formation. This analysis was sensitive to all of the interacting cellular and plasmatic components in a given sample of blood that may affect the rate or structure of the

clotting sample. The procedure was as follows. A clean cup and pin were placed in the instrument and were maintained at 37 °C. Three minutes after being drawn, 360 μL of the blood was placed in the test cup and the pin was lowered into the cup. A thin layer of mineral oil was carefully placed on the blood surface to prevent drying. As a clot forms, the deflection of the pin as it moves in the cup was recorded. Four parameters were measured from the profile: R-time, RK-time, maximum amplitude, and divergence angle. R-time was measured as the distance in millimeters from the start of the tracing until there was a 2 mm divergence. This was the point of reproducible clot formation. RK-time was the distance in millimeters from the beginning of the tracing until a 20 mm divergence was reached. This was the time to a standard clot firmness. The maximum amplitude corresponded to the maximum shear modulus of the clot. Divergence angle was measured by drawing a tangent line from the point of initial divergence along the maximum curvature of the tracing. The size of the angle was directly related to the kinetics of clot formation.

5. *Ex Vivo* Analysis of Functional TFPI in Rabbits

Levels of TFPI activity in rabbit plasma samples were measured using an amidolytic assay based on that of Sandset (Sandset et al., 1987). In this assay, 300 to 400 μL of test plasma was heated in a plastic test tube at 56 °C in a water bath for 15 minutes to inactivate endogenous coagulation enzymes and to precipitate fibrinogen. Following heating, the samples were cooled for 1 minute on ice and then centrifuged for 10 minutes at 1200 x g. The supernatant plasma was diluted 1 to 20 in TFPI buffer (0.1

M NaCl, 0.05 M Tris HCl, 0.01 M trisodium dicitrate; pH = 8.0). These dilutions were then kept on ice until assayed. The following reagents were placed into each reaction tube: 50 μL of 0.025 U/mL human factor VII, 50 μL of Thromboplastin C+ diluted 1 to 20 in TFPI buffer, 50 μL of 0.025 U/mL human factor X, and 50 μL of 0.075 M $CaCl_2$. Each tube was vortexed and incubated at 37 °C. After 5 minutes, 50 μL of the plasma dilution was added to each tube. After vortexing, the tubes were incubated for 40 minutes at 37 °C. 50 μL of 0.4 U/mL factor X was added to each tube. Following vortexing, the tubes were incubated for 20 minutes at 37 °C. 50 μL of 3.25 mM Spectrozyme Xa was added to each tube. Following a 15 minute incubation period, 200 μL of 50 % acetic acid to each tube was added to stop the amidolytic reaction. 200 μL of each sample was pipetted onto a microtiter plate and the optical density was read at 410 nm. Percent inhibition was measured relative to the appropriate baseline sample.

F. Other *In Vivo* Animal Models

Primate studies, using *Macaca fascicularis* and *Macaca mulatta*, provided the opportunity to measure TFPI antigen levels utilizing a modified ELISA technique.

1. Effect of GL-522-Y-1 on TFPI Levels in Non-human Primates

Three male Cynomolgus monkeys (*Macaca fascicularis*), weighing 2.5 to 5.0 kg, received 10 mg/kg GL-522-Y-1 intravenously via the cephalic vein. Blood was drawn via a 12 inch catheter placed in the saphenous vein under ketamine (10 mg/kg) anesthesia using a double syringe technique. Following the draw, blood was placed into a tube

containing 3.8 % sodium citrate. The final ratio of blood to citrate was 9:1. Within 30 minutes of collection, the blood sample was centrifuged to make platelet poor plasma. The sample was aliquoted and frozen at - 70 °C. Samples were collected at baseline, 5, 15, 30, 60, 120, 240, and 360 minutes post-administration. TFPI levels were measured using functional and immunologic assays.

2. Effect of Pentasaccharide on TFPI Levels in Non-human Primates

Three monkeys (*Macaca mulatta*), anesthetized with 10 mg/kg ketamine and ranging in weight from 7 to 13 kg, received pentasaccharide at doses of either 100, 250, or 500 μg/kg intravenously via the saphenous vein. Blood was drawn using a double syringe technique via a butterfly needle placed in the saphenous vein. Following the draw, the blood was placed into a tube containing 3.8 % sodium citrate. The final ratio of blood to citrate was 9:1. The blood was kept on ice until centrifugation to make platelet poor plasma. The plasma samples were aliquoted and frozen at - 70 °C. Samples were collected at baseline and at 5 and 60 minutes post-administration. TFPI levels were measured using an immunologic assay.

G. Human Trials with Aprosulate

In conjunction with two phase I studies carried out in collaboration with Luitpold-Pharma, TFPI levels were measured after aprosulate administration. To relate the TFPI levels with the anticoagulant effects, Heptest® and APTT were also measured.

1. Dose Finding Study with Bis-Lactobionic Acid Amide - Phase I (DELPHI)

Plasma samples used in this study were obtained in the DELPHI phase-I clinical trial of aprosulate conducted by Inveresk Clinical Research, Ltd. (Edinburgh, Scotland) under the sponsorship of Luitpold Pharma GmbH (Munich, Germany). This study was performed for the purpose of assessing the local and systemic tolerance of different doses of aprosulate after subcutaneous application and to measure the kinetics of this agent as well as any changes in coagulation parameters (Papoulias et al., 1993).

The DELPHI study was an open, non-randomized tolerance study in which twelve normal, healthy male volunteers received increasing dosages of aprosulate over a period of 16 days. On day 0 of the study, screenings were performed to confirm the eligibility of the volunteers. On Day 1 of the study, each volunteer received an injection of placebo in order to assess the normal circadian variations in each person's baseline measurements. Aprosulate dosing began on day 2 with a dose of 0.25 mg/kg. Blood samples for the determination of coagulation and clinical chemistry parameters were drawn 1, 2, 4, and 10 hours post-administration. On the ensuing washout day, blood samples were obtained at 4 and 10 hours. Increasing doses of aprosulate (0.5, 0.75, 1.0, 1.5, and 2.0 mg/kg) were subsequently administered on alternate days, separated by intervening washout days.

Blood samples were collected in citrated tubes (1 part 3.8 % citrate to 9 parts whole blood) and centrifuged within 20 minutes at 2000 x g for 20 minutes at 4 to 8 °C. Plasma was separated, aliquoted, and frozen at -70 °C for future analysis. Aliquots were shipped to Loyola University Medical Center (Maywood, IL) on dry ice and were stored

at -70 °C. Samples were later thawed and batch analyzed for TFPI antigen levels.

2. Phase I-Study for the Assessment of the Laboratory Values after Repeated Daily Application of Aprosulate (PALLAS)

Plasma samples used in this study were obtained in the PALLAS phase-I clinical trial of aprosulate conducted by Inveresk Clinical Research, Ltd. (Edinburgh, Scotland) under the sponsorship of Luitpold Pharma GmbH (Munich, Germany). This study was performed for the purpose of assessing the effects of repeated administration of aprosulate on hematologic parameters.

Four dosing regimens were utilized in this study, with 6 healthy, human, male volunteers randomized into each group. The treatments included : 35 mg aprosulate b.i.d., 70 mg aprosulate b.i.d., 70 mg aprosulate o.d. + placebo (saline) o.d., and 40 mg Enoxaparin® o.d. + placebo. The average age for the treatment groups ranged from 25.50 $\pm$ 3.72 to 27.83 $\pm$ 3.24 years (mean $\pm$ S.E.M.). The difference in the ages of the groups was not statistically significant. The injectable drugs were administered subcutaneously every 12 hours for a duration of 7 days. Volunteers could be removed from the study prior to 7 treatment days according to preset safety criteria. Blood samples were drawn at baseline, 15, 30, 45, 60 minutes, 2, 4, 8, and 12 hours on days 1 and 7 of the study. Samples were drawn at 2 and 12 hours on days 2 and 3 and at 12 hours on days 4 through 6 of the study. The majority of the blood samples were drawn on the first and seventh days of the trial in order to determine the effects of repeated aprosulate administration on various hematologic parameters.

Blood samples were collected in citrated tubes (1 part 3.8 % citrate to 9 parts whole blood) and centrifuged within 20 minutes at 2000 x g for 20 minutes at 4 to 8 °C. Plasma was separated, aliquoted, and frozen at -70 °C for future analysis. Aliquots were shipped to Loyola University Medical Center (Maywood, IL) on dry ice and were stored at -70 °C. Samples were later thawed and batch analyzed for TFPI antigen levels.

a. ELISA TFPI Assay

TFPI antigen levels were determined using Imubind® TFPI ELISA kits (American Diagnostica, Greenwich, CT). This was a sandwich, enzyme-linked immunoassay which utilized a murine anti-TFPI monoclonal as the capture antibody and a biotinylated antibody / streptavidin conjugated horseradish peroxidase complex for detection of the captured TFPI. The detection limit of the kit was stated as 200 pg TFPI/mL. Six TFPI standards ranging in concentration from 0 to 10 ng/mL diluted in deionized water were used to construct a calibration curve. A full-length recombinant TFPI which was expressed in *E. coli* was used as the calibration standard. This rTFPI contained a full carboxy tail, but was lacking the native glycosylation. The molecular weight of the rTFPI was approximately 32 kDa. rTFPI was provided by Dr. T.C. Wun (Monsanto Inc, St. Louis, MO). Optical density readings and standard curve calculations were made using a Dynatech MR7000 system (Dynatech Laboratories, Inc, Chantilly, VA).

H. Data Processing and Statistical Analysis

The experimental data obtained in this dissertation is presented as the mean ± standard deviation for the biochemical results and mean ± SEM for the pharmacologic results in order to indicate the precision of the data and to facilitate the comparison between each agent. The results obtained from the biochemical experiments were analyzed using a repeated measures two way analysis of variance. Data which did not meet the criteria for parametric statistics (normality of distibution and equality of variance) were analyzed using the Friedman two way analysis of variance by ranks. This analysis was used to determine statistically significant differences between the effects of the different heparin analogues. If a significant difference was noted using the two way analysis of variance in a given assay system, the concentration response data for each individual agent was analyzed by one way analysis of variance followed by a post hoc Newman Keuls analysis to determine the statistical significance of the effect of each concentration relative to a vehicle control. Designations of potency in a given assay system such as IC_{50}, ED_{50}, etc. were compared using a one way analysis of variance with a post hoc Newman Keuls analysis. In all cases a p value < 0.05 was considered statistically significant. Degrees of freedom, F-ratios, and computed p values are presented for each analysis.

For the analysis of the data obtained from the stasis-thrombosis model, the Kruskal-Wallis H test with a post hoc Mann-Whitney U test was utilized. Standard parametric analysis of variance techniques were not applicable with this data because the clot scores assigned in this model are subjectively designated; there were not necessarily

equal differences between the numerical scores used to grade the clots.

CHAPTER IV

RESULTS

A. Physicochemical Characterization of Various Agents Used in this Research

The three synthetic analogues used in this investigation present diverse chemical and molecular characteristics. Unlike heparin, these agents are homogeneous and obtained by synthetic methods. Molecular mass distribution studies were carried out using HLPC. NMR analysis utilizing both ^{1}H and ^{13}C detection modes was performed on all agents. Since pentasaccharide and heparin represent multicomponenet agents, mass spectral analysis was only performed on GL-522-Y-1 and aprosulate.

1. High Performance Liquid Chromatography (HPLC) Profile

The molecular weights of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were determined by gel permeation chromatography using three different calibrations of the columns. Elution profiles of each agent are depicted in Appendix 2. The first calibration consisted of 19 heparin fractions with previously characterized molecular weights. The agent was detected in the mobile phase by either an ultraviolet or a refractive index detector. The results obtained with the UV detector are depicted in Table 10. The molecular weights of the synthetic analogues determined by HPLC were all lower than the known formula weights. The molecular weight of aprosulate was closest

to its true formula weight, lower by only 1.3 %. The molecular weights of GL-522-Y-1 and pentasaccharide were somewhat farther from their true weights. The molecular weight of heparin was calculated to be 10.5 kDa in this analysis. Dispersity, a measure of the homogeneity of the sample, was highest for heparin.

The higher dispersity of heparin was more dramatically seen in the data obtained with the nineteen calibrators using the refractive index detector. This is depicted in Table 11. The dispersities of the synthetic analogues were less than 1.1 whereas that of heparin was greater than 1.3. The molecular weights obtained with the RI detector were all lower than those obtained with the UV detector, and thus further from the known formula weights.

Two other calibrators developed for the determination of the molecular weight of low molecular weight heparins were also used to analyze the molecular weight profiles of these agents. The results of these analyses are depicted in Tables 12 and 13. GL-522-Y-1 was not analyzed with these calibrants due to the unusual elution profile it exhibited. The molecular weights of aprosulate and pentasaccharide were much different from their formula weights (> 25 %). The molecular weight determined for heparin with these two calibrants varied widely from 9.9 kDa with the HMC calibrant to 12.8 kDa with the F913B calibrant. Dispersity values were consistent with those obtained with the 19 heparin fraction calibrators. The dispersity of heparin was high in both cases, consistent with the polycomponent nature of heparin. The dispersity values for aprosulate and pentasaccharide were less than 1.1.

2. Nuclear Magnetic Resonance (NMR) Spectra

Figure 5 depicts the ^{13}C spectra of unfractionated heparin. This spectra contains signals typical of a heparin-like agent. Between 55 and 60 ppm are signals indicative of the presence of C-N bonds of glucosamine residues. Between 60 and 69 ppm are signals indicative of the presence of primary alcohol groupings of the aminosugar rings. From 70 to 80 ppm are signals from secondary alcohols on both the uronic and amino sugar rings. Anomeric carbon signals appear between 95 and 105 ppm. Methyl groups appear at 24 ppm and carboxyl signals are at 177 ppm. No extraneous signals from potential glycosaminoglycan contaminants such as dermatan sulfate or heparan sulfate are noted. By comparing the signal intensities for the glucuronic N-sulfate at carbon 2 and the glucuronic O-sulfate at carbon 3, it is possible to estimate the relative amount of high affinity ATIII binding sites present in a given sample of heparin. For this preparation, it is estimated that 7 % of the heparin chains contain the 3-O sulfate group required for high affinity ATIII binding. The ^{13}C signal assignments for unfractionated heparin are presented in Table 14.

By integrating the area under the peaks at 62 and 69 ppm under controlled conditions, it is possible to determine the ratio of 6-O-SO_3 to 6-OH. In this heparin, the glucosamine residues are 76 % 6-O sulfated. The ratio of glucosamine N-sulfate to glucosamine N-acetate is determined by comparing the integrals at 60 and 56 ppm, respectively. This heparin is 88 % N-sulfated by this analysis. The % total iduronic acid (sulfated and unsulfated) relative to total uronic acid is 71 %.

Figure 6 depicts the ^{1}H NMR spectrum of heparin. This spectrum contains

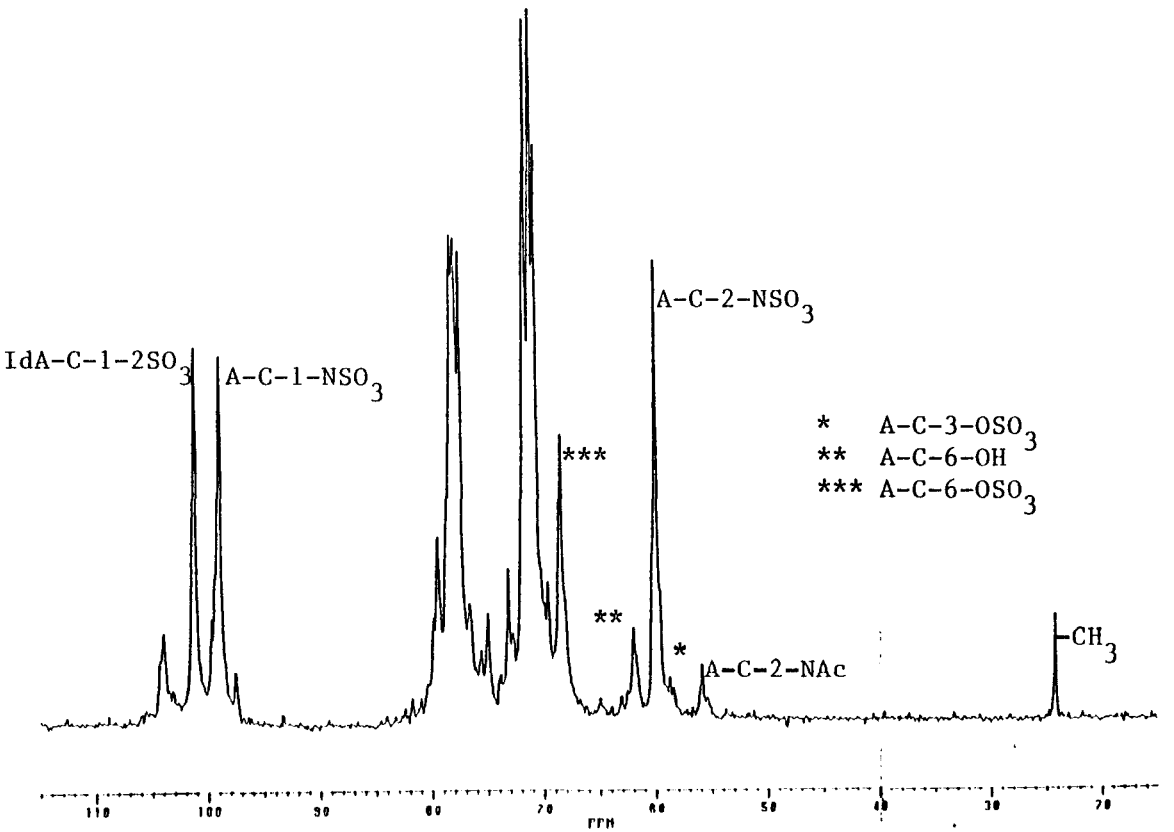


Figure 5. ^{13}C NMR spectrum of unfractionated heparin. This spectrum was determined on a Bruker AC300 NMR spectrometer at 75 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. Specific peak assignments are listed in Table 14. Analysis of the signal intensities for glucosamine N-sulfate (60 ppm) and glucosamine 3-O-sulfate (58.5 ppm) indicates that approximately 7 % of the heparin chains contain the 3-O sulfate group required for high affinity ATIII binding.

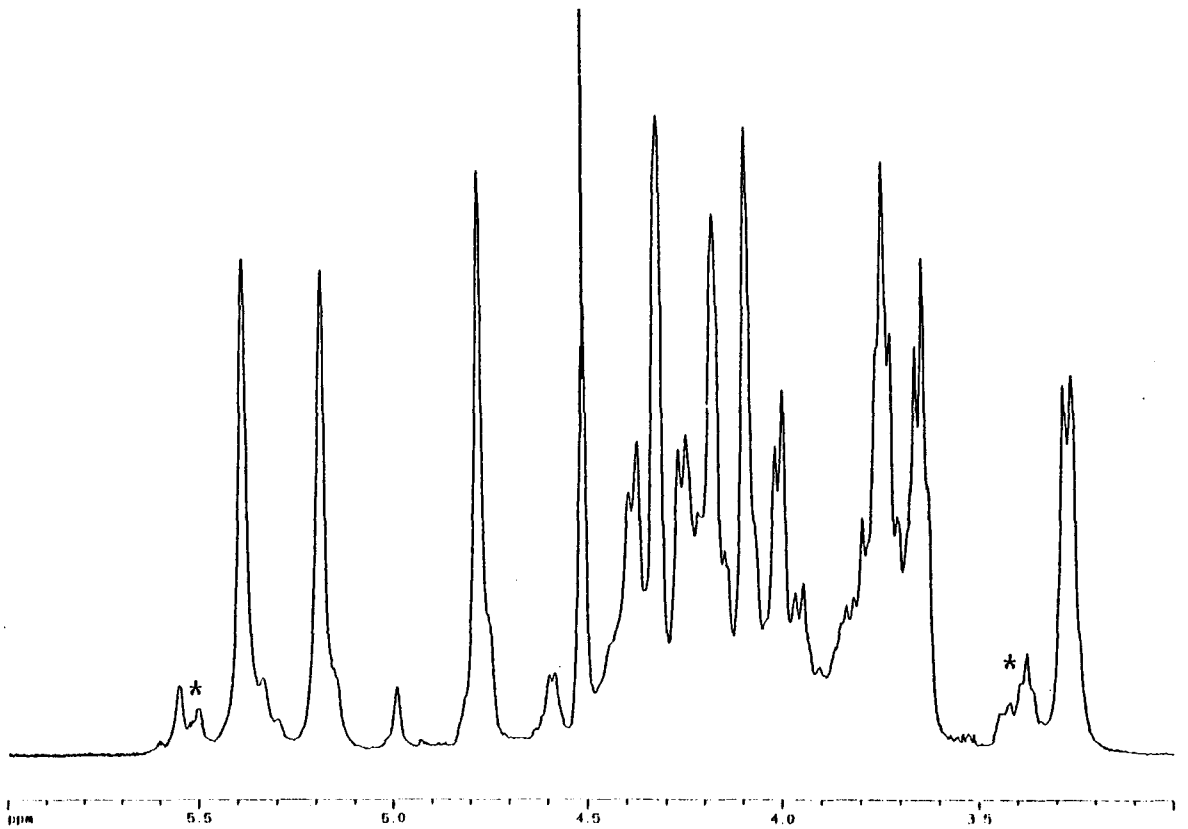


Figure 6. Proton NMR spectrum of unfractionated heparin. This spectrum was determined on an Bruker AMX500 NMR spectrometer at 500 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. The signal at approximately 4.5 ppm is that of residual protons in the deuterated solvent. Peaks labeled with an asterisk belong to the 3-O sulfated amino sugar required for high affinity ATIII binding. The peak at 5.5 ppm. is that of the proton on carbon 1 and the peak at 3.4 ppm is that of the proton on carbon 2.

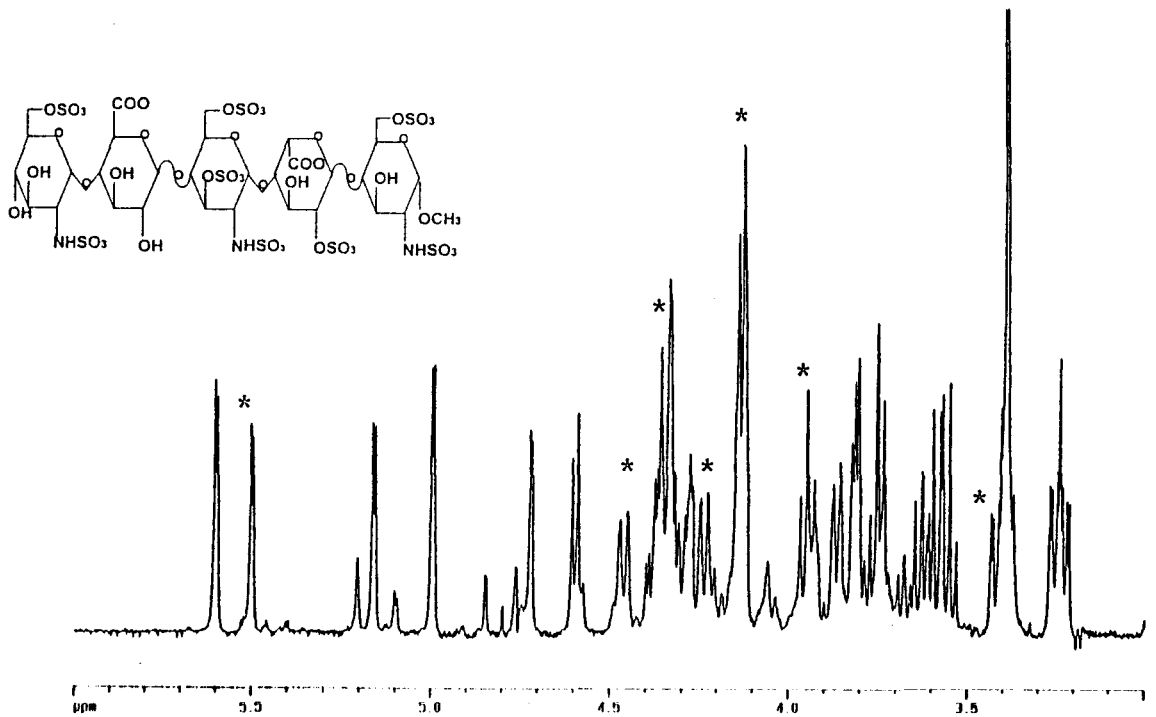


Figure 7. Proton NMR spectrum of the synthetic pentasaccharide. This spectrum was determined on an Bruker AMX500 NMR spectrometer at 500 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. The signal at approximately 4.8 ppm is that of residual protons in the deuterated solvent. No signs of impurities are evident. Peaks labeled with an asterisk belong to the 3-O sulfated amino sugar required for high affinity ATIII binding. Peak assignments are listed in Table 15.

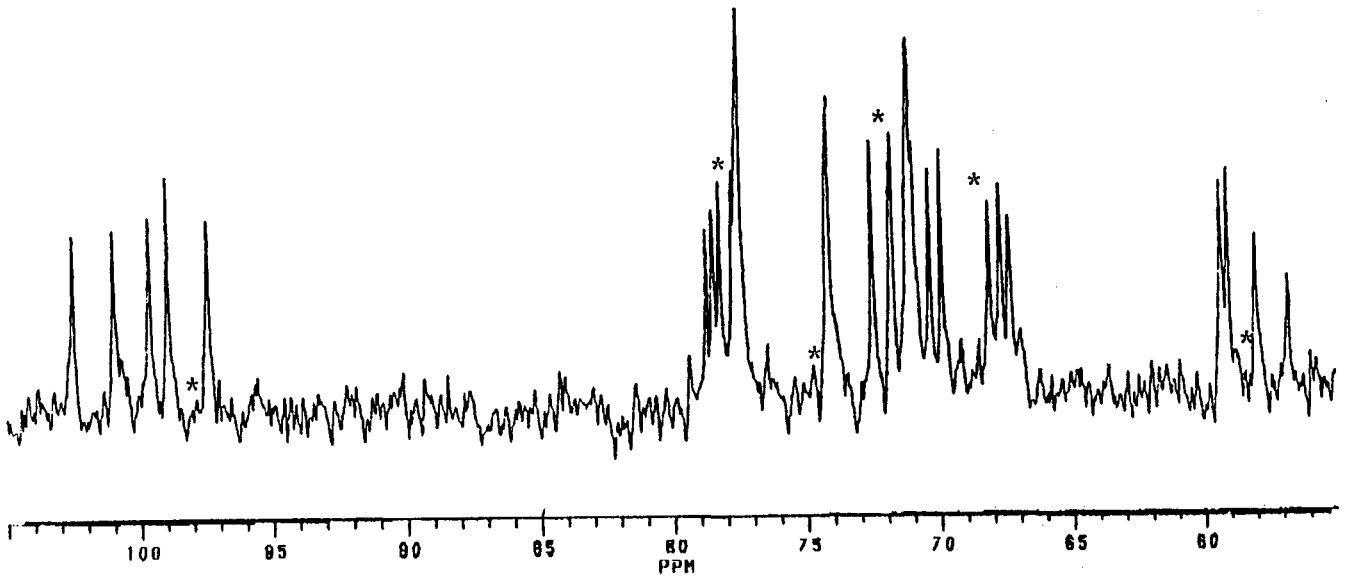


Figure 8. ^{13}C NMR spectrum of the synthetic methylated pentasaccharide. This spectrum was determined on a Bruker AC300 NMR spectrometer at 75 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. The labelled peaks represent the carbons of the 3-O sulfated glucosamine of pentasaccharide. C-1 = 98 ppm, C-2 = 59 ppm, C-3 = 78 ppm, C-4 = 75 ppm, C-5 = 72 ppm, C-6 = 68 ppm.

peaks typically associated with heparin. The assignments of some of the major peaks are found in Table 15. The strong peaks at 4.5 ppm is that of the solvent, HOD. Of note are two peaks labelled with an asterisk which derive from the protons attached to C-1 and C-2 of the 3-O sulfated glucosamine. The intensity of these peaks is markedly less than of the same peaks in the pentasaccharide spectrum.

Figure 7 depicts the ^{1}H NMR spectrum of the synthetic ATIII binding pentasaccharide. It is important to note the signals for the 3-O sulfated aminosugar residue required for high affininy ATIII binding which are labelled by asterisks in the figure. These chemical shifts are listed in Table 16.

Figure 8 depicts the ^{13}C NMR spectrum of the ATIII binding pentasaccharide. Most notable are the peaks labelled by asterisks which belong to the carbons of the 3-O sulfated glucosamine residue. These chemical shifts are listed in Table 17.

In the aprosulate molecule, there are eleven distinct protons on the sugar moieties, five are located on the open chain sugar and six are located on the closed ring sugar. Assignment of the proton spectra is made by homonuclear correlation. With this technique, magnetization transfer is limited to pairs of protons which are located on adjacent carbon atoms.

The ^{13}C spectra of aprosulate is depicted in Figure 10. Peaks belonging to each of the carbon atoms in the open and closed ring sugar moieties are present. No extraneous signals are observed, indicating that the material is pure. A table relating chemical shift to ^{13}C nucleus assignment is presented in Table 18.

In Figure 11, the ^{1}H spectra are placed on both the x and y axes. The series of

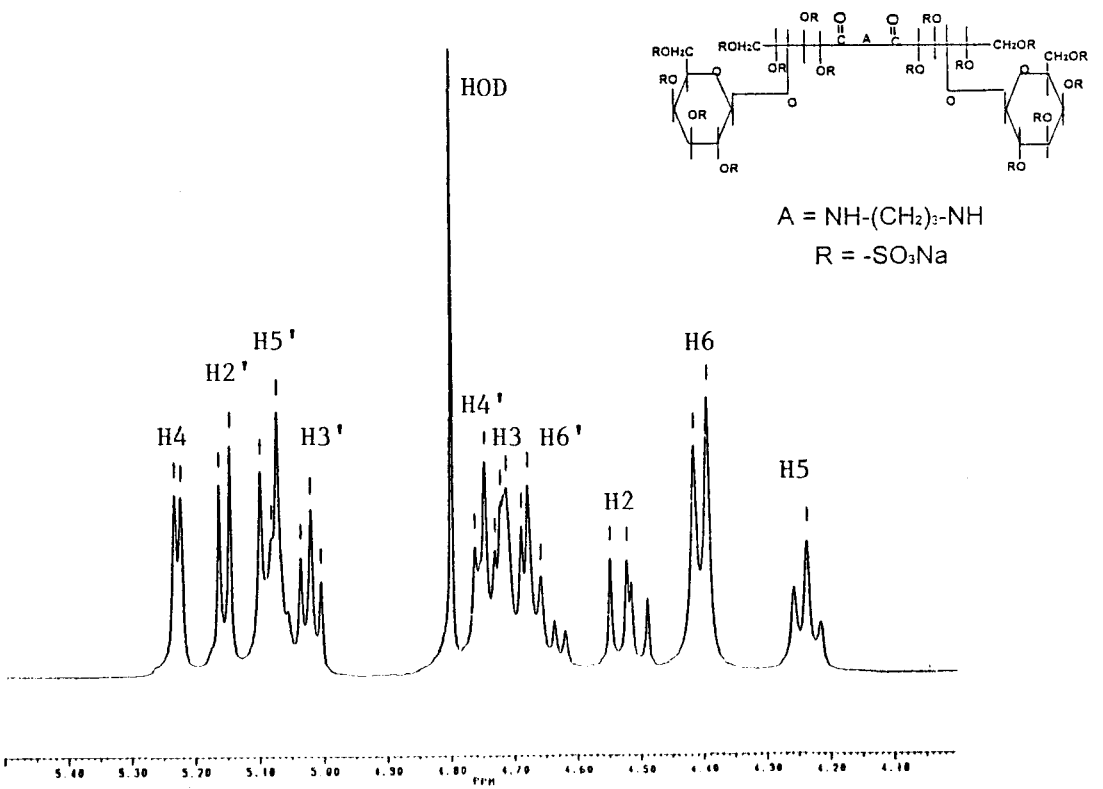


Figure 9. Proton NMR spectrum of aprosulate. This spectrum was determined at 300 Mhz using a Bruker AC300 NMR spectrometer. Peak assignments along with their associated chemical shifts relative to sodium-3-(trimethylsilyl)-propionate are tabulated in Table 17. The large signal at 4.8 ppm is that of residual protons in the deuterated solvent. No additional signals suggestive of contamination are observed.

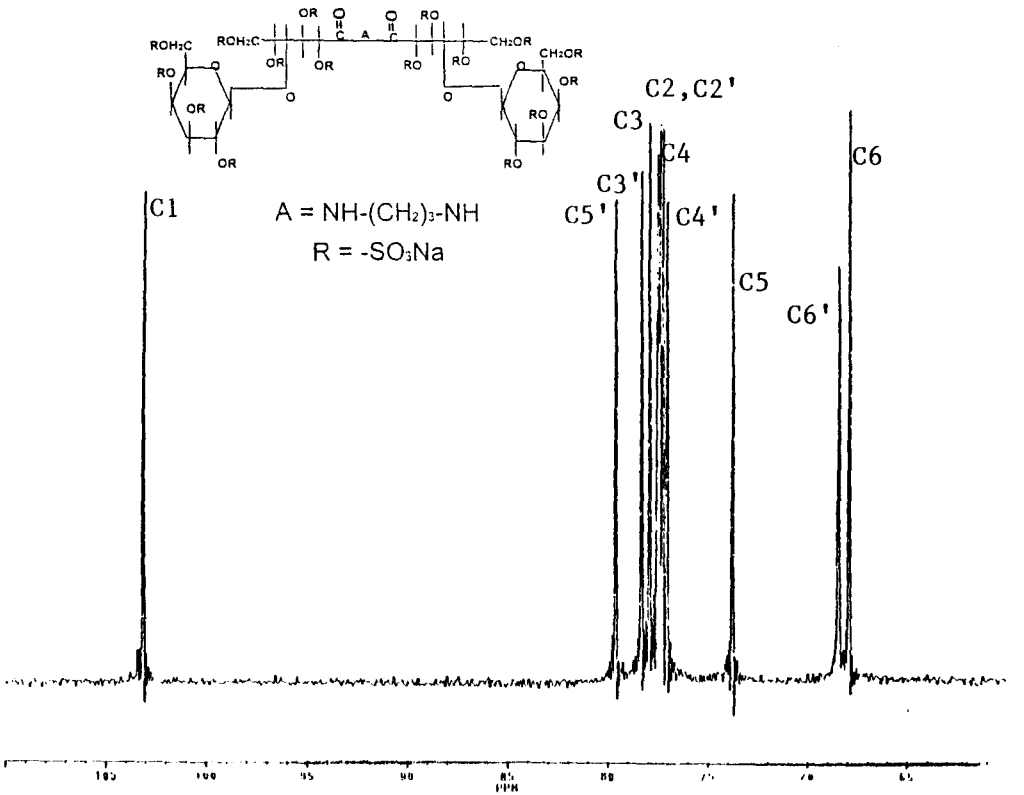


Figure 10. ^{13}C NMR spectrum of aprosulate. This spectrum was determined at 75 Mhz using a Bruker AC300 NMR spectrometer. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. No additional signals suggestive of contamination are observed. Peak assignments are listed in Table 16.

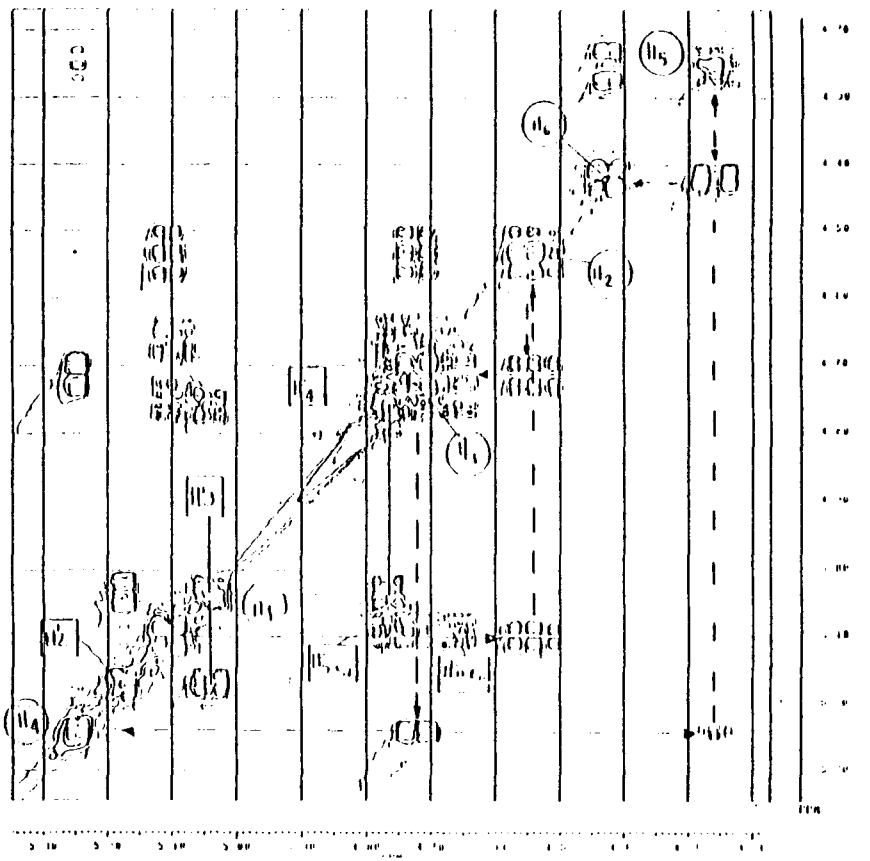


Figure 11. Homonuclear shift-correlated 2-D NMR spectrum of aprosulate. This spectrum was obtained at 300 Mhz using a Bruker AC300 NMR spectrometer. This analysis, ^{1}H spectra are placed on both the x and y axes. The series of peaks on the diagonal correspond to those of the original spectra. Groups of peaks off the diagonal illustrate linkage between those peaks on the diagonal. This analysis is used to make the assignments of the proton spectra.

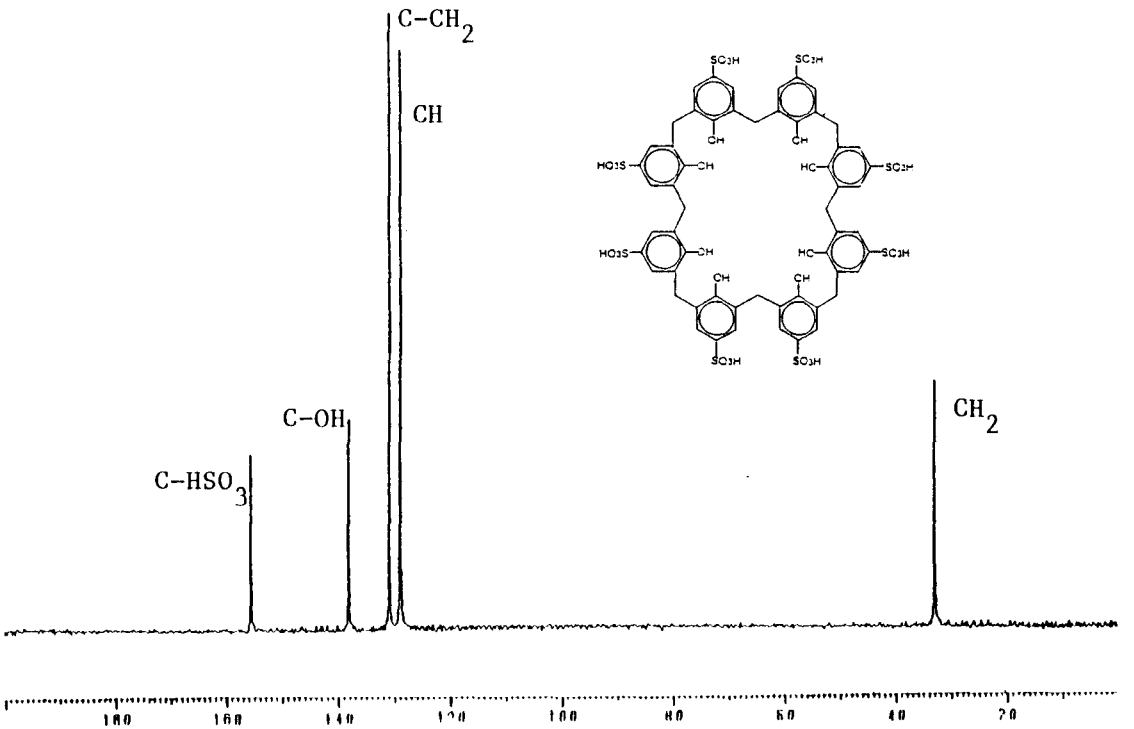


Figure 12. ^{13}C NMR spectrum of GL-522-Y-1. This spectrum was determined on an Bruker AC300 NMR spectrometer at 75 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. Five distinct carbon signals are observed. No signs of impurities are evident. Specific peak assignments are listed in Table 18.

peaks on the diagonal correspond to those of the original ^{1}H spectra. Groups of peaks off the diagonal illustrate linkage between those peaks on the diagonal. By analyzing the linkage pattern, it is possible to make the proton assignments as labelled in Figure 9. A table relating chemical shift to proton assignments is presented in Table 19.

The ^{13}C spectra of GL-522-Y-1 is relatively simple due to the symmetry in the molecular structure. There are only five distinct carbon signals observed for this agent. The ^{13}C spectra is pictured in Figure 12. The ^{13}C NMR signal assignments are tabulated in Table 20. The signal for the methylene bridge carbon atom is located at 33 ppm. The benzyl ring carbons are shifted further downfield. The C-H benzyl carbon exhibits a signal at 128.8 ppm. The C-CH_2 benzyl carbon exhibits a peak at 130.8 ppm. The C-OH benzyl carbon exhibits a peak at 138 ppm. The C-HSO_3 benzyl carbon is shifted furthest downfield, with a signal at 155.5 ppm.

The ^{1}H spectra for GL-522-Y-1 is given in Figure 13. Two distinct signals for protons are observed. These are tabulated in Table 21. The protons of the methylene bridging group are located at approximately 4.2 ppm. The benzyl protons are shifted farther downfield at approximately 7.7 ppm. The peak at 4.7 ppm belongs to residual protons present in the deuterium oxide solvent.

3. Mass Spectral Analysis

In mass spectral analysis, samples are bombarded with a stream of electrons. This bombardment results in a fragmentation of the sample. The molecular fragments are detected based on their mass to charge ratio. As most species are singly charged, the

fragment mass can be determined. The mass spectrum of GL-522-Y-1 is presented in Figure 14. From this spectrum it is observed that the molecular ion peak is at 1489.1 Da and corresponds to the intact GL-522-Y-1 (C8) molecule. The masses of some of the more prominent peaks are labelled in the figure. The next most prominent peak is that of the C4 species. Peaks are also observed at 926 and 1118 Da corresponding to the 5 and 6 benzyl ring species. A small degree of desulfonation is also evident. The peak at 1410 corresponds to the loss of one $-SO_3$ group from the intact GL-522-Y-1 molecule.

Figure 15 depicts the mass spectrum of aprosulate. Aprosulate is a symmetrical molecule consisting of two fully sulfated disaccharides linked by a three member carbon chain. Upon bombardment, aprosulate is cleaved between the amide nitrogen and the alkyl linker region. The peak at 1172.8 Da is that of one half the aprosulate molecule, the fully sulfated disaccharide. The peak at 1132 corresponds to the loss of the amide group from this disaccharide. The regularly spaced peaks at 545, 687, 829, and 971 Da represent progressive desulfation of the disaccharide components of aprosulate.

B. *In Vitro* Study Results

Heparin is both a functionally and chemically heterogeneous drug. It exhibits multiple interactions with plasmatic proteins, platelets, and other cells. The endogenous interactions of heparin are largely mediated by antithrombin III and heparin cofactor II, resulting in direct antiprotease activity and protease generation inhibition. Heparin also directly or indirectly influences the function of endothelial cells and platelets. In this section, defined experimental systems have been used to determine the effects of various

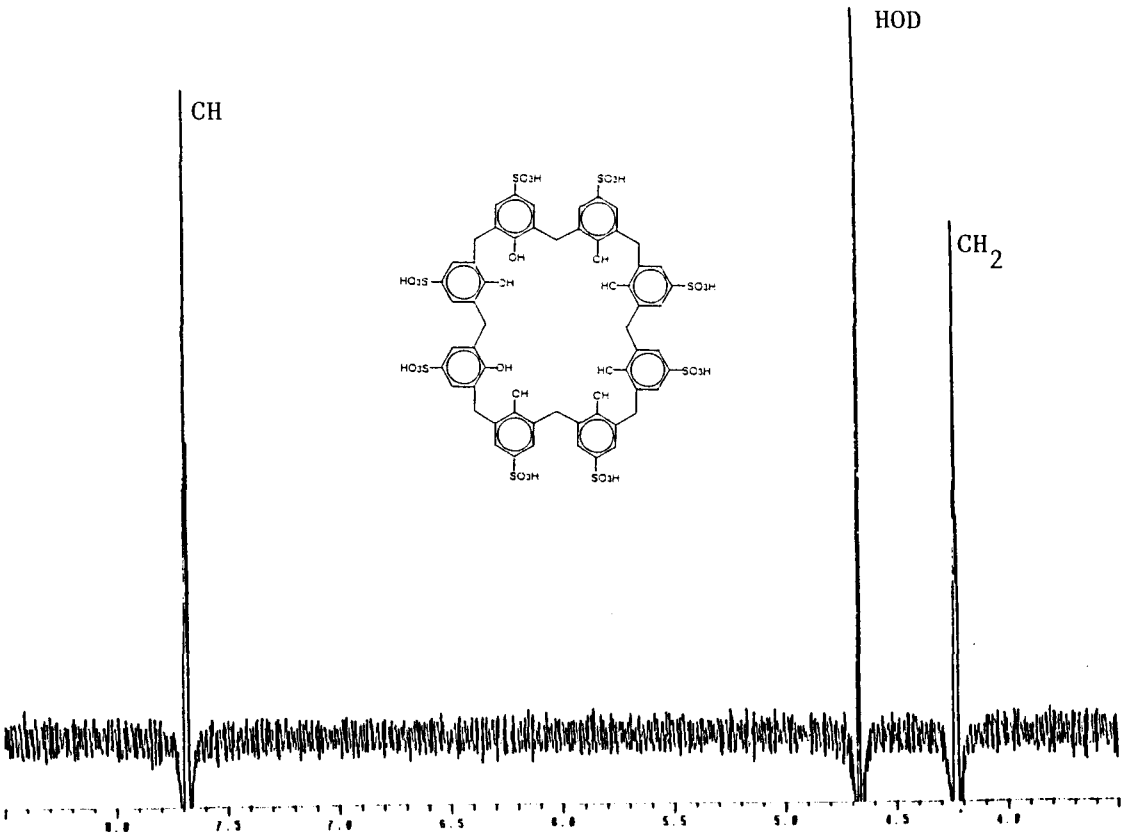


Figure 13. Proton NMR spectrum of GL-522-Y-1. This spectrum was determined on an Bruker AC300 NMR spectrometer at 300 MHz. The *x* axis represents chemical shift from a sodium-3-(trimethylsilyl)-propionate standard in ppm. Three distinct proton signals are observed. The signal at approximately 4.7 ppm is that of residual protons in the deuterated solvent. No signs of impurities are evident. Specific peak assignments are listed in Table 19.

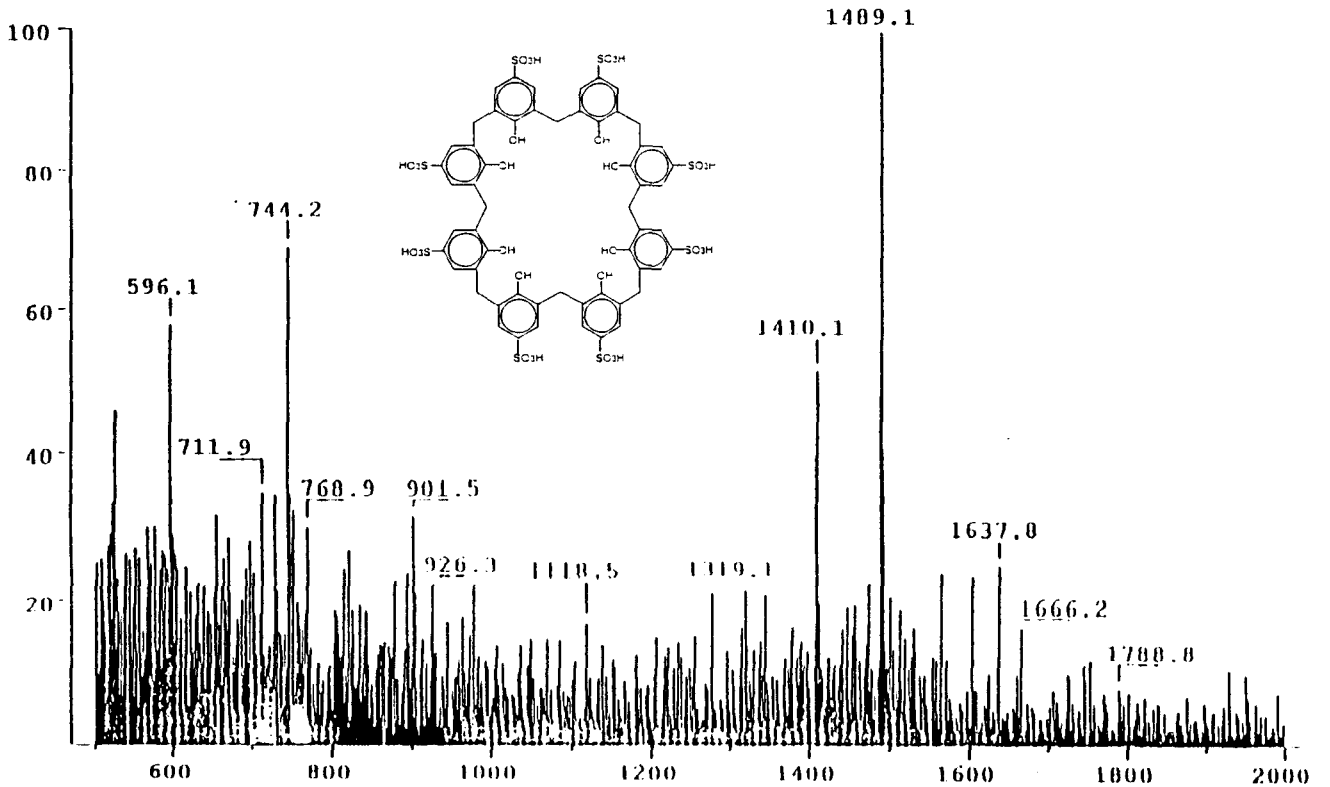


Figure 14. Mass spectrum of GL-522-Y-1. The molecular ion peak is observed at 1489 Da.

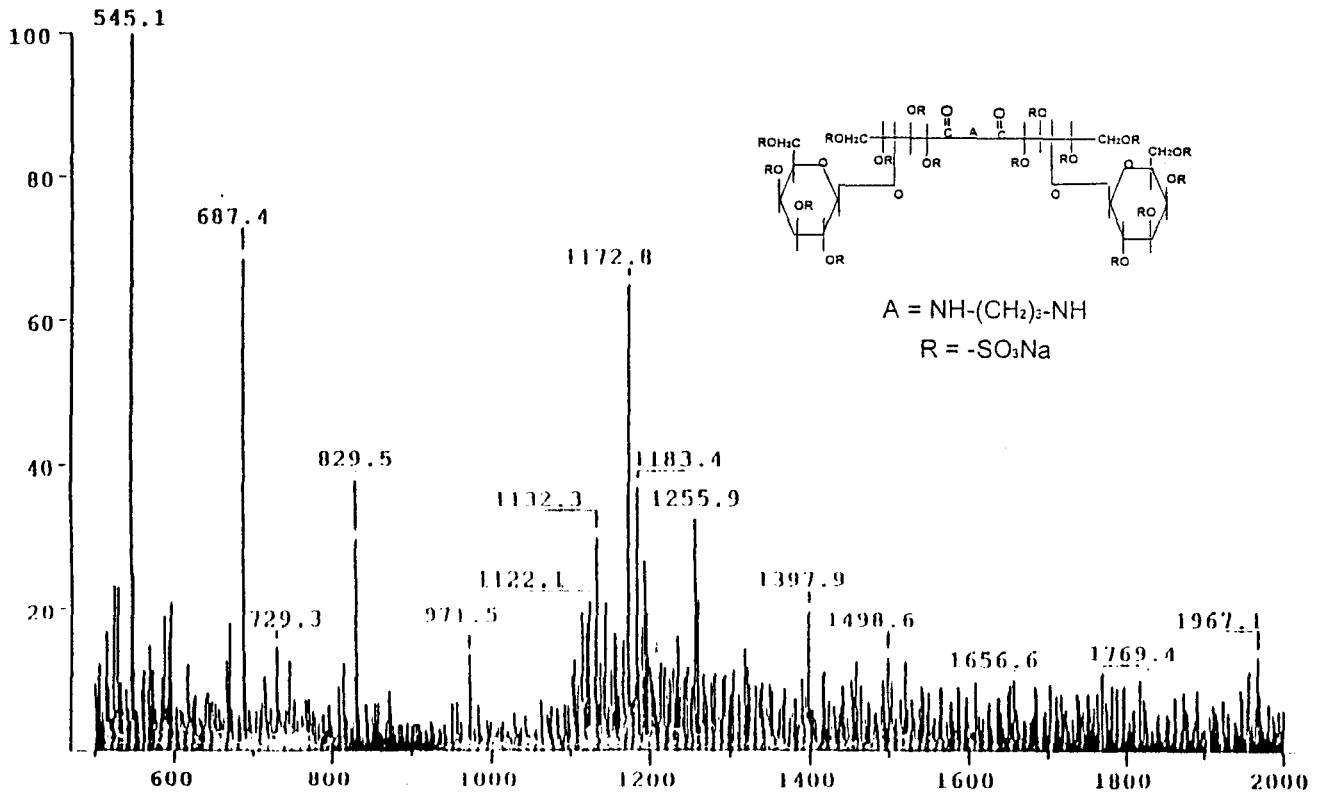


Figure 15. Mass spectrum of aprosulate.

heparin analogues and to demonstrate the relative contribution of HCII and ATIII in mediating various plasmatic and cellular effects in *in vitro* settings.

1. ATIII Mediated Antiprotease Actions

Serpin mediated inhibition of thrombin and factor Xa was determined using amidolytic assay systems. These plasma-free systems contained purified enzyme (thrombin or factor Xa), purified serpin (ATIII or HCII), amidolytic substrate, and varying concentrations of heparin or synthetic heparin analogue. IC_{50} values were determined by linear regression of the straight line portion of the concentration response curves. Concentrations producing 50 % maximal inhibition were extrapolated from the best fit lines. Individual data from these assays are presented in Tables 20 to 23.

The antithrombin III mediated inhibition of thrombin was only potentiated by unfractionated heparin as seen in Figure 16 (Friedman two way ANOVA; chi-square = 73.89, dF = 31, $p < 0.0001$). Heparin potently inhibited the amidolytic activity of thrombin in this assay with an IC_{50} value calculated as 0.87 ± 0.12 nM. Pentasaccharide produced a small (< 15 %) but statistically significant inhibition of thrombin via antithrombin III at concentrations above 0.18 μM. Neither aprosulate nor GL-522-Y-1 exhibited a significant inhibition of thrombin via antithrombin III.

Concentration response curves for the antithrombin III mediated inhibition of factor Xa are depicted in Figure 17. Both heparin and pentasaccharide were observed to potently inhibit the amidolytic activity of factor Xa (Friedman two way ANOVA; chi-square = 85.50, dF = 31, $p < 0.0001$). Heparin was three fold more potent than

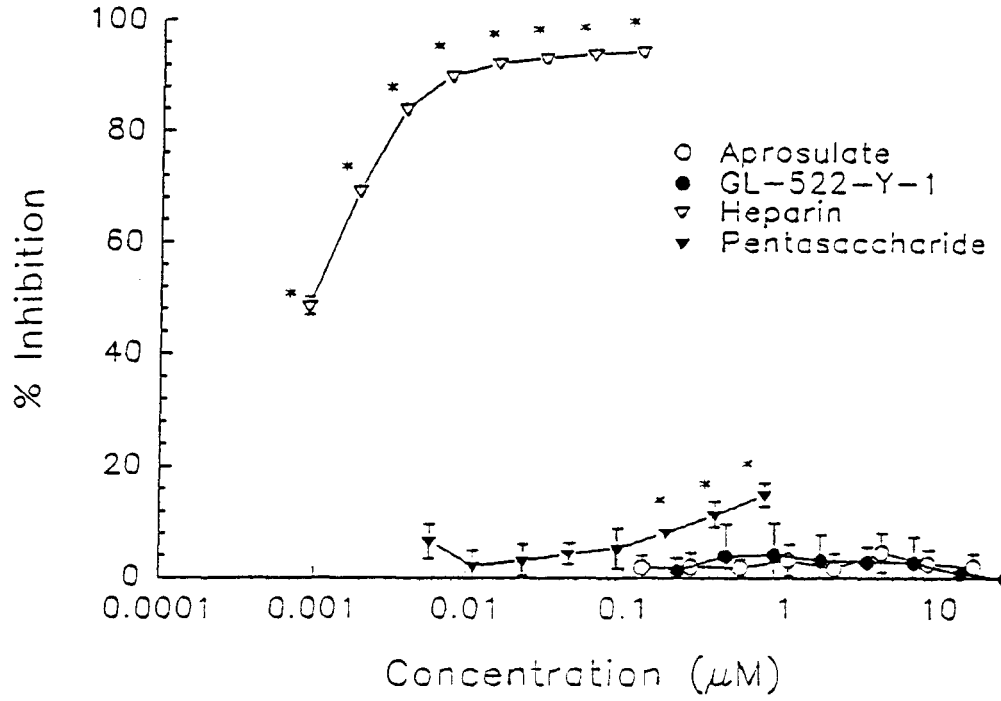


Figure 16. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the ATIII mediated inhibition of thrombin. The ATIII mediated inhibition of thrombin was determined using a plasma-free amidolytic assay system. All results represent the mean ± one standard devation of three observations. Heparin was the only agent observed to inhibit thrombin via ATIII. Statistical differences between agents was determined using the Friedman two ANOVA (Friedman chi-squared = 75.89, dF = 31, $p < 0.0001$). Statistical differences relative to control were determined by one way ANOVA followed by the Newman Keuls multiple comparison test for each agent. $^{*}p < 0.05$ was considered to be statistically significant. IC_{50} values were calculated where possible to compare the potencies of the various agents. Data are compiled in Tables 20 through 23.

Heparin : $IC_{50} = 0.87 \pm 0.12$ nM

Aprosulate : $p = 0.636$; ANOVA

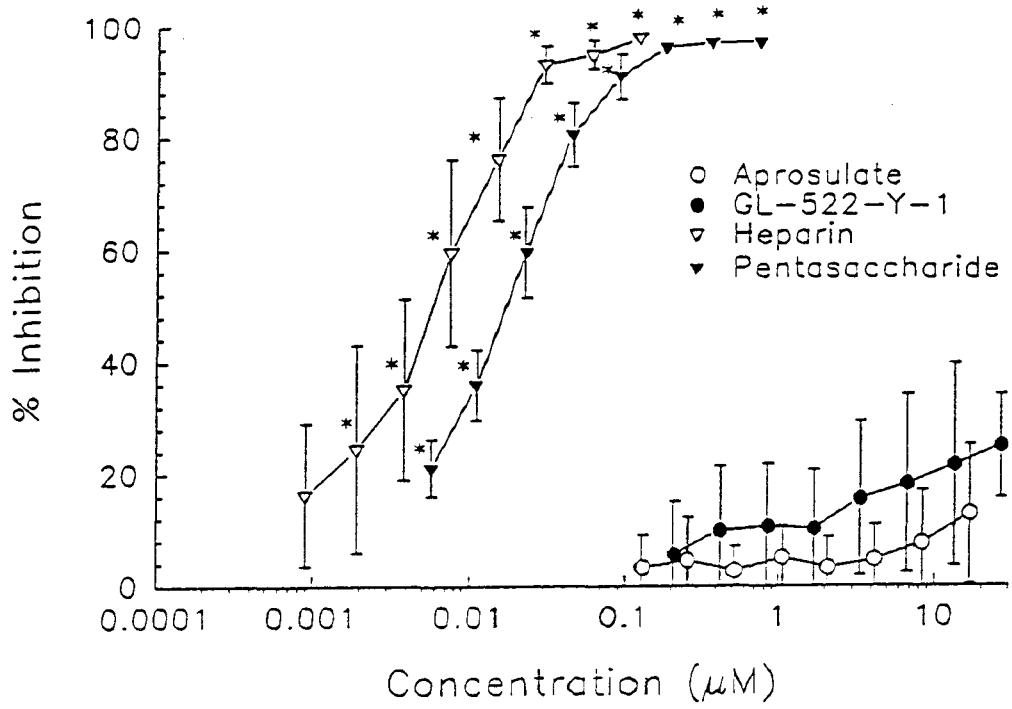


Figure 17. Effect of aprosulate, GL-522-Y-1. heparin and pentasaccharide on the ATIII mediated inhibition of factor Xa. The ATIII mediated inhibition of factor Xa was determined using a plasma-free amidolytic assay system. All results represent the mean ± one standard deviation of three observations. Heparin more potently inhibited factor Xa than did pentasaccharide. Neither aprosulate not GL-522-Y-1 were observed to significantly inhibit Xa. Statistical differences between the agents were determined using the Friedman two ANOVA (Friedman chi-square = 85.50, dF = 31, $p < 0.0001$). Statistical differences relative to control were determined by one way ANOVA followed by the Newman Keuls multiple comparison test for each agent. * $p < 0.05$ was considered to be statistically significant. IC_{50} values were calculated where possible to compare the potencies of the various agents. Data are compiled in Tables 20 through 23.

Heparin : $IC_{50} = 0.007 \pm 0.003$ μM; $p = 0.004$ *vs.* pentasaccharide

Pentasaccharide : $IC_{50} = 0.024 \pm 0.004$ μM

GL-522-Y-1 : $p = 0.316$; ANOVA

Aprosulate : $p = 0.640$; ANOVA

pentasaccharide with a calculated IC_{50} value of 0.007 ± 0.003 μM *vs.* 0.024 ± 0.004 μM for pentasaccharide (t-test, $p = 0.004$). The inhibition by each agent plateaued at 97 to 98 % relative to control. GL-522-Y-1 promoted a weaker concentration dependent inhibition of factor Xa. At an assay concentration of 27 μM, a 25 percent inhibition of factor Xa activity was noted. The inhibition of factor Xa by GL-522-Y-1 was not statistically significant (ANOVA; $p = 0.316$). Aprosulate did not promote the antithrombin III mediated inhibition of factor Xa (ANOVA; $p = 0.640$).

2. HCII Mediated Antiprotease Actions

Concentration response curves for the heparin cofactor II mediated inhibition of thrombin are depicted in Figure 18. Heparin, aprosulate, and GL-522-Y-1 exhibited a concentration dependent inhibition of thrombin activity (Friedman two way ANOVA; chi-square = 91.97, dF = 31, $p < 0.0001$). Heparin exhibited the highest potency (IC_{50} = 0.051 ± 0.003 μM) of the three agents and was approximately 20 fold more potent than either aprosulate or GL-522-Y-1 (ANOVA, Newman Keuls; $p < 0.05$). Aprosulate and GL-522-Y-1 exhibited similar potencies (IC_{50} = 1.23 ± 0.22 *vs.* 0.84 ± 0.09 μM, respectively; $p < 0.05$) Maximal inhibition of thrombin activity plateaued at approximately 80 percent for each agent. No significant differences in the maximal effect of any agent were noted (ANOVA; $p = 0.239$). Only the highest concentration of pentasaccharide tested exhibited a significant inhibition of thrombin via heparin cofactor II. It should be noted that the concentrations of heparin required to promote thrombin inhibition by heparin cofactor II were nearly two orders of magnitude higher than those

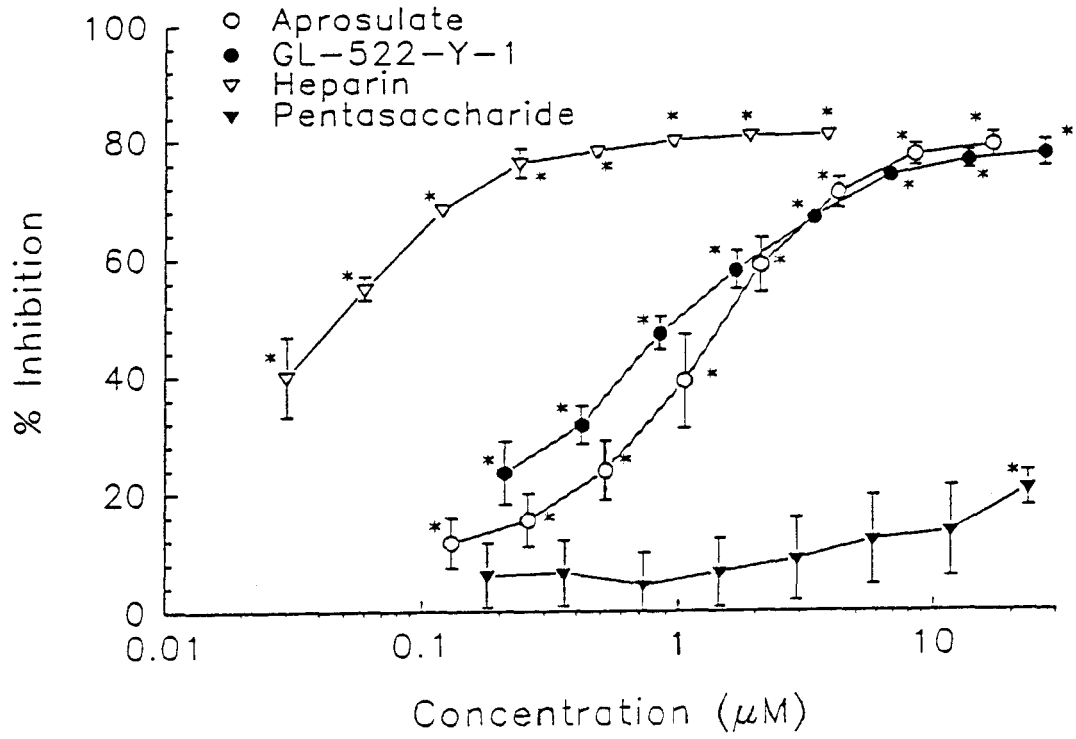


Figure 18. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the HCII mediated inhibition of thrombin. The HCII mediated inhibition of thrombin was determined using a plasma-free amidolytic assay system. All results represent the mean ± one standard deviation of three observations. The rank order potency for thrombin inhibition was observed to be heparin > GL-522-Y-1 > aprosulate > pentasaccharide. Statistical differences between agents were determined using the Friedman two way ANOVA (Friedman chi-square = 91.97, dF = 31, $p < 0.0001$). Statistical differences relative to control were determined by one way ANOVA followed by the Newman Keuls multiple comparison test for each agent. $^{*}p < 0.05$ was considered to be statistically significant. IC_{50} values were calculated where possible to compare the potencies of the various agents. Data are compiled in Tables 20 through 23.

Heparin : $IC_{50} = 0.051 \pm 0.003\ \mu M$; $p < 0.05$ *vs.* aprosulate and GL-522-Y-1

GL-522-Y-1 : $IC_{50} = 0.84 \pm 0.09\ \mu M$; $p < 0.05$ *vs.* aprosulate

Aprosulate : $IC_{50} = 1.23 \pm 0.22\ \mu M$

TABLE 1

POTENCY COMPARISON OF APROSULATE, GL-522-Y-1, HEPARIN, AND PENTASACCHARIDE IN SERPIN ACTIVITY ASSAYS

	IC_{50} (μM)		
	ATIII/IIa	ATIII/Xa	HCII/IIa
Aprosulate	> 16.8	> 16.8	1.2 ± 0.2
GL-522-Y-1	> 26.9	> 26.9	0.8 ± 0.1
Heparin	0.87 ± 0.12#	7 ± 3#	0.51 ± 0.01
Pentasaccharide	> 0.7	0.24 ± 0.004	> 23.2

All values represent the mean ± one standard deviation of three individual trials. IC_{50} values were determined by extrapolation from the regression line of the straight line portion of the concentration reponse curve. # results are expressed in nM.

required for thrombin inhibition by antithrombin III.

3. Global Anticoagulant Profile

a. Studies in Supplemented Normal Human Plasma Systems

The global anticoagulant effects of heparin, pentasaccharide, aprosulate, and GL-522-Y-1 were determined following supplementation to normal human pooled plasma using the prothrombin time (PT), activated partial thromboplastin time (APTT), Heptest®, and 5 U thrombin time (5U TT). Each drug was supplemented to pooled plasma in four separate trials and the clotting times were determined as described in "Materials and Methods". Pooled plasma was made by mixing citrated platelet poor plasma from at least five healthy volunteers. Agent concentrations were expressed as micromolar (μM) amounts of agent supplemented to plasma. In the PT, APTT, and Heptest® assays, the final assay concentration of each agent was one third that of the plasma concentration. In the 5U TT, the final assay concentration is two thirds that of the plasma concentration. Micromolar concentrations for the synthetic heparin analogues were determined using their known formula weights. The weight average molecular weight of heparin as determined by gel permeation chromatography was used to calculate the micromolar concentrations of heparin. Individual clotting and antiprotease data is tabulated in Tables 24 to 27. In each assay, statistically significant differences between agents was determined using the Friedman two way ANOVA. If a significant difference was found, significant differences for treatment relative to control for each agent were determined using one way ANOVA followed by the Newman-Keuls multiple comparison test.

Figure 19 describes the anticoagulant effects of heparin and the synthetic heparin analogues as measured by the prothrombin time assay (PT). Only heparin was observed to prolong the clotting time (Friedman chi-square = 114.70, dF = 35, $p < 0.0001$). Potency of each agent was determined by comparing the plasma concentrations which prolonged clotting time to 100 seconds. Where possible, these concentrations were extrapolated from the best fit regression curves of the data. Other concentrations were extrapolated from the concentration *vs.* clotting time curves. Potencies of aprosulate, GL-522-Y-1, heparin, and pentasaccharide are compared in Table 2. Doubling of the baseline clotting time was observed at a plasma heparin concentration of approximately 2.5 μM and a 100 second clotting time was achieved at a plasma concentration of 6.2 $\pm$ 2.1 μM. None of the synthetic analogues were able to double the clotting time in the PT despite plasma concentrations 16 to 27 fold higher than the effective heparin concentration.

The APTT was a more sensitive measure of the anticoagulant effects of heparin and the synthetic analogues as observed in Figure 20. A statistically significant difference between agents was observed (Friedman chi-square = 133.46, dF = 35, $p < 0.0001$). As the concentration-response curves were not parallel, the slopes of the straight line portions of the curves were calculated by least squares regression. The slope of the heparin curve was approximately 40 fold higher than that calculated from the aprosulate curve (454 vs 11.5 s/μM). Neither pentasaccharide nor GL-522-Y-1 exhibited a potent anticoagulant effect in this assay. Using the concentration which brought about a 100 second clotting time as an index of potency, it was observed that heparin was 10 fold more potent than aprosulate (0.30 $\pm$ 0.08 *vs.* 3.1 $\pm$ 0.4 μM, respectively). Both

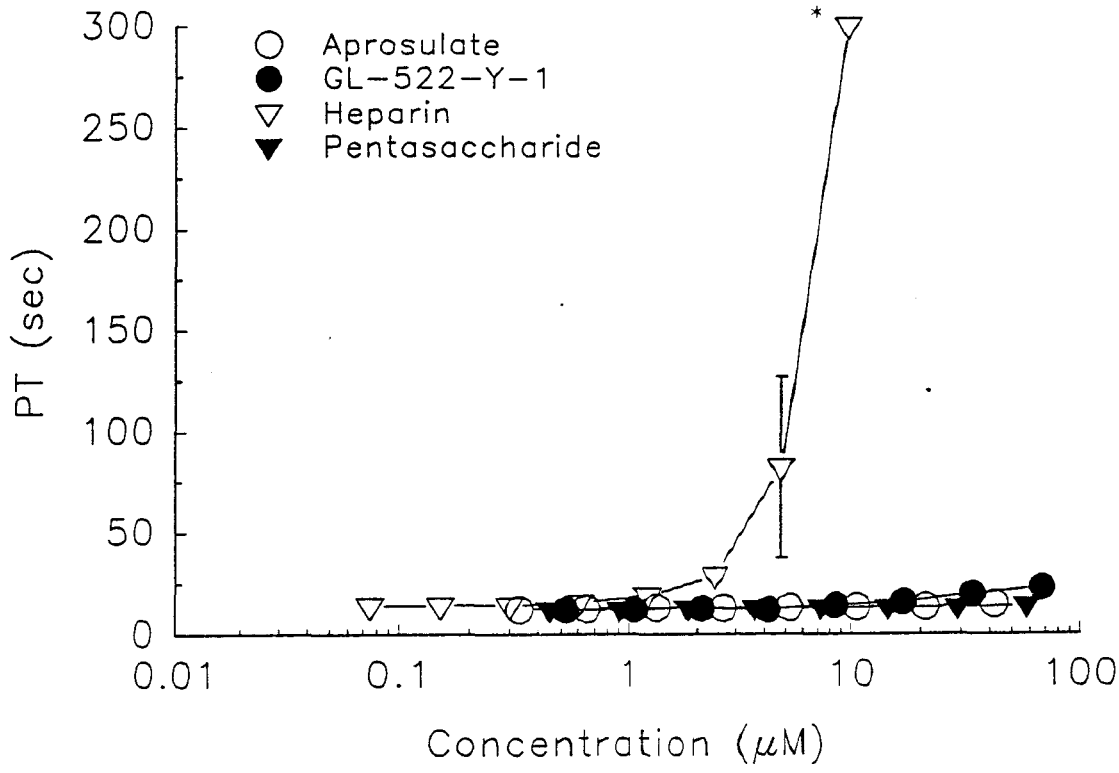


Figure 19. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the prothrombin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. Heparin was the only agent observed to prolong the clotting time in this assay. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 114.90, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

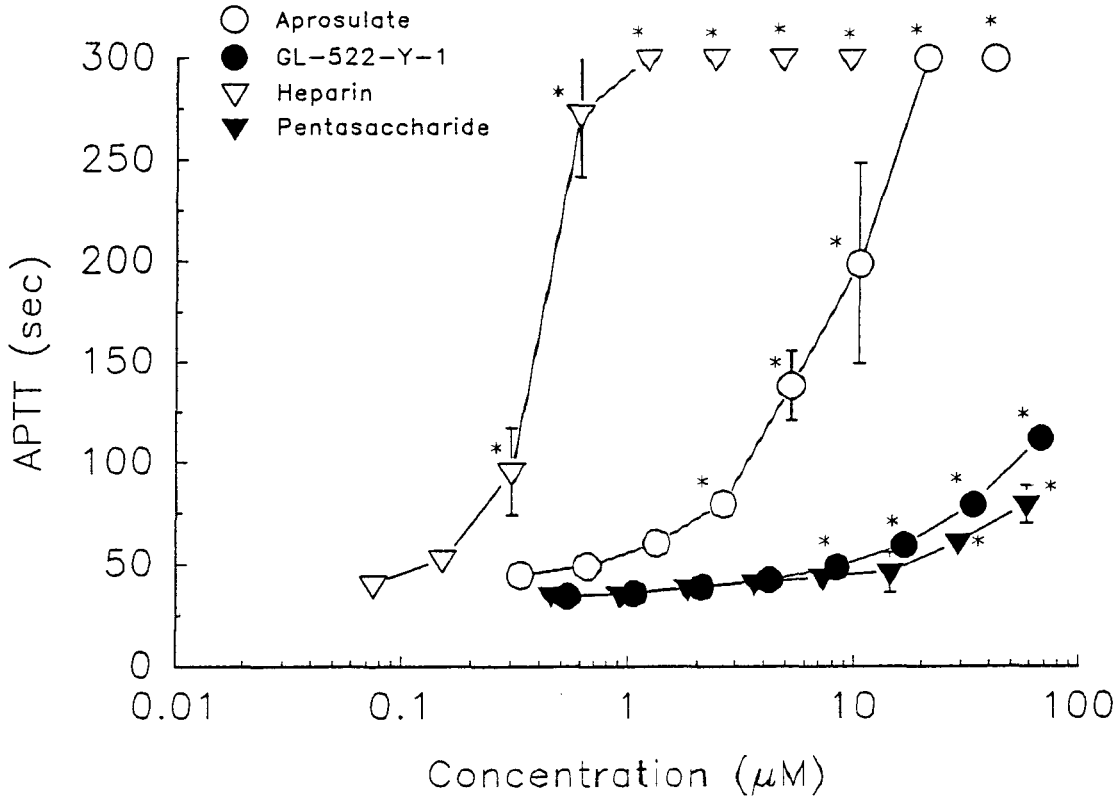


Figure 20. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the activated partial thromboplastin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. Clotting times were dose-dependently prolonged with a rank order potency of heparin > aprosulate > GL-522-Y-1 > pentasaccharide. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 133.46, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

pentasaccharide and GL-522-Y-1 were notably weaker, with $Conc_{100s}$ greater than 50 μM. This data is presented in Table 2.

In the Heptest® assay, all agents exhibited concentration dependent anticoagulant effects with the exception of GL-522-Y-1. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 132.94, dF = 35, $p < 0.0001$). Concentration response curves are depicted in Figure 21. As with the APTT, the log concentration vs. clotting time curves were not parallel. Heparin was the most potent agent on a molar basis (calculated slope = 229 s/μM). The concentration-response curves for heparin and aprosulate appeared to parallel each other though 25 fold more aprosulate than heparin was required to achieve a 100 second clotting time. The curve for pentasaccharide did not parallel those of heparin or aprosulate ($Conc_{100s}$ = 1.1 $\pm$ 0.9 μM). GL-522-Y-1 doubled the Heptest® clotting time only at the highest concentration tested (67 μM).

The 5 unit thrombin time (5U TT) was the most sensitive assay for heparin with a slope of 850 s/μM. The response to aprosulate in this assay was considerably weaker. Comparison of the $Conc_{100s}$ values for heparin and aprosulate indicate that 90 fold more aprosulate was required to achieve a 100 second clotting time. GL-522-Y-1 did not produce a statistically significant increase in thrombin clotting time whereas pentasaccharide significantly increased clotting times only at concentrations above 58 μM. Concentration response curves are depicted in Figure 22. A statistically significant difference between the effects of the various agents was observed (Frieman chi-square = 110.14, dF = 35, $p < 0.0001$).

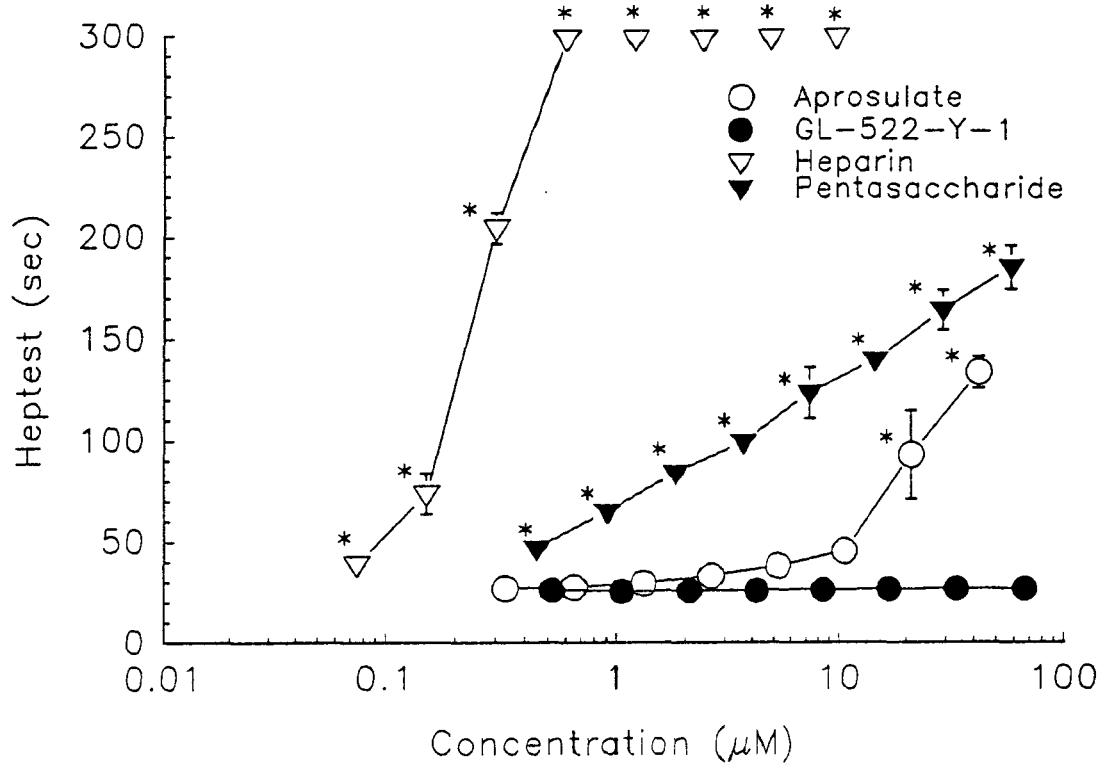


Figure 21. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the Heptest®. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. The rank order potency for Heptest® prolongation was observed to be Heparin > pentasaccharide > aprosulate > GL-522-Y-1. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 132.94, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

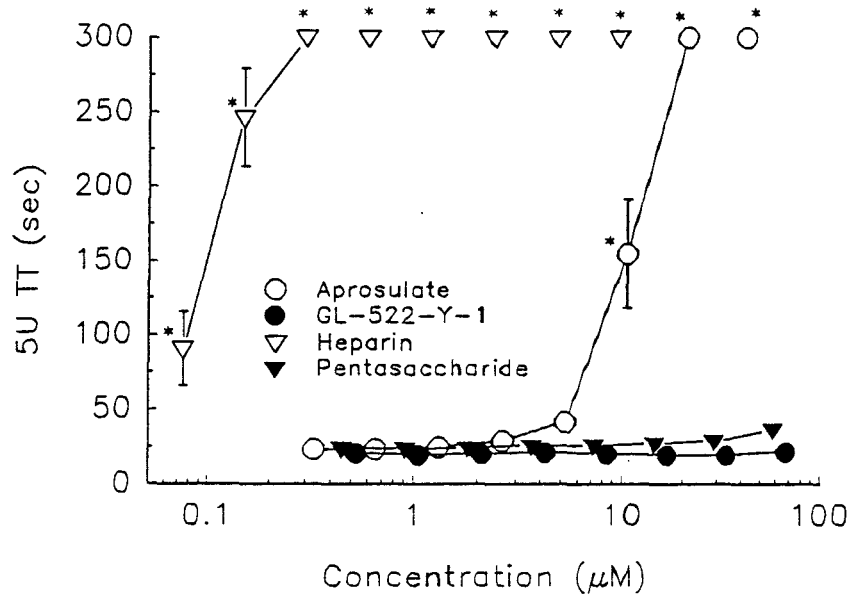


Figure 22. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the 5 unit thrombin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. Heparin was observed to more potently prolong the thrombin time than aprosulate. Pentasaccharide and GL-522-Y-1 had no effect on the clotting time in this assay. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 110.14, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

TABLE 2

PLASMA CONCENTRATIONS OF SYNTHETIC HEPARIN ANALOGUES REQUIRED TO PROLONG NORMAL HUMAN PLASMA CLOTTING TIMES TO 100 SECONDS

	PT	APTT	Heptest	5U TT
Aprosulate	> 42	3.1 ± 0.4**	8.0 ± 0.7	7.2 ± 0.3
GL-522-Y-1	> 67	53.9 ± 6.4	> 67	> 67
Heparin	6.2 ± 2.1*	0.30 ± 0.05**	0.32 ± 0.03#	0.08 ± 0.05##
Pentasaccharide	> 58	> 58	1.1 ± 0.9#	> 58

The potencies of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were compared by extrapolating the concentrations of each agent which caused a prolongation of the clotting time to 100 seconds. Results represent the mean ± 1 standard deviation of 4 determinations. In cases in which agents did not prolong the clotting time to 100 seconds, the highest concentration tested is presented. Statistical Comparison of these values was made by one way ANOVA followed by the Student Newman-Keuls multiple comparison test for each assay. $p < 0.05$ was considered statistically significant.

PT : * $p < 0.05$; heparin *vs.* aprosulate, GL-522-Y-1, and pentasaccharide

APTT : ** $p < 0.05$; aprosulate and heparin *vs.* GL-522-Y-1
$p > 0.05$; aprosulate *vs.* heparin

Heptest® : # $p < 0.05$; heparin and pentasaccharide *vs.* aprosulate
$p > 0.05$; pentasaccharide *vs.* heparin

5U TT : ## $p < 0.05$; heparin *vs.* aprosulate

b. Studies in Supplemented Normal Rabbit Pool Plasma Systems

The global anticoagulant effects of heparin, aprosulate, GL-522-Y-1, and pentasaccharide were determined following supplementation to normal rabbit plasma using the prothrombin time, activated partial thromboplastin time, Heptest®, and 5 unit thrombin time. Each agent was supplemented to pooled normal rabbit plasma on three occasions and clotting times were determined as described in "Materials and Methods". Pooled normal rabbit plasma was made by mixing citrated platelet poor plasma obtained from at least five virgin male New Zealand white rabbits. Agent concentrations were expressed as μM amounts based on the known molecular formula weights for the synthetic analogues and the weight average molecular weight determined by gel permeation chromatography for heparin. Concentration response data for each agent is tabulated in Tables 28 to 31. The potency of each agent was assessed by determining the plasma concentration of each agent which prolonged the various clotting times to 100 seconds. A comparison of these potencies is made in Table 3.

Figure 23 depicts the concentration response curves for aprosulate, GL-522-Y-1, heparin, and pentasaccharide in the prothrombin time assay. The baseline clotting time of rabbit plasma was observed to be 6.3 $\pm$ 0.4 seconds. Only heparin produced a concentration dependent increase in clotting time (Friedman chi-square = 84.01, dF = 35, $p < 0.0001$). At the highest concentration tested, a 2.5 fold increase over baseline was observed. None of the synthetic analogues was able to prolong the PT over the concentration ranges tested.

The APTT was a more sensitive assay to measure the anticoagulant activity of

heparin and the synthetic analogues. Concentration response curves are depicted in Figure 24. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 102.21, dF = 35, $p < 0.0001$). Heparin was the most potent agent in this assay, prolonging the clotting time beyond 300 seconds at concentrations above 0.30 μM. The concentration of heparin required to prolong the clotting time to 100 seconds was estimated from the concentration-response curves to be 0.15 $\pm$ 0.03 μM. Aprosulate also achieved a clotting time greater than 300 seconds over the concentration range tested. The concentration needed to reach 100 seconds was approximately 25 times that of heparin at 3.9 $\pm$ 1.1 μM ($p > 0.05$ *vs.* heparin). Pentasaccharide exhibited a relatively weaker concentration dependent prolongation of clotting time, reaching 100 seconds at a concentration of 32.4 $\pm$ 6.4 μM ($p < 0.05$ *vs.* heparin and aprosulate). GL-522-Y-1 did not significantly prolong the APTT over the concentration range tested.

In the Heptest® assay, heparin produced the most potent anticoagulant activity, reaching 100 seconds at a concentration of 1.9 $\pm$ 0.1 μM and prolonging clotting time beyond 300 seconds at concentrations greater than 0.60 μM. Pentasaccharide produced a linear increase in clotting time with increasing dose, though did not reach 300 seconds over the concentration range tested. The concentration to elevate the clotting time to 100 seconds was determined to be 3.7 $\pm$ 0.2 μM pentasaccharide ($p > 0.05$ *vs.* heparin). Aprosulate did not prolong the Heptest® at plasma concentrations below 10 μM. A 100 second concentration of 23.8 $\pm$ 6.7 μM ($p < 0.05$ *vs.* heparin and pentasaccharide). GL-522-Y-1 did not prolong the Heptest® clotting time at concentrations up to 67 μM.

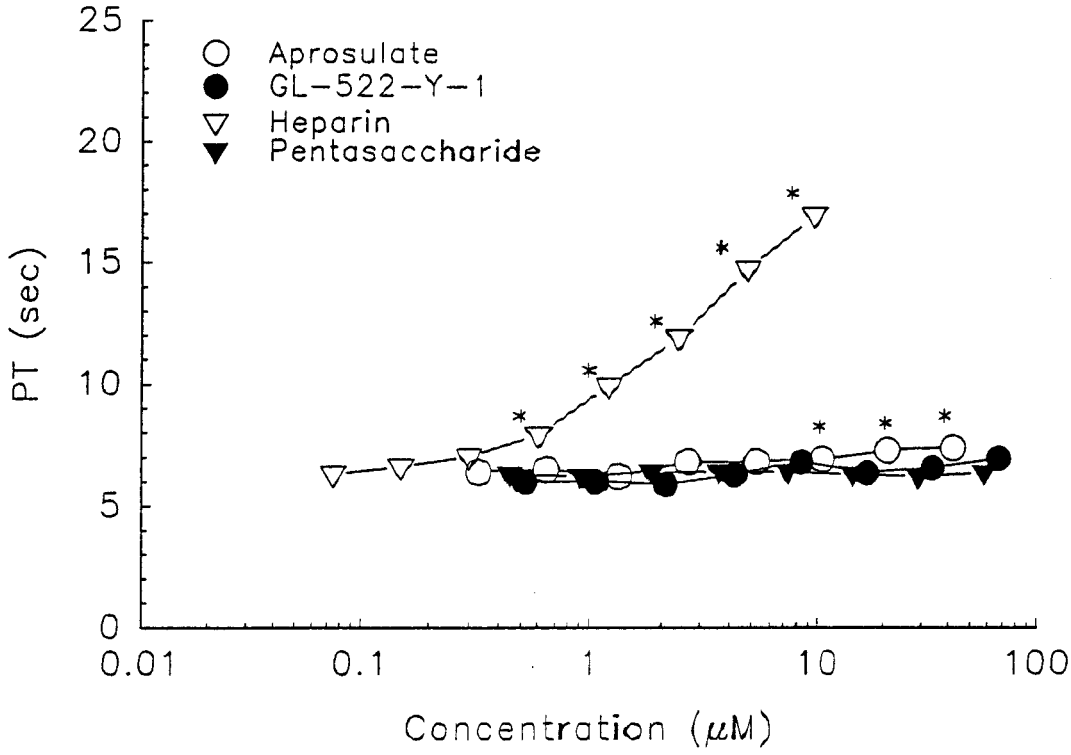


Figure 23. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the prothrombin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of three observations. Heparin was the only agent observed to prolong the clotting time in this assay. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 84.00, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

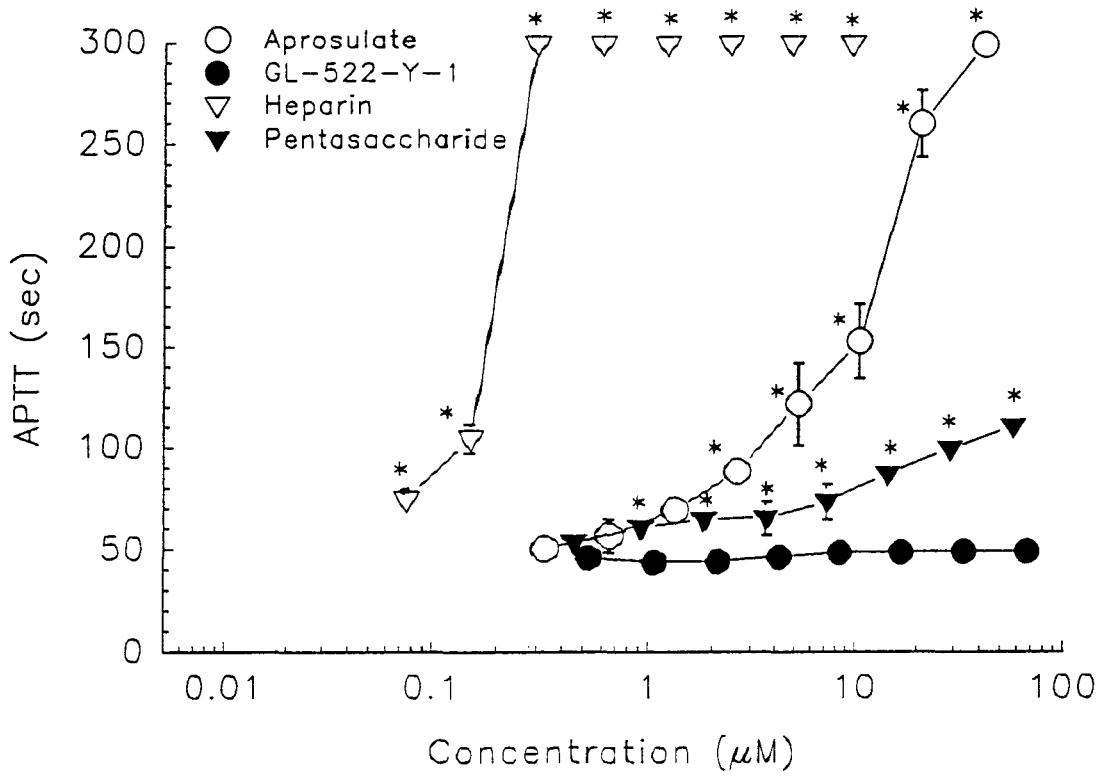


Figure 24. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the activated partial thromboplastin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of three observations. The agents prolonged the clotting time with a rank order potency of heparin > aprosulate > pentasaccharide. GL-522-Y-1 did not prolong the clotting time over the concentration range tested. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 102.21, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

This data is presented in Figure 25. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 94.10, dF = 35, $p < 0.0001$).

Heparin exhibited a strong anticoagulant effect in the 5 U thrombin time assay as depicted in Figure 26. At the lowest concentration tested, 70 nM, clotting times exceeded 300 seconds. Aprosulate was the only other agent capable of prolonging the clotting time beyond 300 seconds. A concentration to prolong clotting time to 100 seconds of 5.5 $\pm$ 0 μM was determined for aprosulate. Pentasaccharide produced a weak prolongation of the clotting time (< 2 fold baseline) at the highest concentrations tested. GL-522-Y-1 did not prolong the thrombin time. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 98.67, dF = 35, $p < 0.0001$).

4. Antiprotease Profile

a. Studies in Normal Human Plasma Systems

Thrombin and factor Xa inhibition by heparin, aprosulate, pentasaccharide, and GL-522-Y-1 was assessed following supplementation of each agent to normal human plasma. Amidolytic assays using specific substrates were utilized such that enzyme inhibition was determined by measuring changes in optical density of the sample. Percent inhibition was calculated relative to unsupplemented NHP. IC_{50} and IC_{25} values were determined by performing linear regression on the straight line portion of each individual concentration versus percent inhibition curve. IC_{50}/IC_{25} values were determined by

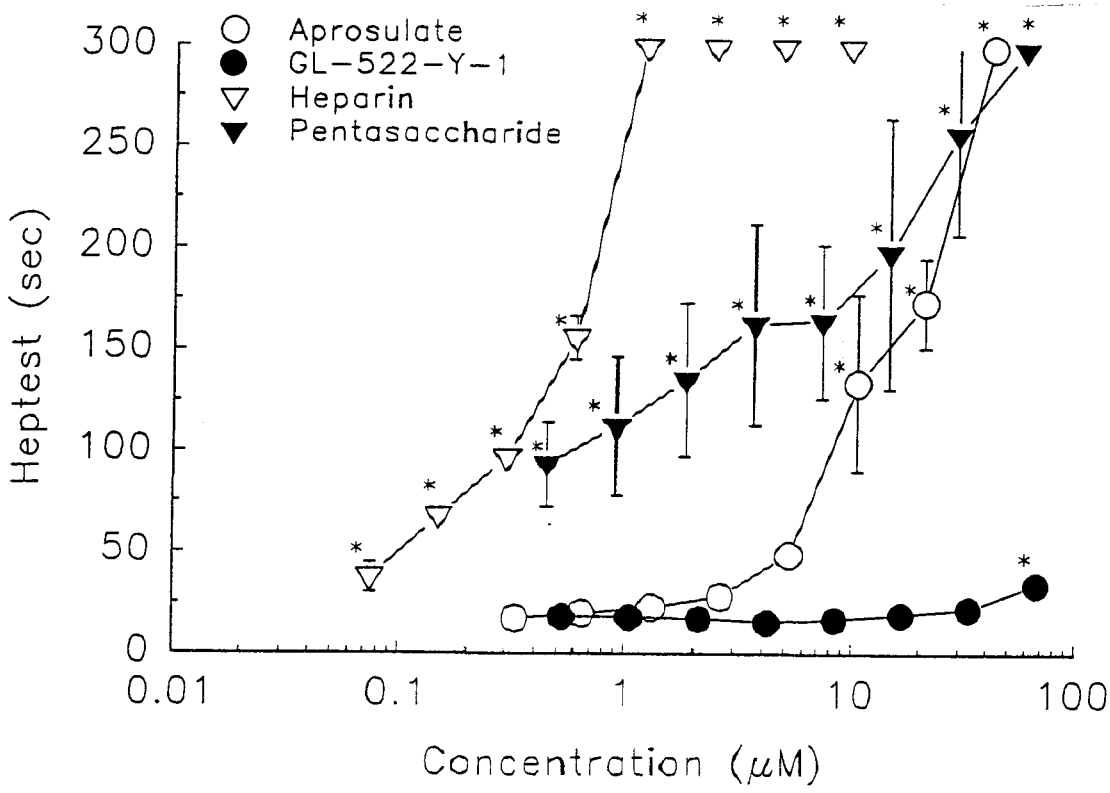


Figure 25. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the Heptest®. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean ± one standard deviation of three observations. The agents prolonged the clotting time with a rank order potency of heparin > pentasaccharide > aprosulate. GL-522-Y-1 did not prolong the clotting time in this assay. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 94.10, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

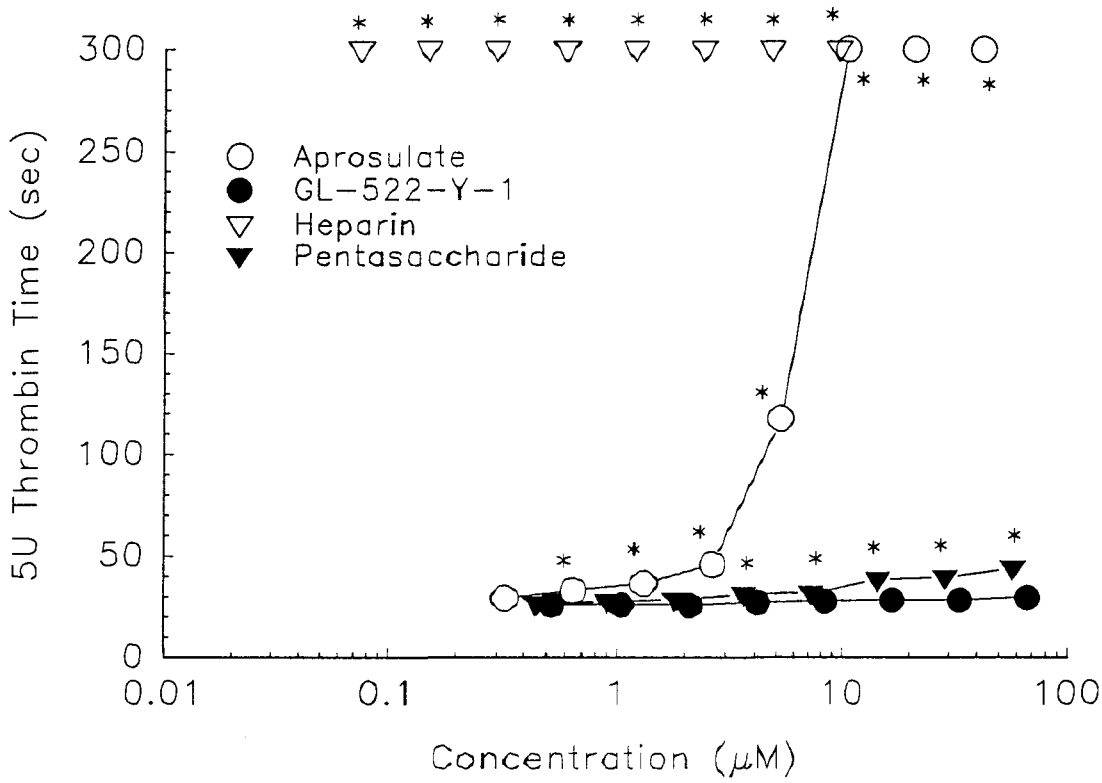


Figure 26. Comparative anticoagulant effect of heparin and three synthetic heparin analogues as measured by the 5 unit thrombin time. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of three observations. The agents prolonged the clotting time with a rank order potency of heparin > aprosulate > pentasaccharide. GL-522-Y-1 did not prolong the clotting time in this assay. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 98.67, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

TABLE 3

PLASMA CONCENTRATIONS OF SYNTHETIC HEPARIN ANALOGUES REQUIRED TO PROLONG NORMAL RABBIT PLASMA CLOTTING TIMES TO 100 SECONDS

	PT	APTT	Heptest	5U TT
Aprosulate	> 42	3.9 ± 1.1*	23.8 ± 6.7	5.5 ± 0
GL-522-Y-1	> 67	> 67	> 67	> 67
Heparin	> 10	0.15 ± 0.03*	1.9 ± 0.1#	< 0.8
Pentasaccharide	> 58	32.4 ± 6.4	3.7 ± 0.2#	> 58

The potencies of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were compared by extrapolating the concentrations of each agent which caused a prolongation of the clotting time to 100 seconds. Results represent the mean ± 1 standard deviation of 3 determinations. In cases in which agents did not prolong the clotting time to 100 seconds, the highest concentration tested is presented. Statistical Comparison of these values was made by one way ANOVA followed by the Student Newman-Keuls multiple comparison test for each assay. $p < 0.05$ was considered statistically significant.

APTT : * $p < 0.05$; aprosulate and heparin *vs.* pentasaccharide
$p > 0.05$; aprosulate *vs.* heparin

Heptest® : # $p < 0.05$; heparin and pentasaccharide *vs.* aprosulate
$p > 0.05$; pentasaccharide *vs.* heparin

interpolation from the regression curves. A correlation coefficient greater than 0.92 was obtained for each regression line. Potencies of aprosulate, Gl-522-Y-1, heparin, and pentasaccharide are compared in Table 4.

Heparin was the most potent thrombin inhibitor in this assay system, with an IC_{50} value calculated to be 0.144 ± 0.003 μM. Aprosulate was the only heparin analogue to inhibit thrombin, though its effects were much weaker than those of heparin. At concentrations as high as 40 μM, the inhibition of thrombin by aprosulate was less than 50 % (Figure 27). The IC_{25} value for aprosulate was determined to be 1.95 ± 0.05 μM. This was 28 fold higher than the value determine for heparin (0.069 ± 0.002 μM). Neither pentasaccharide nor GL-522-Y-1 displayed a significant inhibition of thrombin. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 89.29, dF = 31, $p < 0.0001$).

Heparin and pentasaccharide inhibited factor Xa amidolytic activity in a concentration dependent manner. This is depicted in Figure 28. The inhibition of factor Xa by pentasaccharide was observed to be slightly weaker than that for heparin. The IC_{50} value for pentasaccharide was two fold higher than that for heparin (0.83 ± 0.03 *vs.* 0.40 ± 0.02 μM). Neither aprosulate nor GL-522-Y-1 promoted the inhibition of factor Xa. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 86.76, dF = 31, $p < 0.0001$).

b. Studies in Normal Rabbit Pool Plasma Systems

Amidolytic assays were preformed to assess each agents antiprotease activity.

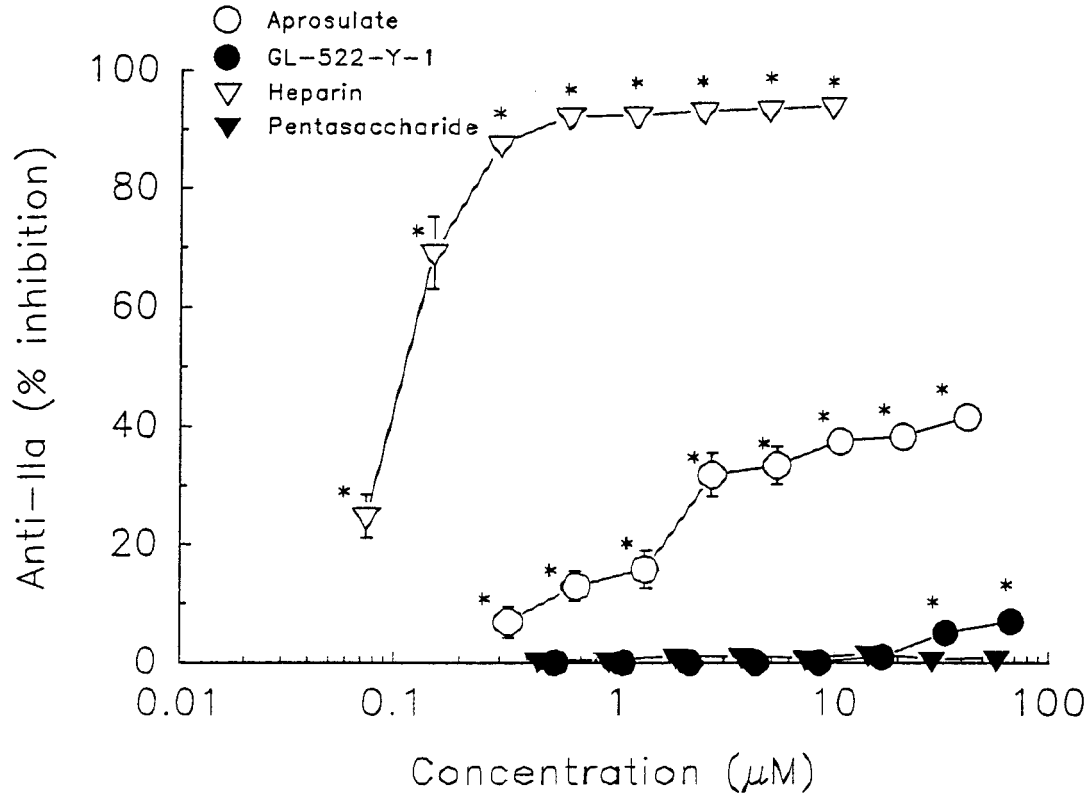


Figure 27. Comparative antiprotease effect of heparin and three synthetic heparin analogues as measured by the amidolytic anti-IIa assay. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. The rank order potency of thrombin inhibition was heparin > aprosulate > GL-522-Y-1. Pentasaccharide did not promote the inhibition of thrombin. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 89.29 dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

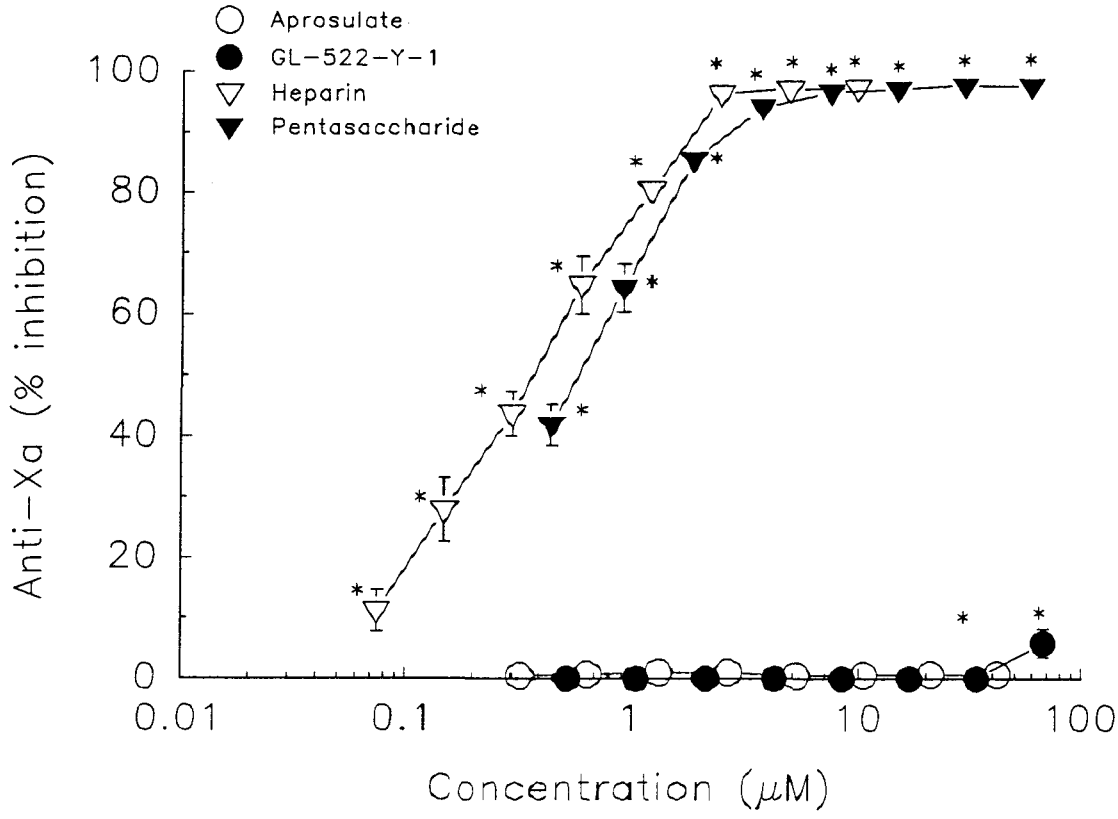


Figure 28. Comparative antiprotease effect of heparin and three synthetic heparin analogues as measured by the amidolytic anti-Xa assay. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal human plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of four observations. Heparin exhibited a higher anti-Xa potency than pentasaccharide. GL-522-Y-1 and aprosulate were not observed to inhibit Xa activity. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 86.76 dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 24 through 27.

TABLE 4

IC_{50} VALUES FOR SYNTHETIC HEPARIN ANALOGUES IN AMIDOLYTIC ANTIPROTEASE ASSAYS IN NORMAL HUMAN PLASMA

	Anti-IIa	Anti-Xa
Aprosulate	1.05 ± 0.05 μM	> 42 μM
GL-522-Y-1	> 67 μM	> 67 μM
Heparin	0.14 ± 0.01 μM*	0.40 ± 0.02 μM**
Pentasaccharide	> 58 μM	0.83 ± 0.03 μM

The potencies of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were compared in amidolytic antiprotease assays by determining the IC_{50} values for each agent from the concentration *vs.* % inhibition curves. Results represent the mean ± 1 standard deviation of 4 determinations. In cases where the agent did not promote inhibition, the highest concentration tested is presented. Statistical significance was determined using the Student's t-test ($p < 0.05$).

aIIa: $^{*}p < 0.001$; heparin *vs.* aprosulate
aXa: $^{**}p < 0.001$; heparin *vs.* pentasaccharide

Concentration-response curves for the antithrombotic activity of each agent are depicted in Figure 29. Only heparin exhibited a potent inhibition of thrombin. Inhibition plateaued at 90 % at concentrations above 1.2 μM. The IC_{50} for heparin was calculated to be 120 $\pm$ 10 nM. Aprosulate, GL-522-Y-1, and pentasaccharide exhibited small inhibitions of thrombin at the highest concentrations tested. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 77.83, dF = 35, $p < 0.0001$).

In the anti-Xa assay, heparin and pentasaccharide both exhibited potent inhibition of Xa activity. This data is depicted in Figure 30. Maximal inhibition of approximately 95 % was achieved at concentrations greater than 3 μM. Heparin was more potent than pentasaccharide. The IC_{50} for heparin was determined to be 0.36 $\pm$ 0.02 μM. The IC_{50} for pentasaccharide was determined to be 2.10 $\pm$ 0.51 μM (p = 0.004 *vs.* heparin). Neither aprosulate nor GL-522-Y-1 exhibited an anti-Xa activity. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 86.78, dF = 35, $p < 0.0001$). The potencies of each agent in the amidolytic assays are listed in Table 5.

5. FVIII:C Mediated Factor Xa Generation

Figure 31 depicts the concentration response curves for the effect of heparin, aprosulate, GL-522-Y-1 and pentasaccharide on the FVIII:C mediated generation of factor Xa. All agents exhibited a concentration dependent inhibition of factor Xa amidolytic activity. The effects of the different agents were compared using the Friedman

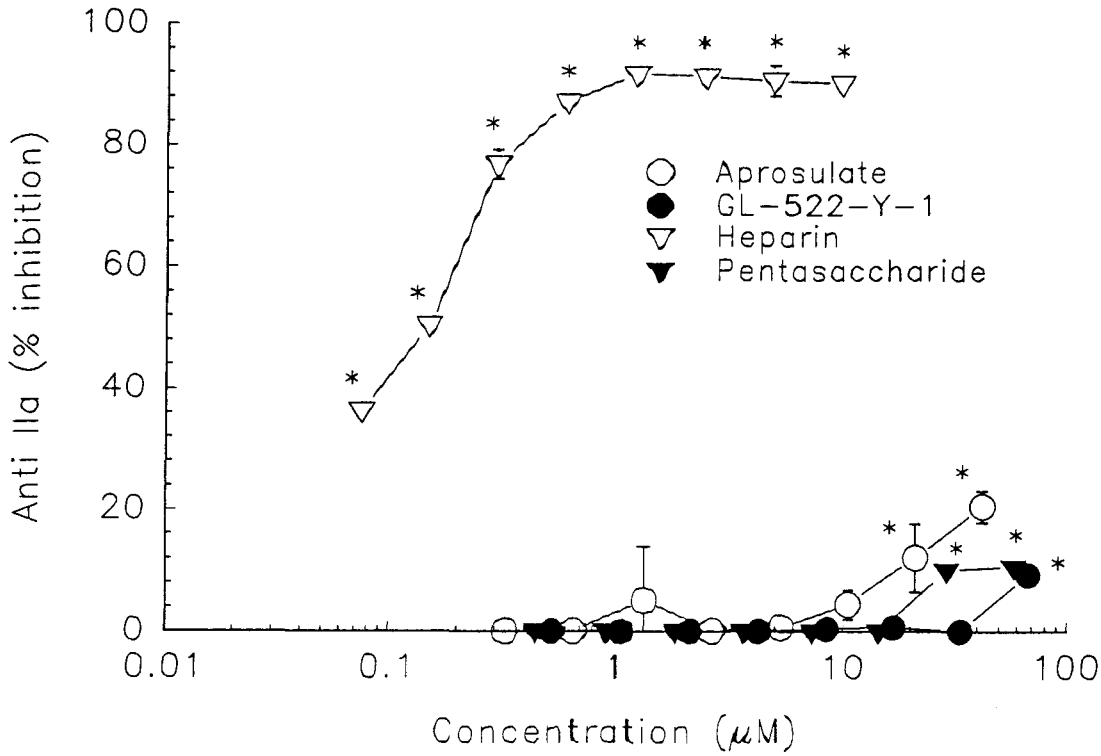


Figure 29. Comparative antiprotease effect of heparin and three synthetic heparin analogues as measured by the amidolytic anti-IIa assay. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of three observations. Thrombin was most potently inhibited by heparin. IC_{50} values for aprosulate, GL-522-Y-1, and pentasaccharide could not be calculated from this data. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 77.83, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

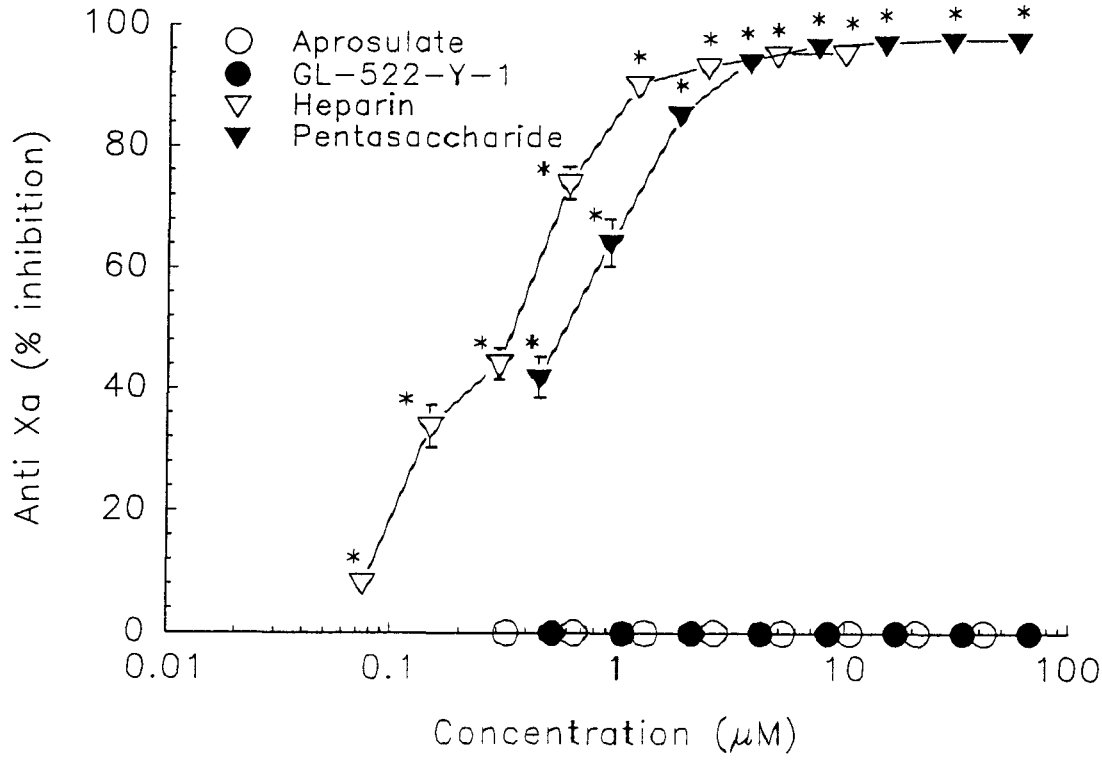


Figure 30. Comparative antiprotease effect of heparin and three synthetic heparin analogues as measured by the amidolytic anti-Xa assay. Aprosulate, GL-522-Y-1, heparin, and pentasaccharide were supplemented to normal rabbit plasma over a concentration range of 0 to 100 μg/mL. Plasma concentrations were converted to μM amounts using the molecular weight of each agent as described previously. All results represent the mean $\pm$ one standard deviation of three observations. Heparin exhibited a higher anti-Xa potency than did pentasaccharide. Aprosulate and GL-522-Y-1 did not promote the inhibition of Xa activity. A statistical difference between agents was determined using the Friedman two way ANOVA (Friedman chi-square = 86.78, dF = 35, $p < 0.0001$). Statistically significant differences between treatment and control for each agent were determined by one way ANOVA followed by the Newman-Keuls test. Results were considered to be statistically significant if $p < 0.05$. * indicates statistical significance relative to unsupplemented control plasma. Data are compiled in Tables 28 through 31.

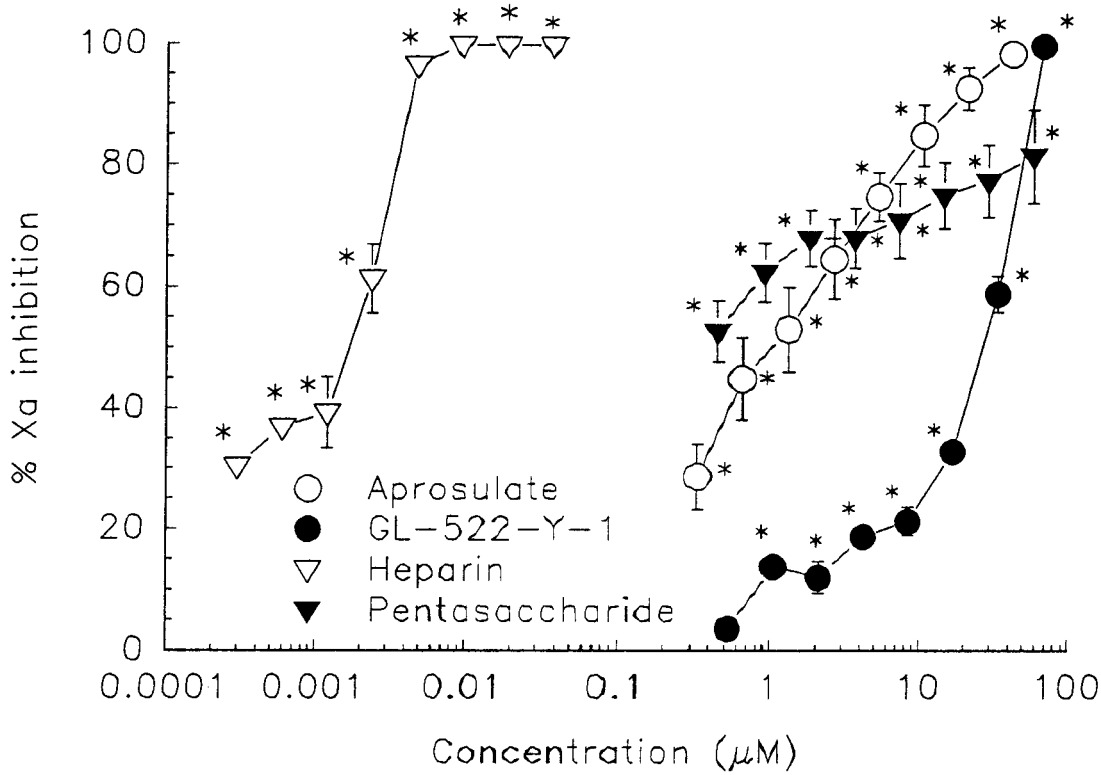


Figure 31. Comparison of the effect of heparin analogues on the FVIII:C mediated generation of factor Xa. This effect was measured with a plasma-free amidolytic assay system in which only agent, FVIII, FIXa, FX and FXa substrate were present. All results represent the mean ± one standard deviation of three observations. All agents inhibited Xa amidolytic activity in a concentration dependent manner. A statistical difference between agents was determined by Friedman two ANOVA (Friedman chi-square = 91.59, dF = 31, $p < 0.0001$). IC_{50}s were calculated in order to compare the potencies of each agent. IC_{50}s were compared using one way ANOVA followed by the Newman-Keuls multiple comparison test. Data are compiled in Tables 32 through 35.

Heparin: $IC_{50} = 0.0016 \pm 0.0003$ μM; $p < 0.05$ *vs.* GL-522-Y-1

Pentasaccharide: $IC_{50} < 0.45$ μM

Aprosulate: $IC_{50} = 1.13 \pm 0.68$ μM; $p < 0.05$ *vs.* GL-522-Y-1

GL-522-Y-1: $IC_{50} = 25.66 \pm 0.94$ μM

two way ANOVA. A significant difference was observed between the agents (Friedman chi-square = 91.59, dF = 31, $p < 0.0001$). In this system, FVIII, FIXa and FX were incubated with a phosphatidylserine containing phospholipid. The generation of factor Xa could then be measured in the absence of the extrinsic and contact pathway enzymes. Heparin strongly inhibited the generation of Xa amidolytic activity, reaching 100 % inhibition at concentrations above 10 nM. IC_{50} values were determined by extrapolation from the regression line of the linear portion of the curve. The IC_{50} for heparin was determined to be 1.6 $\pm$ 0.3 nM ($p < 0.05$ *vs.* GL-522-Y-1). Aprosulate and GL-522-Y-1 were also able to attain maximal inhibition. The IC_{50} values for these agents were significantly higher than for heparin. IC_{50} values of 1.13 $\pm$ 0.68 ($p < 0.05$) and 25.66 $\pm$ 0.94 μM ($p < 0.05$) were determined for aprosulate and GL-522-Y-1. Pentasaccharide also inhibited the generation of factor Xa in this system. The concentration response curve for pentasaccharide was much flatter than for the other agents. A change in % inhibition from 53 % to 82 % was observed over a concentration range of 0.45 to 58 μM. As a plateau in percent inhibition was not achieved with pentasaccharide over the concentration range tested, an IC_{50} value was not determined. Raw data is tabulated in Tables 32 to 35.

6. Protease Generation in Fibrinogen Deficient Plasma

The inhibition of thrombin and factor Xa generation by heparin, aprosulate, GL-522-Y-1, and pentasaccharide was determined using amidolytic assays in which protease formation was initiated by either thromboplastin (extrinsic pathway) or Actin®, an ellagic

TABLE 5

IC_{50} VALUES FOR SYNTHETIC HEPARIN ANALOGUES IN AMIDOLYTIC ANTIPROTEASE ASSAYS IN NORMAL RABBIT PLASMA

	Anti-IIa	Anti-Xa
Aprosulate	> 42 μM	> 42 μM
GL-522-Y-1	> 67 μM	> 67 μM
Heparin	0.12 ± 0.01 μM	0.36 ± 0.02 μM*
Pentasaccharide	> 58 μM	2.10 ± 0.51 μM

The potencies of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were compared in amidolytic antiprotease assays by determining the IC_{50} values for each agent from the concentration *vs.* % inhibition curves. Results represent the mean ± 1 standard deviation of 3 determinations. In cases where the agent did not promote inhibition, the highest concentration tested is presented. Statistical significance was determined using the Student's t-test ($p < 0.05$).

aXa: $^{*}p = 0.004$; heparin *vs.* pentasaccharide

acid reagent (contact system). Concentration response data for each agent is tabulated in Tables 36 to 39. Figure 32 illustrates the concentration response curves for the inhibition of Xa generation following activation of the extrinsic pathway. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 87.84, dF = 35, $p < 0.0001$). Both heparin and GL-522-Y-1 were observed to concentration dependently inhibit Xa formation. Both agents were able to completely inhibit Xa generation. Comparison of the IC_{50} values for heparin and GL-522-Y-1 showed that heparin was significantly more potent than GL-522-Y-1 (0.56 ± 0.04 *vs.* 6.57 ± 1.77 μM; t-test; $p = 0.004$). Neither aprosulate nor pentasaccharide was able to promote Xa generation inhibition following extrinsic pathway activation.

Both heparin and GL-522-Y-1 also inhibited the formation of thrombin following extrinsic pathway activation as shown in Figure 33. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 82.33, dF = 35, $p < 0.0001$). The concentration response curves were not parallel, with that of GL-522-Y-1 showing a more gradual rise. The IC_{50} for heparin (2.10 ± 0.07 μM) was significantly lower than that calculated for GL-522-Y-1 (8.00 ± 1.87 μM; t-test; $p = 0.005$). Aprosulate exhibited a weak inhibition at high concentrations. Pentasaccharide did not inhibit thrombin generation following activation of the extrinsic pathway. Heparin was relatively more potent at inhibiting Xa generation than IIa generation following extrinsic activation ($p < 0.001$). While the IC_{50} for GL-522-Y-1 mediated Xa generation inhibition was lower than that for IIa generation inhibition, this difference was not statistically significant.

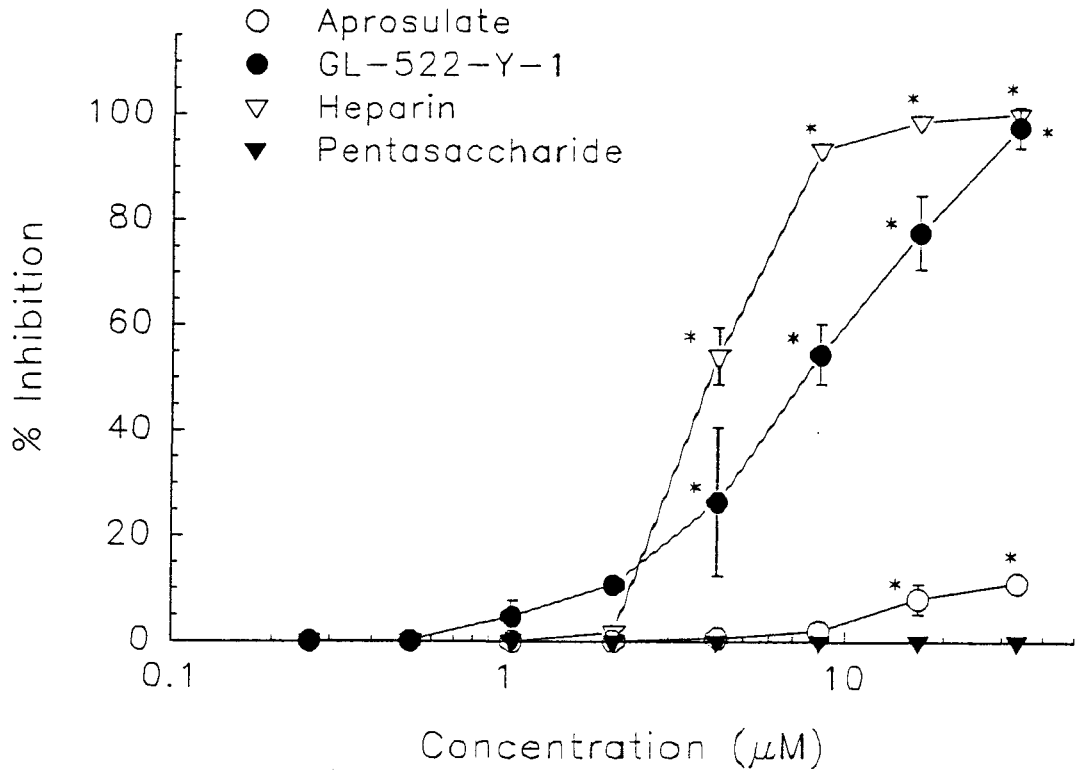


Figure 32. The effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on thrombin generation following activation of the extrinsic pathway. Thrombin generation was measured using an amidolytic assay in fibrinogen deficient plasma. Percent inhibition was calculated relative to unsupplemented control. All results represent the mean ± one standard deviation of three observations. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 82.33, dF = 35, $p < 0.0001$). Heparin was observed to more potently inhibit thrombin generation than GL-522-Y-1 in this system. Neither aprosulate not pentasaccharide exhibited an inhibitory effect in this assay. IC_{50} values were determined to compare the potency of the various agents. Statistical comparison of the IC_{50}s was made by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant *vs.* control. Data are compiled in Tables 36 through 39.

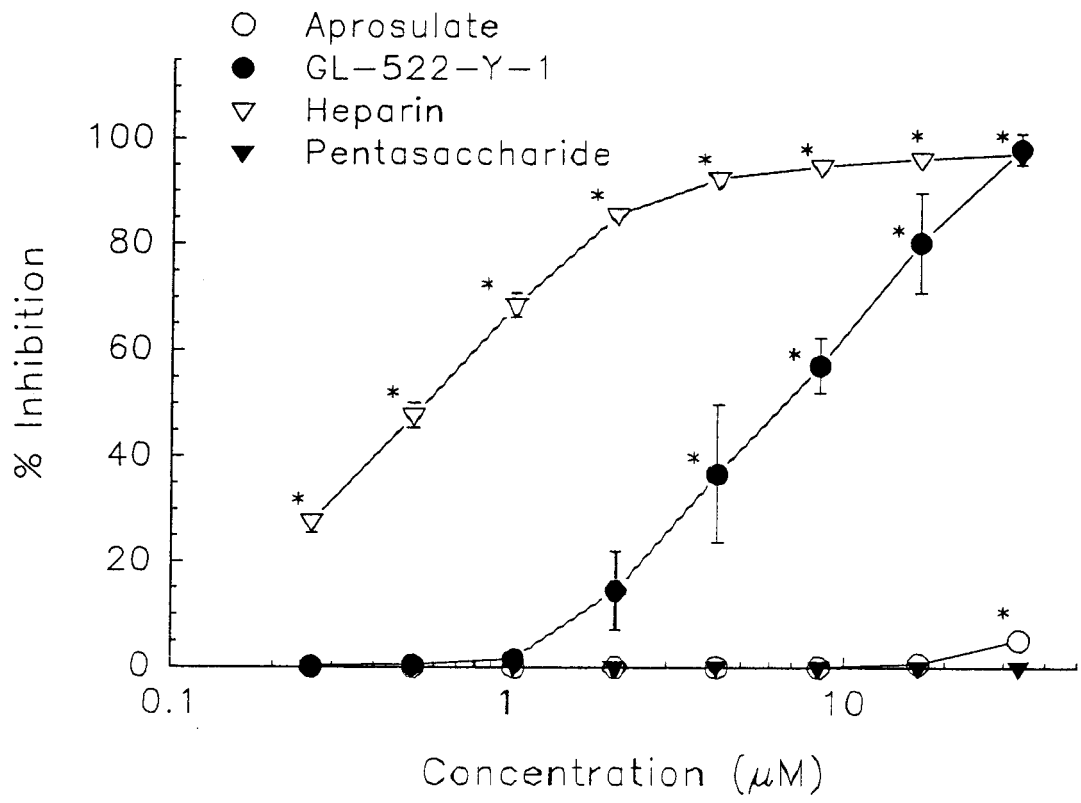


Figure 33. The effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on factor Xa generation following activation of the extrinsic pathway. Factor Xa generation was measured using an amidolytic assay in fibrinogen deficient plasma. Percent inhibition was calculated relative to unsupplemented control. IC_{50} values were determined to compare the potency of the various agents. All results represent the mean $\pm$ one standard deviation of three observations. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 87.84, dF = 35, $p < 0.0001$). Heparin was observed to more potently inhibit thrombin generation than GL-522-Y-1 in this system. Neither aprosulate not pentasaccharide exhibited an inhibitory effect in this assay. Statistical comparison of the IC_{50} values was made by one way ANOVA followed by the Newman-Keuls multiple comparison test. * $p < 0.05$ was considered statistically significant *vs.* control. Data are compiled in Tables 36 through 39.

Heparin (IC_{50} = 0.56 $\pm$ 0.04 μM) *vs.* GL-522-Y-1 (IC_{50} = 6.57 $\pm$ 1.77 μM) ; p = 0.004

Thrombin generation following intrinsic pathway activiation was inhibited by GL-522-Y-1, aprosulate and heparin in a concentration dependent manner as shown in Figure 34. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 85.76, **dF** = 35, $p < 0.0001$). GL-522-Y-1 was the most efficacious agent, completely inhibiting thrombin generation at concentrations above 10 μM. Inhibition by aprosulate plateaued at approximately 75 % over the concentration range tested. The potency of these agents was not statistically different (3.51 $\pm$ 1.22 μM aprosulate *vs.* 2.89 $\pm$ 0.83 μM GL-522-Y-1; $p = 0.507$). Heparin exhibited less activity in this assay with maximal inhibition peaking at 30 % at concentrations greater than 4 μM ($IC_{50} \approx 0.7$ μM).

All agents were active at inhibiting factor Xa generation following intrinsic pathway activation. A statistically significant difference between the effects of the various agents was observed (Friedman chi-square = 101.50, **dF** = 35, $p < 0.0001$). Both aprosulate and GL-522-Y-1 inhibited Xa generation greater than 90 % relative to unsupplemented control. The potencies of these agents were not statistically different (IC_{50} = 1.28 $\pm$ 0.66 μM aprosulate *vs.* 1.46 $\pm$ 0.33 μM GL-522-Y-1; t-test; p = 0.694). Pentasaccharide also exhibited a concentration dependent inhibition of Xa generation, though weaker than either aprosulate or GL-522-Y-1. IC_{25} values were calculated assuming that pentasaccharide would reach the same maximal level of inhibition as aprosulate or GL-522-Y-1 at the appropriate concentration. No statistical differences were noted between the IC_{25} values for aprosulate and GL-522-Y-1 (0.95 $\pm$ 0.43 *vs.* 0.93 $\pm$ 0.32 μM, respectively). Both were significantly lower than that

determined for pentasaccharide (12.44 ± 6.19 μM; $p < 0.05$). This data is depicted in Figure 35.

The potencies of the test agents in the various systems are compared in Table 6. Following activation of the intrinsic pathway, aprosulate was more potent at inhibiting the generation of Xa than IIa (IC_{50} = 1.28 ± 0.66 *vs.* 3.51 ± 1.22 μM; $p = 0.050$). The IC_{50} for GL-522-Y-1 mediated Xa generation inhibition was lower than for IIa generation, though not statistically significant. Heparin was more efficacious at inhibiting Xa generation compared to IIa generation. In the intrinsically activated systems, heparin was more than 100 fold more potent at inhibiting Xa generation compared with thrombin generation. Overall, aprosulate and GL-522-Y-1 were more potent at inhibiting intrinsically activated generation than following extrinsic activation. Pentasaccharide demonstrated minimal ability to inhibit protease generation in these systems. IC_{50} values for pentasaccharide were greater than 33 μM in all four systems.

7. Protease Generation in Non-plasmatic Systems

The inhibition of thrombin and factor Xa generation by aprosulate, GL-522-Y-1, heparin and pentasaccharide was examined in non-plasmatic systems in which native and activated prothrombin complex concentrates provided the necessary coagulation factors. Two different prothrombin complex concentrates were used, one containing factor VII (Konyne®) and one containing factor VIIa (FEIBA®). Figure 36 depicts the concentration response curves for thrombin generation inhibition in the FEIBA® based system. Both heparin and GL-522-Y-1 were able to concentration dependently inhibit thrombin

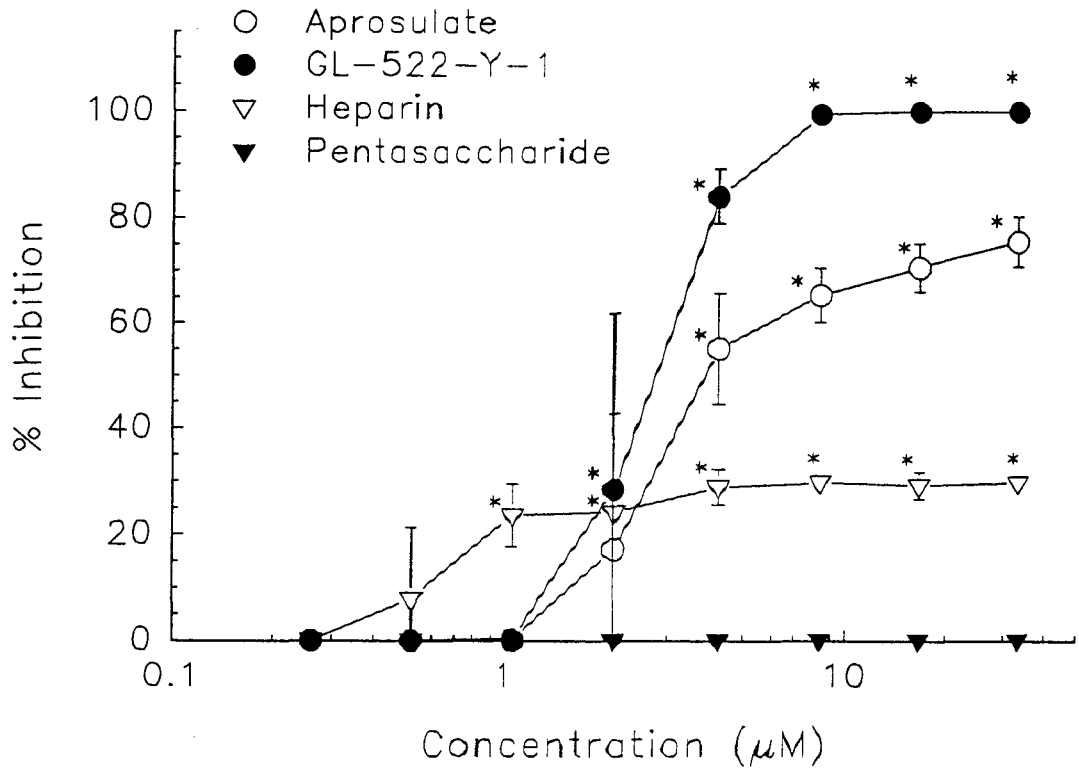


Figure 34. The effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on thrombin generation following activation of the intrinsic pathway. Thrombin generation was measured using an amidolytic assay in fibrinogen deficient plasma. Percent inhibition was calculated relative to unsupplemented control. IC_{50} values were determined to compare the potency of the various agents. All results represent the mean $\pm$ one standard deviation of three observations. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 85.76, dF = 35, $p < 0.0001$). Statistical comparison of the IC_{50} values was made by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant *vs.* control. Data are compiled in Tables 36 through 39.

Aprosulate (IC_{50} = 3.51 $\pm$ 1.22 μM) *vs.* GL-522-Y-1 (IC_{50} = 2.89 $\pm$ 0.83 μM) ; p = 0.507

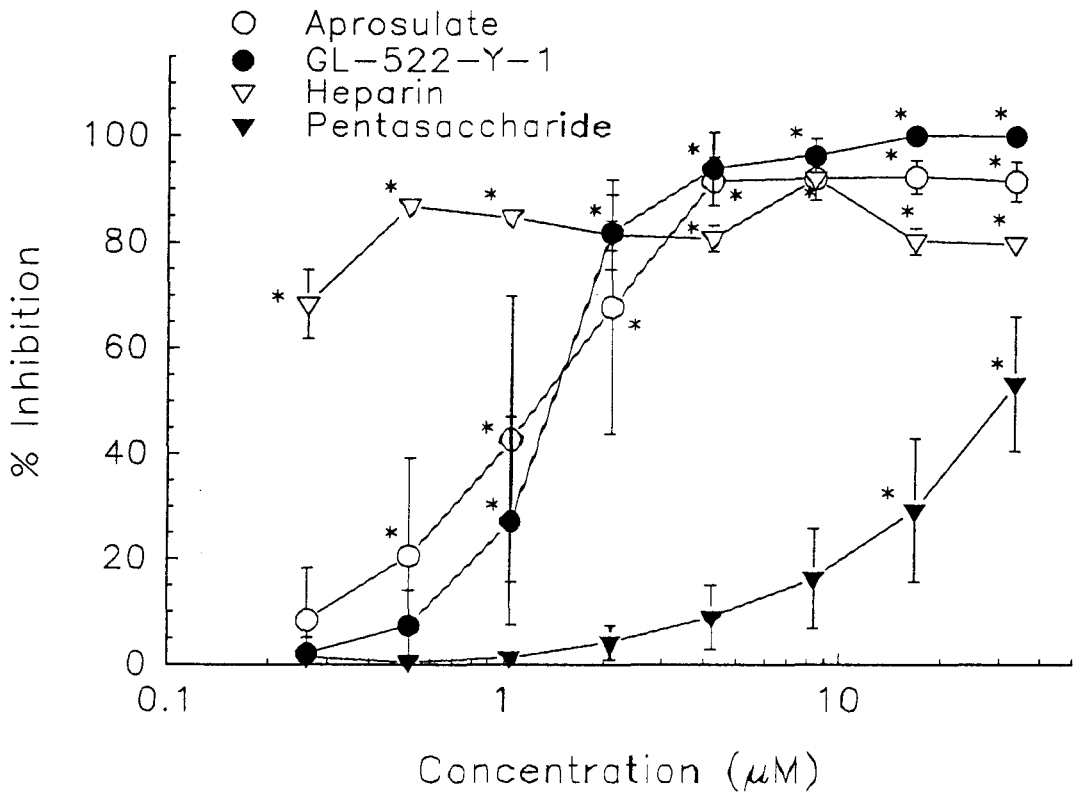


Figure 35. The effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on factor Xa generation following activation of the intrinsic pathway. Factor Xa generation was measured using an amidolytic assay in fibrinogen deficient plasma. Percent inhibition was calculated relative to unsupplemented control. IC_{50} values were determined to compare the potency of the various agents. All results represent the mean $\pm$ one standard deviation of three observations. A statistically significant difference between the effects of the various agents was noted (Friedman chi-square = 101.50, dF = 35, $p < 0.0001$). Statistical comparison of the IC_{50} values was made by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant *vs.* control. Data are compiled in Tables 36 through 39.

Aprosulate (IC_{50} = 1.28 $\pm$ 0.66 μM) *vs.* GL-522-Y-1 (IC_{50} = 1.46 $\pm$ 0.33 μM) ; p = 0.694

TABLE 6

POTENCY EVALUATION OF HEPARIN ANALOGUES IN PROTEASE GENERATION SYSTEMS IN FIBRINOGEN DEFICIENT PLASMA

	Extrinsic		Intrinsic	
	IIa	Xa	IIa	Xa
Aprosulate	> 33.3	> 33.3	3.51 ± 1.22	1.28 ± 0.66
GL-522-Y-1	8.00 ± 1.87	6.57 ± 1.77	2.89 ± 0.83	1.46 ± 0.33
Heparin	2.10 ± 0.07	0.56 ± 0.04	> 33.3	< 0.26
Pentasaccharide	> 33.3	> 33.3	> 33.3	> 33.3

IC_{50} values were determined by extrapolation from the best fit regression lines of the straight line portion of the concentration response curves.

formation in this system. Both heparin and GL-522-Y-1 reached levels of thrombin inhibition of approximately 85 %. Heparin was observed to be approximately 10 fold more potent than GL-522-Y-1. Neither aprosulate nor pentasaccharide demonstrated substantial inhibition of thrombin generation in this system.

Figure 37 depicts the concentration response curves for factor Xa generation in the FEIBA® based system. GL-522-Y-1 was the only agent to demonstrate significant inhibition of Xa generation. At concentrations greater than 10 μM GL-522-Y-1, more than 75 % inhibition was observed. Neither aprosulate, pentasaccharide, nor heparin produced any inhibition of Xa generation.

Figure 38 depicts the concentration response curves for the inhibition of thrombin generation in the Konyne® based system. Aprosulate, GL-522-Y-1 and heparin all inhibited thrombin generation in a dose-dependent fashion. Heparin produced the most potent inhibition of thrombin generation, reaching 50 % inhibition at a concentration of 3 μM. Aprosulate and GL-522-Y-1 produced somewhat weaker inhibitory effects than heparin. Neither agent reached a level of 50 % inhibition at concentrations up to 70 μM. Pentasaccharide did not produce an inhibition of thrombin generation.

Figure 39 depicts the concentration response curves for the inhibition of factor Xa generation in the Konyne® based system. As in the other non-plasmatic systems, GL-522-Y-1 and heparin produced a concentration dependent inhibition of Xa generation. The slope of the heparin response curve was flatter than that of the GL-522-Y-1 response curve. Neither aprosulate nor pentasaccharide were able to inhibit Xa generation.

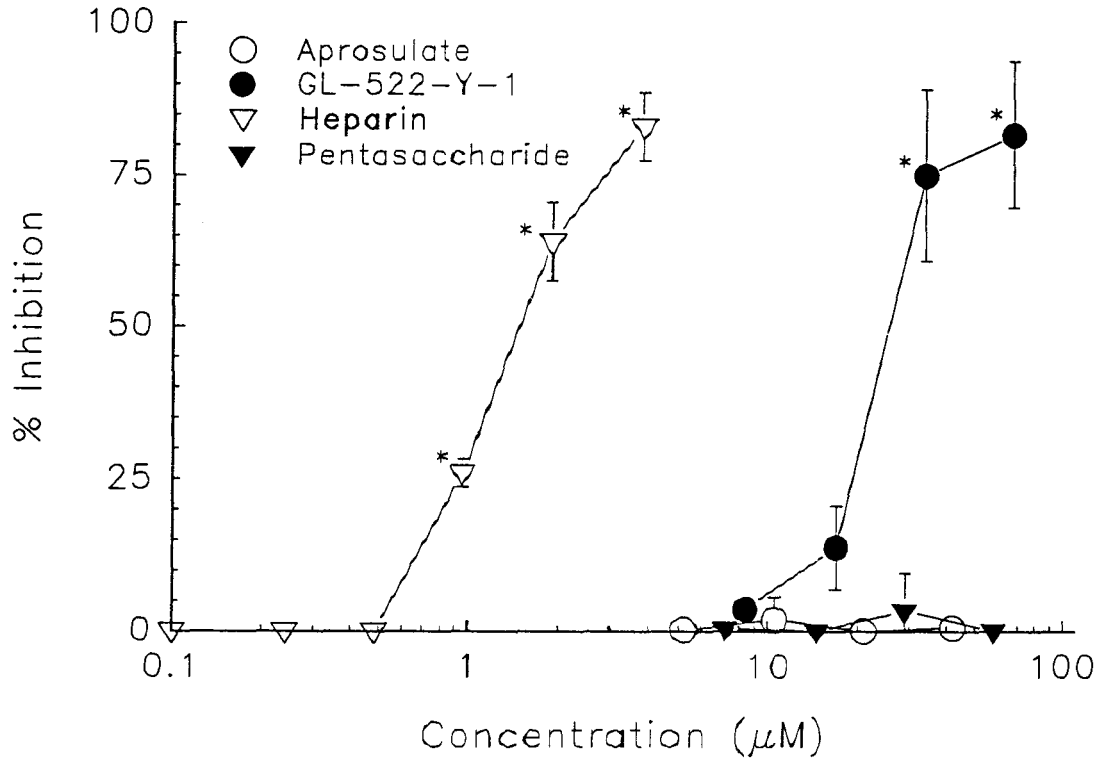


Figure 36. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the inhibition of thrombin generation in a FEIBA® based system. All results represent the mean (± one standard deviation) percent inhibition relative to unsupplemented control of three determinations. Heparin more potently inhibited thrombin generation in this system than did GL-522-Y-1. Neither aprosulate nor pentasaccharide exhibited an inhibitory effect in this assay. Statistical comparisons versus control were made by one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA,

aprosulate; $p = 0.704$
GL-522-Y-1; $p < 0.001$
heparin; $p < 0.001$
pentasaccharide; $p = 0.548$

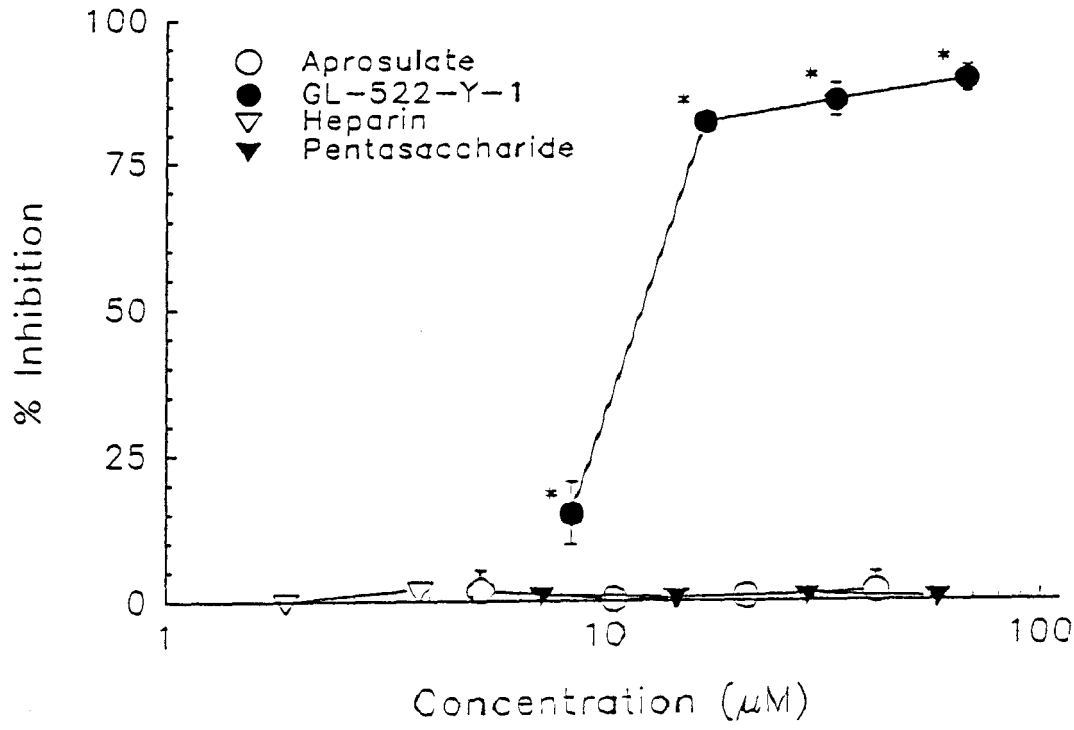


Figure 37. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the inhibition of factor Xa generation in a FEIBA® based system. All results represent the mean (± one standard deviation) percent inhibition relative to unsupplemented control of three determinations. GL-522-Y-1 was the only agent to exhibit an inhibitory effect in this assay. Statistical comparisons versus control were made by one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA,

aprosulate; $p = 0.823$
GL-522-Y-1; $p < 0.001$
heparin; $p = 0.013$
pentasaccharide; $p = 0.911$

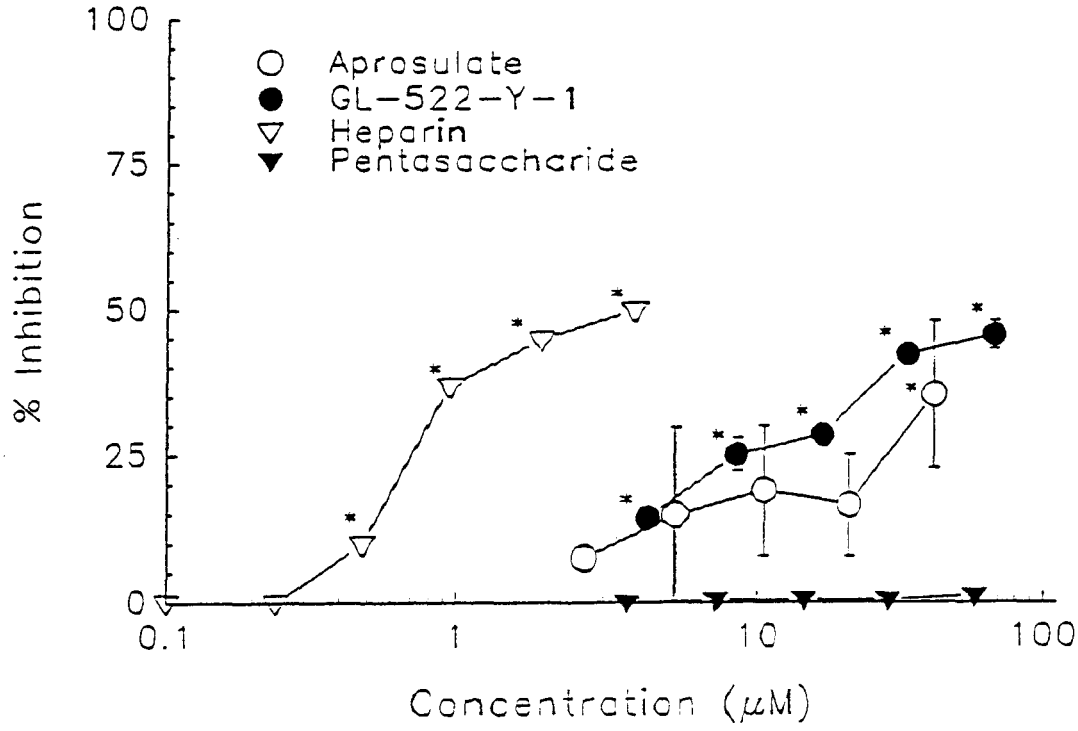


Figure 38. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the inhibition of thrombin generation in a Konyne® based system. All results represent the mean (± one standard deviation) percent inhibition relative to unsupplemented control of three determinations. Thrombin generation was inhibited in this system with a rank order potency of heparin > GL-522-Y-1 > aprosulate. Pentasaccharide did not exhibit an inhibitory effect in this assay. Statistical comparisons versus control were made by one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA,

aprosulate; $p = 0.017$
GL-522-Y-1; $p < 0.001$
heparin; $p < 0.001$
pentasaccharide; $p = 0.543$

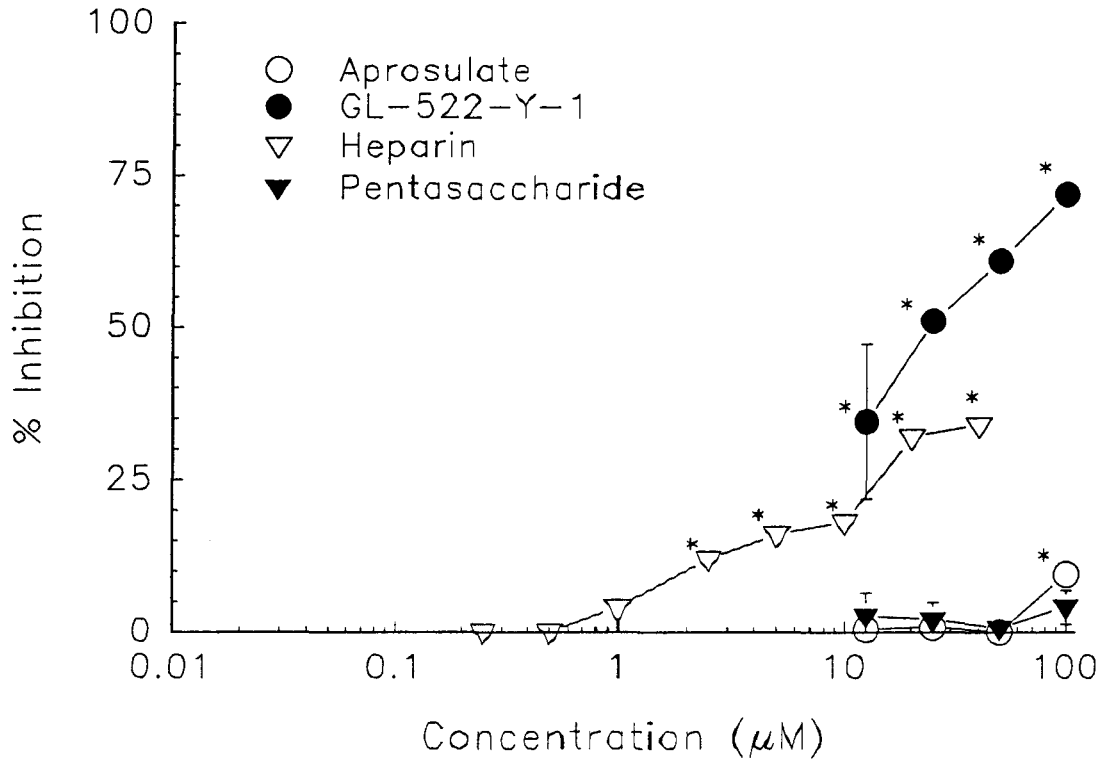


Figure 39. Effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the inhibition of factor Xa generation in a Konyne® based system. All results represent the mean (± one standard deviation) percent inhibition relative to unsupplemented control of three determinations. Heparin and GL-522-Y-1 exhibited an inhibitory effect in this assay. Aprosulate and pentasaccharide displayed no effect on Xa generation. Statistical comparisons versus control were made by one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA,

aprosulate; $p < 0.001$
GL-522-Y-1; $p < 0.001$
heparin; $p < 0.001$
pentasaccharide; $p = 0.352$

8. Effect of Analogues on Heparan Sulfate / Dermatan Sulfate Synthesis in Cell Culture

The effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the modulation of glycosaminoglycan synthesis by endothelial cells was examined in cultured rabbit endothelial cells. Agents were supplemented to the culture media for 18 hours prior to analysis. Glycosaminoglycan synthesis was quantitated by measuring the amount of ^{35}S present in the chondroitin sulfate and heparan sulfate bands of the culture media and cell homogenates separated by gel electrophoresis. ^{35}S was measured by scintillation counting. Concentration response data is presented in Tables 40 to 43.

Figure 40 depicts the concentration-response curves for aprosulate on the synthesis of chondroitin and heparan sulfates in cultured rabbit endothelial cells. In panel A it is observed that incubation of endothelial cells with aprosulate concentration dependently increased the amount of chondroitin sulfate in the culture media. None of the levels measured in the aprosulate treated cultures reached statistical significance relative to control. Panel B depicts the concentration-response curves for the synthesis of heparan sulfate as measured in the culture media of rabbit endothelial cells. A concentration-dependent increase in CPM's was observed. A concentration of 41 μM was determined to double the amount of CPM's relative to baseline. Aprosulate increased the amount of chondroitin sulfate associated with the endothelial cells at concentrations greater than 4.2 μM (panel C). Aprosulate had no effect on cell associated heparan sulfate levels (panel D).

The effect of GL-522-Y-1 on the synthesis of glycosaminoglycans by cultured

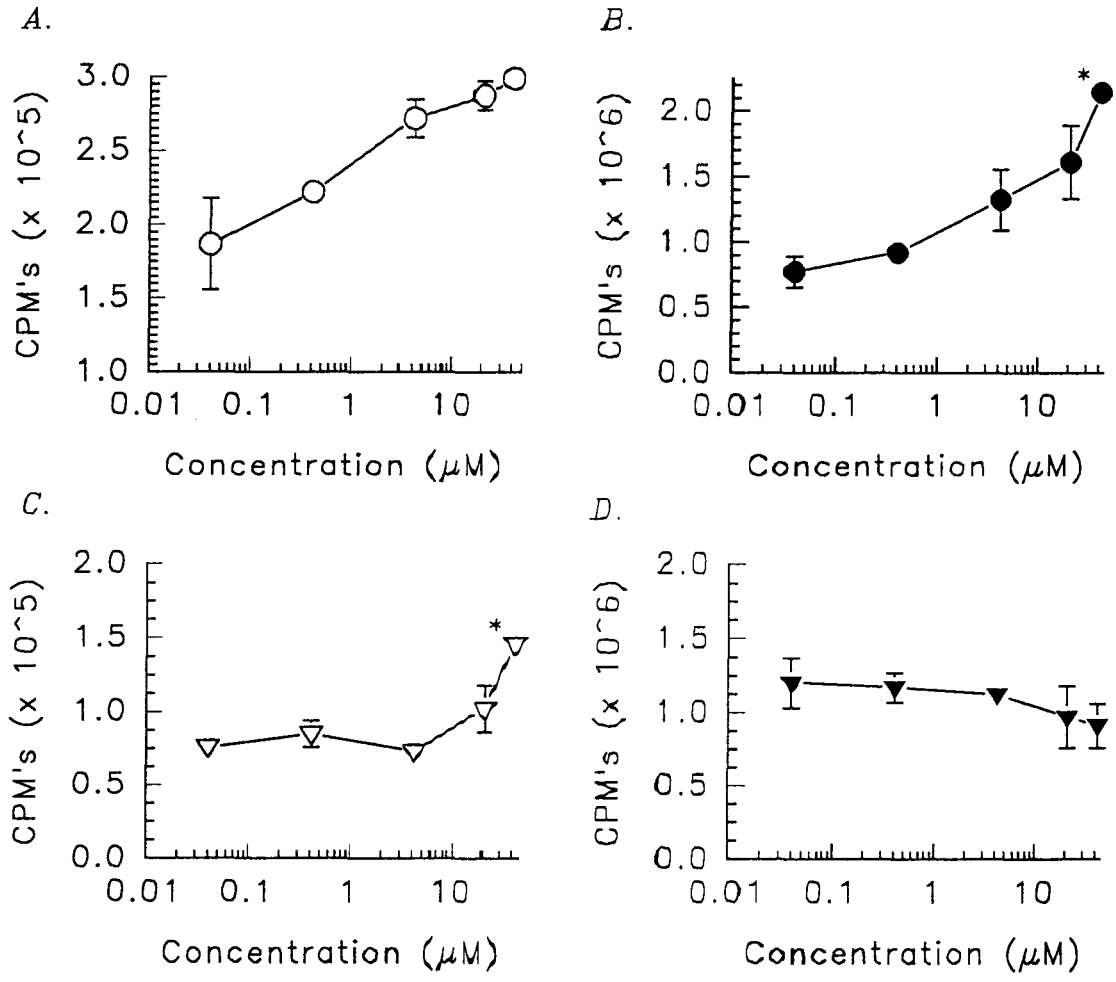


Figure 40. Effect of aprosulate on glycosaminoglycan synthesis in endothelial cell culture. Concentration-response curves for the effect of aprosulate on the synthesis of chondroitin sulfate in culture media (panel A), heparan sulfate in culture media (panel B), chondroitin sulfate associated with endothelial cells (panel C), and heparan sulfate associated with endothelial cells (panel D) are depicted. The results represent the mean (± SEM) C.P.M.'s of three independent measurements as determined by scintillation counting. Aprosulate was observed to concentration dependently increase the synthesis of chondroitin sulfate and heparan sulfate as measured in the culture media. The synthesis of chondroitin sulfate associated with the endothelial cells was increased at concentrations greater than 10 μM. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls test. (CS Med; dF = 17, F = 10.5, p = 0.0005. HS Med; dF = 17, F = 21.2, $p < 0.0001$. CS Cell; dF = 17, F = 25.4, $p < 0.0001$. HS Cell; dF = 17, F = 2.56, $p = 0.0847$). *$p < 0.05$ *vs.* unsupplemented control was considered statistically significant. Data are compiled in Table 40.

endothelial cells is depicted in Figure 41. GL-522-Y-1 supplementation did not have an effect on the amount of chondroitin sulfate measured in the cell culture media. Heparan sulfate was greatly increased by incubation with GL-522-Y-1. In panel B it is observed that GL-522-Y-1 produced a concentration-dependent increase in heparan sulfate over a concentration range of 0.07 to 33.5 μM. Increase in the GL-522-Y-1 concentration above 33.5 μM did not further enhance heparan sulfate production. Heparan sulfate levels at 33.5 and 67 μM were signifcantly greater than control. A concentration of 4.5 μM was determined to double the number of CPM's relative to unsupplemented control. As seen in panels C and D, GL-522-Y-1 also increased the synthesis of chondroitin and heparan sulfate associated with the endothelial cells. In both instances, significant increases in synthesis were observed at concentrations above 0.67 μM.

Figure 42 depicts the concentration-response curves for the modulation of glycosaminoglycan synthesis by heparin. Heparin did not significantly alter the production of chondroitin sulfate as measured in the culture media or associated with the endothelial cells or the production of heparan sulfate associated with the endothelial cells. As depicted in panel B, heparin concentration-dependently increased the amount of heparan sulfate released to the culture media. Concentrations greater than 0.95 μM were observed to significantly increase heparan sulfate production compared to control. A concentration of 3.3 μM was determined to double the number of CPM's relative to unsupplemented control.

The concentration-response curves for the modulation of glycosaminoglycan synthesis in endothelial cell culture by pentasaccharide are depicted in Figure 43.

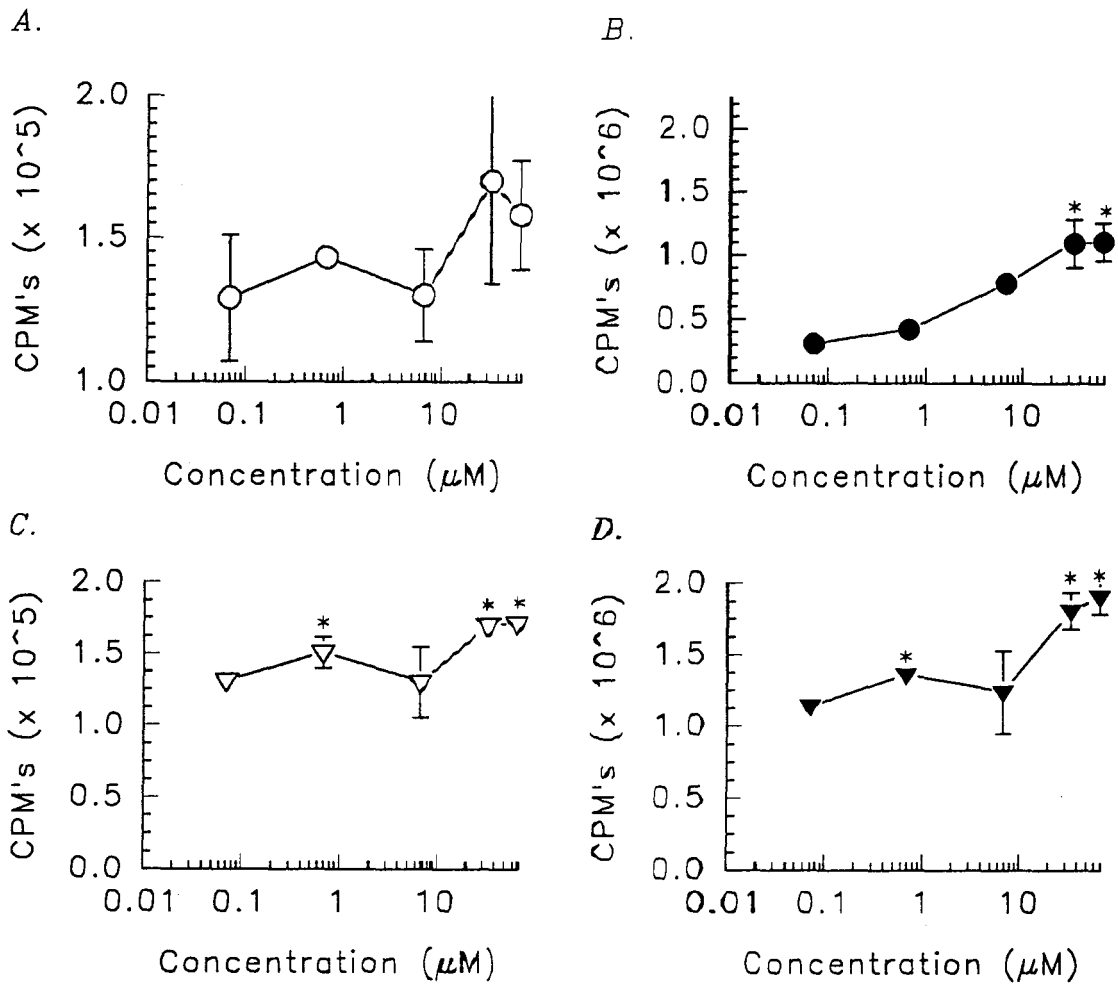


Figure 41. Effect of GL-522-Y-1 on glycosaminoglycan synthesis in endothelial cell culture. Concentration-response curves for the effect of GL-522-Y-1 on the synthesis of chondroitin sulfate in culture media (panel A), heparan sulfate in culture media (panel B), chondroitin sulfate associated with endothelial cells (panel C), and heparan sulfate associated with endothelial cells (panel D) are depicted. The results represent the mean (± SEM) C.P.M.'s of three independent measurements as determined by scintillation counting. GL-522-Y-1 significantly increased the production of chondroitin sulfate and heparan sulfate associated with the endothelial cells and heparan sulfate content in the culture media at concentrations above 10 μM. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls test. (CS Med; dF = 17, F = 1.80, $p = 0.1867$. HS Med; dF = 17, F = 31.6, $p < 0.0001$. CS Cell; dF = 17, F = 16.5, $p < 0.0001$. HS Cell; dF = 17, F = 28.3, $p < 0.0001$). *$p < 0.05$ *vs.* unsupplemented control was considered statistically significant. Data are compiled in Table 41.

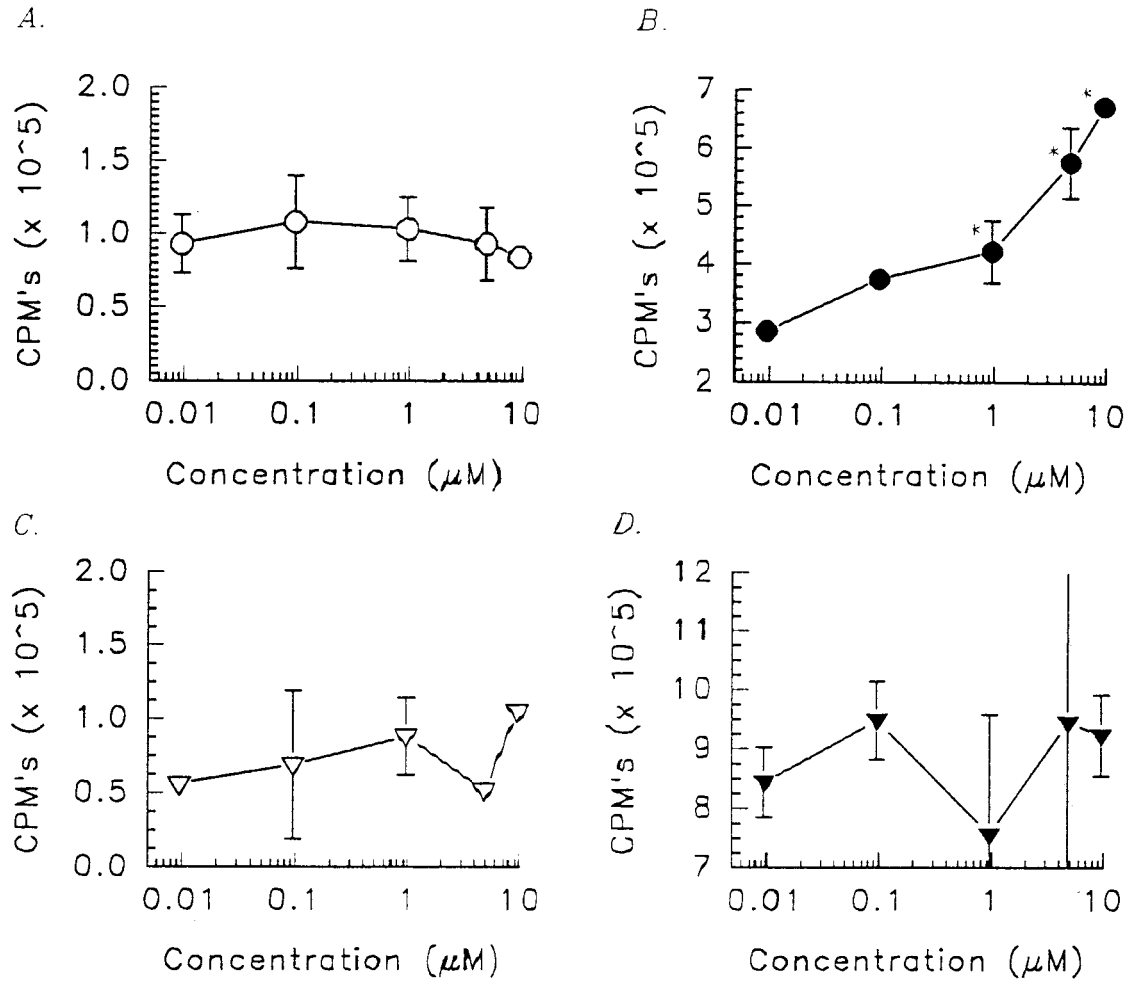


Figure 42. Effect of heparin on glycosaminoglycan synthesis in endothelial cell culture. concentration-response curves for the effect of heparin on the synthesis of chondroitin sulfate in culture media (panel A), heparan sulfate in culture media (panel B), chondroitin sulfate associated with endothelial cells (panel C), and heparan sulfate associated with endothelial cells (panel D) are depicted. The results represent the mean (± SEM) C.P.M.'s of three independent measurements as determined by scintillation counting. Heparin dose-dependently increased heparan sulfate content as measured in the culture media. Statistically significant increases were observed at concentrations greater than 1 μM. Chondroitin sulfate synthesis was not effected by heparin supplementation. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls test (CS Med; dF = 17, F = 0.959, p = 0.4793. HS Med; dF = 17, F = 59.9, $p < 0.0001$. CS Cell; dF = 17, F = 2.44, p = 0.0950. HS Cell; dF = 17, F = 0.655, p = 0.6636). $^{*}p < 0.05$ *vs.* unsupplemented control was considered statistically significant. Data are compiled in Table 42.

Pentasaccharide did not significantly effect the production of chondroitin or heparan sulfate as measured in the culture media (panels A and B). While trends for increasing levels of chondroitin and heparan associated with the endothelial cells were observed, no statistically significant differences relative to unsupplemented control were determined.

9. Studies in Native Human Whole Blood

Whole blood was drawn into syringes containing representative amounts of aprosulate, pentasaccharide, or GL-522-Y-1 to result in a final concentration of 25 μM. The anticoagulant activity of these agents was determined by ACT and TEG. Figure 44 depicts the results of the celite ACT analysis. The effect of heparin at 0.25 μM was included for comparison. All agents were observed to significantly increase the clotting time compared with saline supplementation. A baseline clotting time 110 $\pm$ 12 seconds was observed. Pentasaccharide and GL-522-Y-1 prolonged clotting times to nearly 200 seconds. Aprosulate produced a significantly higher clotting time compared to pentasaccharide and GL-522-Y-1 ($p < 0.05$). Supplementation of 25 μM aprosulate resulted in a clotting time of 290 seconds. Heparin was not included in the statistical analysis due to the 100 fold lower concentration used.

Two parameters of the TEG were used to compare the effects of aprosulate, GL-522-Y-1, or pentasaccharide supplementation. Figure 45 depicts the effect on K-time, or time to standard clot firmness. The K-time of saline supplemented whole blood was measured as 10.5 mm. Aprosulate doubled the K-time ($p < 0.05$). GL-522-Y-1 did not prolong the K-time relative to control. The K-time for pentasaccharide supplemented

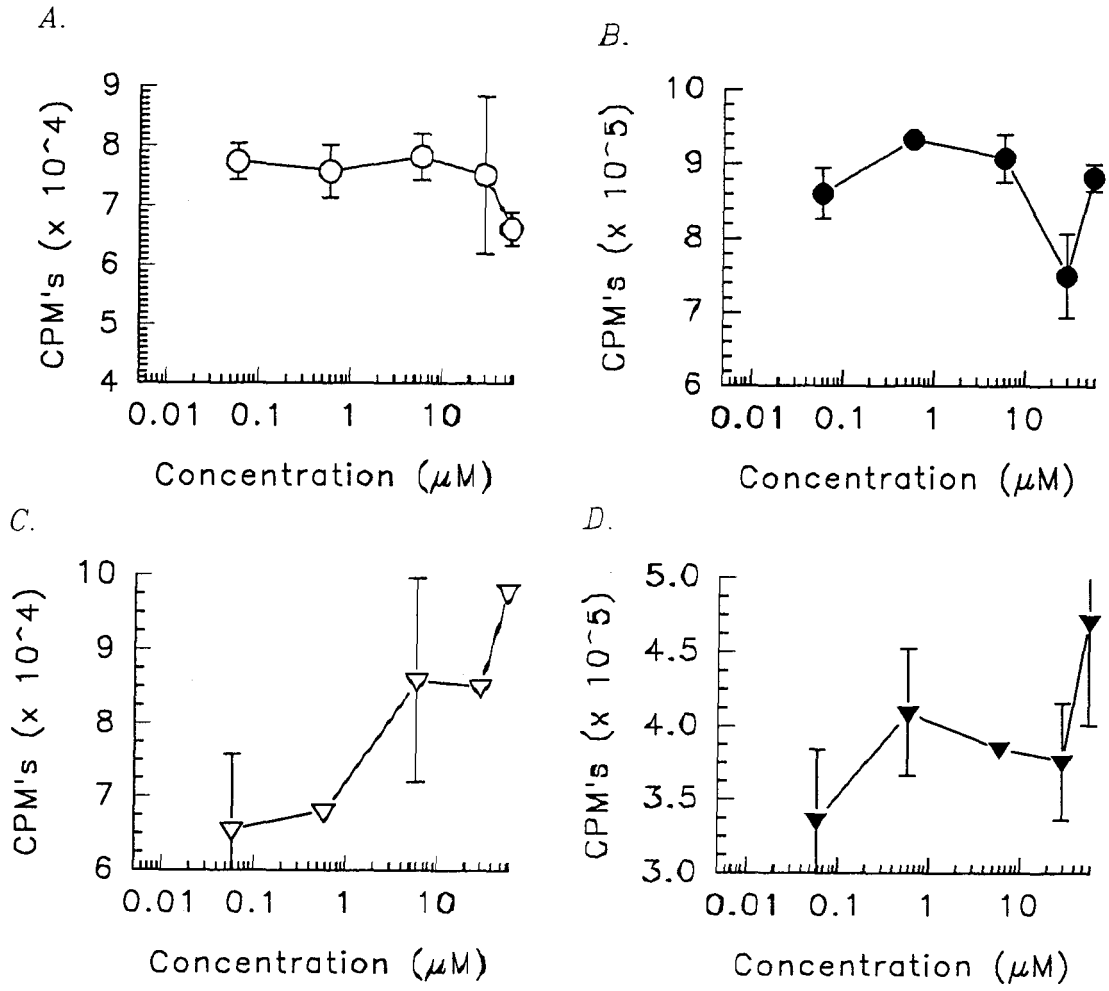


Figure 43. Effect of pentasaccharide on glycosaminoglycan synthesis in endothelial cell culture. Concentration-response curves for the effect of pentasaccharide on the synthesis of chondroitin sulfate in culture media (panel A), heparan sulfate in culture media (panel B), chondroitin sulfate associated with endothelial cells (panel C), and heparan sulfate associated with endothelial cells (panel D) are depicted. The results represent the mean (± SEM) C.P.M.'s of three independent measurements as determined by scintillation counting. Pentasaccharide supplementation was not observed to effect the synthesis of heparan sulfate. Chondroitin sulfate associated with the cells was observed to be concentration dependently increased, though statistical significance was not achieved. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls test. (CS Med; dF = 17, F = 0.964, p = 0.4770. HS Med; dF = 17, F = 11.0, p = 0.0004. CS Cell; dF = 17, F = 9.86, p = 0.0006. HS Cell; dF = 17, F = 3.63, p = 0.0313). $^{*}p < 0.05$ *vs.* unsupplemented control was considered statistically significant. Data are compiled in Table 43.

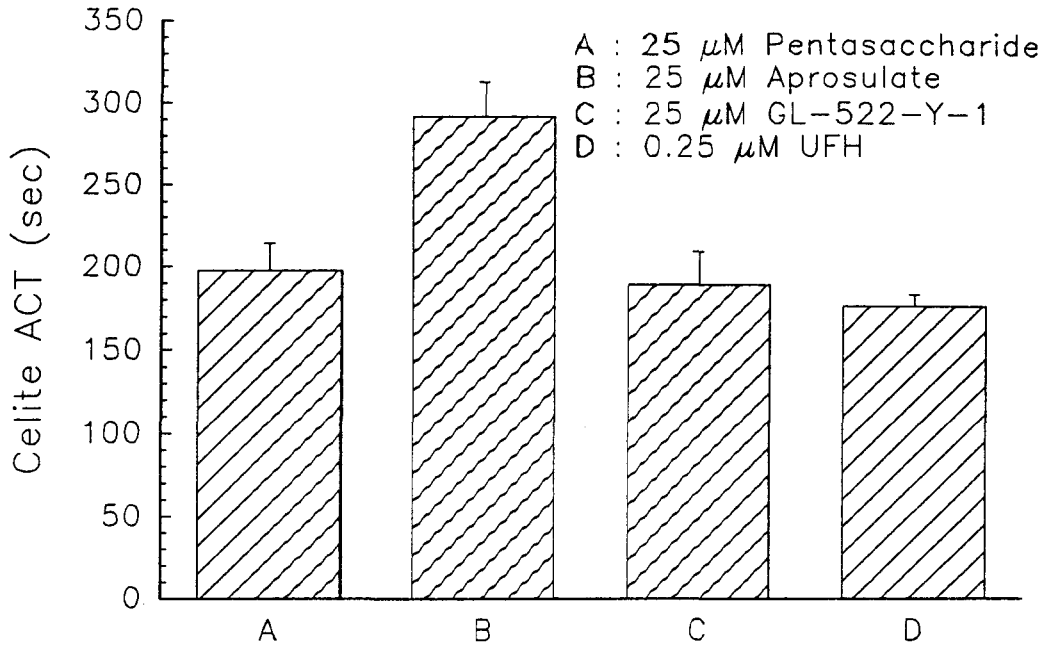


Figure 44. Anticoagulant effect of aprosulate, GL-522-Y-1, heparin and pentasaccharide on freshly drawn whole blood supplemented with each agent at a final concentration of 25 μM (0.25 μM for heparin) using the celite ACT. Blood from 10 volunteers was used to test each agent. All results represent the mean $\pm$ one standard deviation. A control clotting time of 110 $\pm$ 12 seconds was observed. Statistical comparisons were made by one way ANOVA followed by the Newman Keuls test. All treatments produced significant increases in clotting time compared to saline supplementation. Aprosulate produced the strongest anticoagulant action of the synthetic analogues, increasing clotting times approximately 100 seconds longer than pentasaccharide or GL-522-Y-1.

Aprosulate *vs.* pentasaccharide; $p < 0.05$
Aprosulate *vs.* GL-522-Y-1; $p < 0.05$

samples was not determinable as the blood did not begin to clot over the 45 minute test period.

Figure 46 depicts the effects of heparin analogue supplementation on the angle measured on the TEG tracings. The angle in control samples was 47.4 ± 4.1°. Both aprosulate and GL-522-Y-1 significantly reduced the angle relative to control ($p < 0.05$) indicating a slower clot formation. The angle in aprosulate supplemented blood was 26.0 ± 2.0° compared with 43.8 ± 4.1° for GL-522-Y-1 supplemented blood. The angle determined from aprosulate treated samples was significantly lower than that determined for GL-522-Y-1 treated blood. ($p < 0.05$). The angle was not determinable for pentasaccharide treated samples.

10. Studies in Platelet Based Systems

a. Agonist Induced Platelet Aggregation

The effects of aprosulate, GL-522-Y-1, heparin and pentasaccharide on agonist induced platelet aggregation were examined in a platelet rich plasma system in response to epinephrine, ADP, arachidonic acid, thrombin, and collagen. All test agents were supplemented to the plasma at a final concentration of 10 μg/mL. Agonists were used at concentrations listed in "Materials and Methods". Maximal percent aggregation as well as the slope of the aggregation response were determined from the tracings. These results are tabulated in Tables 44 and 45. Figure 47 depicts a comparison of the effect of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on thrombin induced aggregation. In panel A, a comparison of the aggregation response is observed. Thrombin caused a

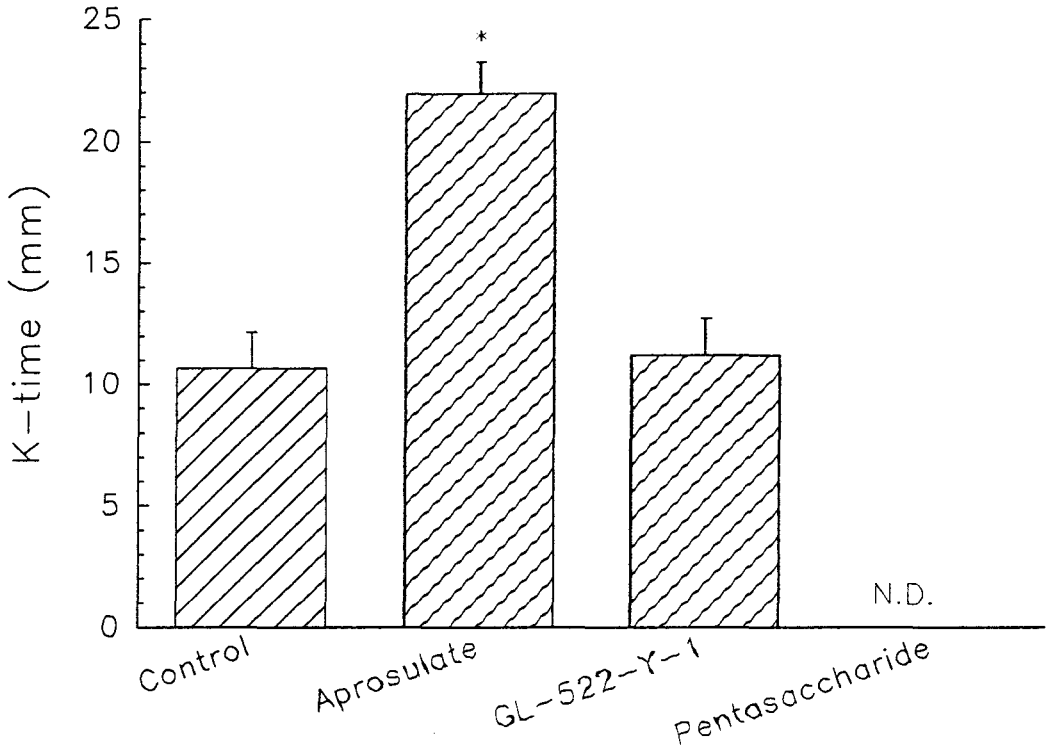


Figure 45. Effect of heparin analogues on the TEG following *ex vivo* supplementation. TEG analysis was performed on freshly drawn blood supplemented with either aprosulate, GL-522-Y-1, or pentasaccharide at a final concentration of 25 μM. The results represent the mean $\pm$ one standard deviation of the K-time determined on 10 volunteers in each treatment group. Statistical comparisons were made by one way ANOVA followed by the Newman Keuls multiple comparison test. GL-522-Y-1 did not prolong the K-time relative to control. Aprosulate doubled the K-time relative to saline supplemented controls. Blood supplemented with pentasaccharide did not clot over the course of the experiment, thereby preventing a determination of K-time on these samples.

Aprosulate *vs.* control; $p < 0.05$
Aprosulate *vs.* GL-522-Y-1; $p < 0.05$
GL-522-Y-1 *vs.* control; $p > 0.05$

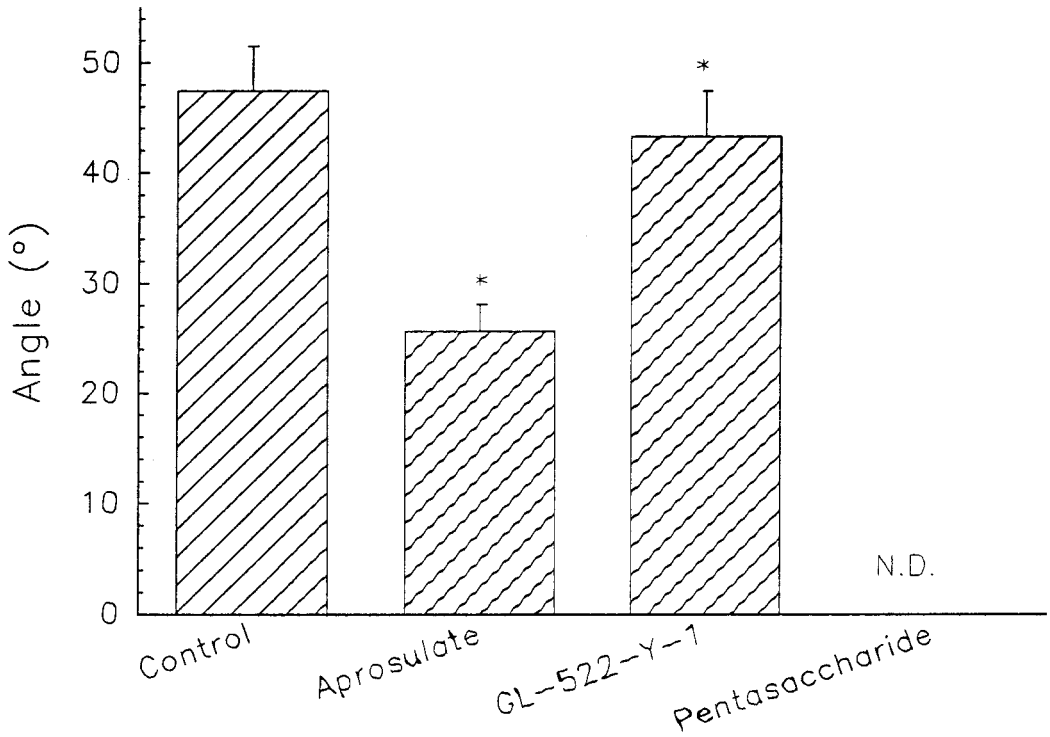


Figure 46. Effect of heparin analogues on the TEG following *ex vivo* supplementation. TEG analysis was performed on freshly drawn blood supplemented with either aprosulate, GL-522-Y-1, or pentasaccharide at a final concentration of 25 μM. The results represent the mean $\pm$ one standard deviation of the angle determined on 10 volunteers in each treatment group. Statistical comparisons were made by one way ANOVA followed by the Newman Keuls multiple comparison test. Aprosulate supplementation resulted in a larger decrease in angle than did supplementation of GL-522-Y-1. The angle could not be calculated on pentasaccharide supplemented samples as the blood did not clot over the course of the experiment.

Aprosulate *vs.* control; $p < 0.05$
Aprosulate *vs.* GL-522-Y-1; $p < 0.05$
GL-522-Y-1 *vs.* control; $p < 0.05$

94 % aggregation response in saline supplemented PRP. Both aprosulate and heparin significantly attenuated the aggregation response ($p < 0.05$) relative to saline supplemented plasma. Addition of 10 μg/mL aprosulate to the PRP resulted in a 50 % decrease in the aggregation response. 10 μg/mL heparin almost completely inhibited the proaggregatory actions of thrombin. In the heparinized PRP, only 7 % aggregation was noted. Neither GL-522-Y-1 nor pentasaccharide were able to inhibit thrombin induced platelet aggregation at the concentration tested. In panel B, the slopes of the aggregation responses in the presence of test agent are compared. As with the aggegation response, only aprosulate and heparin were able to significantly reduce the slope of the aggregation response. The aggregation response in aprosulate supplemented plasma exhibited a slope which was one third that measured in control PRP. The responses to epinephrine, ADP, arachidonic acid, and collagen were not significantly effected by analogue supplementation.

b. Heparin Induced Thrombocytopenia Screening

The effect of aprosulate, GL-522-Y-1, heparin and pentasaccharide in a heparin induced thrombocytopenia screening assay is depicted in Figure 48. In this assay, platelet rich plasma is incubated with the test agent and serum from a known HIT positive pool. Platelet aggregation upon addition of the test agent and the serum indicates a positive HIT potential. Both heparin and aprosulate concentration dependently increased the degree of platelet aggregation. In PRP supplemented with only HIT positive serum, 8.3 $\pm$ 1.1 % aggregation was observed. At concentrations above 11 μg/mL, aprosulate and

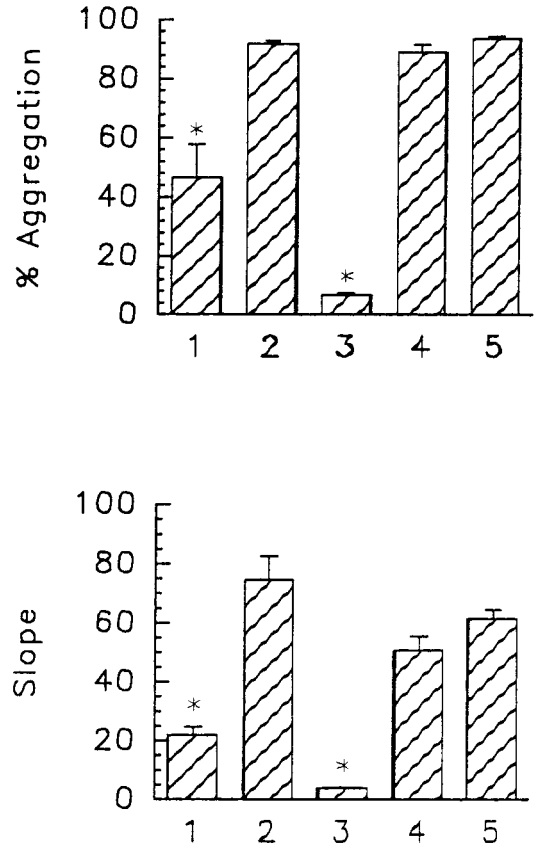


Figure 47. Effect of aprosulate, GL-522-Y-1, heparin and pentasaccharide on platelet aggregation in a platelet rich plasma system. The results represent the mean ± SEM of 10 donors for each agent. All test agents were supplemented to plasma at a final concentration of 10 μg/mL. Statistical comparisons were made by one way ANOVA followed by the Newman Keuls multiple comparison test (aggregation; dF = 49, F = 532.0, $p < 0.0001$. slope; dF = 49, F = 410.6, $p < 0.0001$). *$p < 0.05$ *vs.* control was considered statistically significant. Data are compiled in Tables 44 and 45.

1 = aprosulate
2 = GL-522-Y-1
3 = Heparin
4 = Pentasaccharide
5 = Control

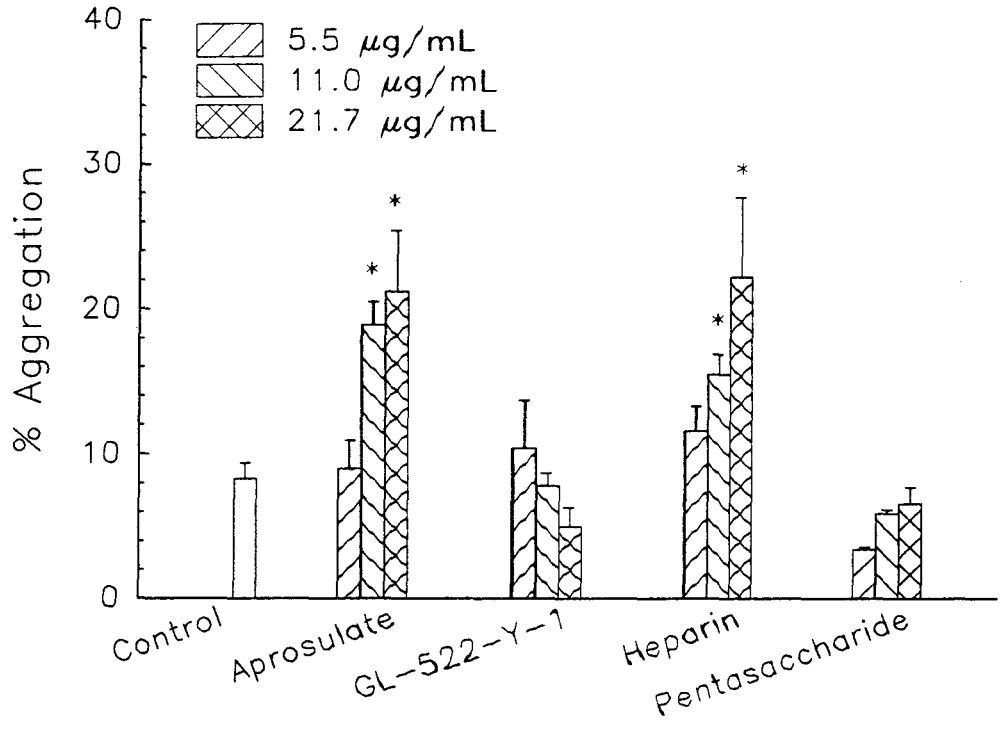


Figure 48. Comparative effect of synthetic heparin analogues ina heparin induced thrombocytopenia screening assay. In this assay, platelet aggregation is induced by the test agent and serum from a heparin induced thrombocytopenic individual. All results represent the mean ± SEM of 10 blood donors. Statistical comparisons between agent and control were made using one way ANOVA followed by the Newman Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant. Data are compiled in Table 46.

heparin produced significantly higher levels of aggregation than control ($p < 0.05$). Neither pentasaccharide nor GL-522-Y-1 produced a significant increase in the amount of platelet aggregation measured in this assay. At a concentration of 22 μg/mL, heparin and aprosulate produced a comparable 22% level of aggregation. At equigravimetric concentrations, aprosulate produced a stronger aggregation response than either GL-522-Y-1 or pentasaccharide ($p < 0.05$; ANOVA). Aggregation levels determined in these studies are tabulated in Table 46.

C. *In Vivo* Study Results

The *in vivo* studies are designed to demonstrate the relative antithrombotic and hemorrhagic effects of these analogues after intravenous and subcutaneous adminstration. Well defined animal models were used to determine the dose response of the antithrombotic and bleeding effects. In addition, the plasmatic pharmacodynamic response which is related to the ATIII and HCII mediated effects was also investigated. The role of functional TFPI in relation to the observed pharmacologic action was investigated by measuring the release of this agent.

1. Dose-response in the Rabbit Stasis thrombosis Model Following Intravenous Administration

A rabbit model of stasis thrombosis was used to assess the antithrombotic activity of heparin, aprosulate, GL-522-Y-1, and pentasaccharide. In this model, clots were formed in response to a stasis of blood flow in the jugular vein segment and a

hypercoagulable state induced by injection of an activated prothrombin complex concentrate.

In the intravenous dose-response study, test agents were administered via the marginal ear vein following drawing of the baseline blood sample. Control animals were administered saline at a dose of 0.1 mL/kg. The thrombogenic challenge was administered after a 5 minute drug circulation time. Clots were scored visually after 10 or 20 minutes stasis time according to the scale described in "Materials and Methods". Statistical differences between the clot scores obtained in treated and control animals were assessed using the Kruskal-Wallis non-parametric analysis of variance. Specific comparisons were made using the Mann-Whitney U test. All agents produced a dose-dependent decrease in clot score. Potency was evaluated by fitting the individual data points to a straight line by least squares regression. ED_{50} values were extrapolated from these regression lines based on a clot score at 10 minutes stasis time of 2.9 in saline treated control animals. Clot scores for each intravenous treatment are tabulated in Table 47.

Dose response curves for the antithrombotic activity measured after a 10 minute stasis time following intravenous administration are depicted in Figure 49. Heparin was observed to be the most potent of the agents tested with an ED_{50} value of 1.7 nmol/kg. Doses greater than or equal to 2.4 nmol/kg produced a statistically significant reduction in thrombus formation ($p < 0.05$). Pentasaccharide was the most potent of the synthetic analogues with an ED_{50} value of 20 nmol/kg. Statistical significance was achieved relative to control at doses higher than 14 nmol/kg. The ED_{50} in aprosulate treated animals was

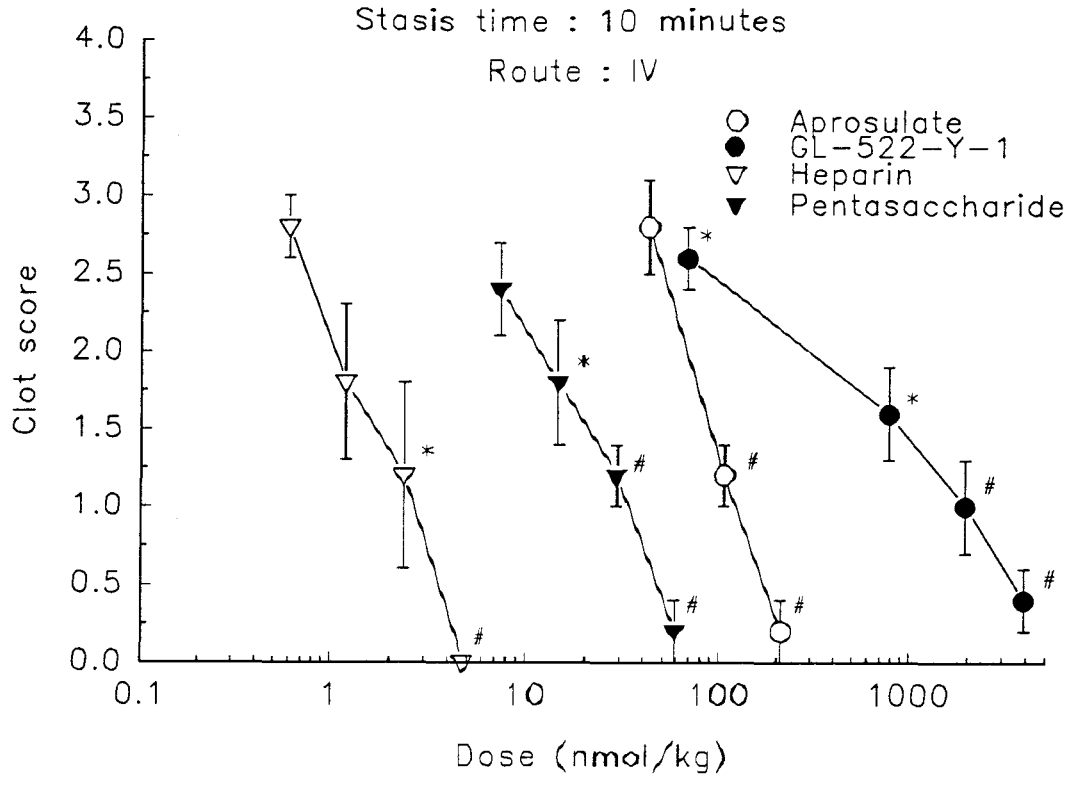


Figure 49. Comparison of the antithrombotic actions of heparin analogues in a rabbit model of stasis thrombosis. The antithrombotic activity of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were determined using the rabbit stasis thrombosis model. Dose-response curves of the clot scores obtained following a stasis time of 10 minutes are depicted. Drug was allowed to circulate for 5 minutes prior to the administration of the thrombogenic challenge. 7.5 U/kg FEIBA was used as a thrombogenic challenge. The results represent the mean $\pm$ S.E.M. of 5 rabbits per treatment group. Saline treatment resulted in a mean clot score of 2.9 $\pm$ 0.1. All agents produced a dose-dependent antithrombotic action. The rank order potency of antithrombotic activity was observed to be heparin > pentasaccharide > aprosulate > GL-522-Y-1. Statistical comparisons were made using the Kruskal-Wallis non-parametric analysis of variance following by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 47.

determined to be 93 nmol/kg. Aprosulate doses greater than 104 nmol/kg produced significant antithrombotic effects. GL-522-Y-1 was the weakest of the agents tested with an ED_{50} equal to 662 nmol/kg. All doses tested produced a significant antithrombotic action. The slope of the GL-522-Y-1 dose-response curve was noted to be different from those of the other agents.

Figure 50 presents the dose-response curves for the antithrombotic actions of the synthetic heparin analogues after a 20 minute stasis time. A much weaker antithrombotic activity was observed following 20 minutes stasis time compared to the 10 minute stasis time point. Additionally, a weak dose-response was observed where only the highest doses of heparin and pentasaccharide resulted in clot scores less than 2. Aprosulate produced a statistically significant reduction in clot score after 20 minutes of stasis only at a dose of 209 nmol/kg ($p < 0.05$ *vs.* control). Both heparin and pentasaccharide also significantly inhibited clot formation after 20 minutes stasis at the highest dose tested ($p < 0.01$ *vs.* control). GL-522-Y-1 was ineffective at inhibiting thrombus formation after 20 minutes stasis. ED_{50} values based on this data were not calculated.

2. Dose-response in the Rabbit Stasis Thrombosis Model Following Subcutaneous Administration

Relatively higher doses of each agent were required to prevent thrombus formation following subcutaneous administration. Dose response curves for the antithrombotic activity are depicted in Figure 51. The antithrombotic activity was

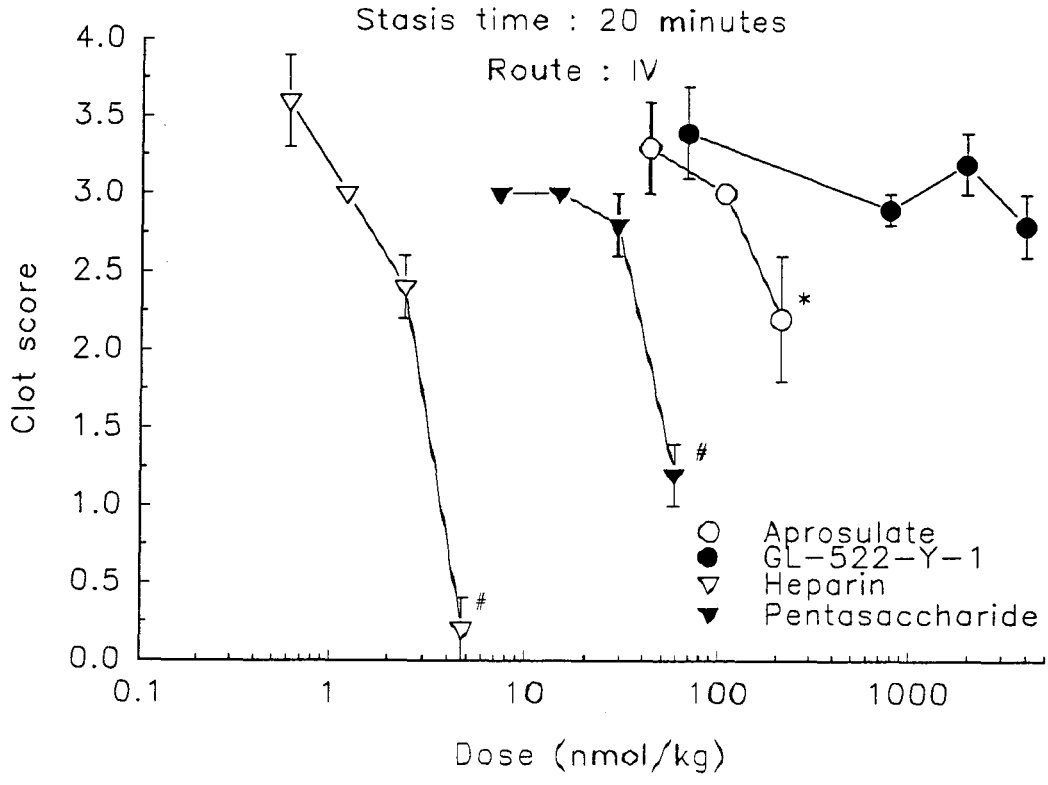


Figure 50. Comparison of the antithrombotic actions of heparin analogues in a rabbit model of stasis thrombosis. The antithrombotic activity of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were determined using the rabbit stasis thrombosis model. Dose-response curves of the clot scores obtained following a stasis time of 20 minutes are depicted. Drug was allowed to circulate for 5 minutes prior to the administration of the thrombogenic challenge. 7.5 U/kg FEIBA was used as a thrombogenic challenge. The results represent the mean $\pm$ S.E.M. of 5 rabbits per treatment group. Saline treatment resulted in a mean clot score of 3.6 $\pm$ 0.3. The rank order potency of antithrombotic activity was observed to be heparin > pentasaccharide > aprosulate > GL-522-Y-1. Statistical comparisons were made using the Kruskal-Wallis non-parametric analysis of variance following by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 47.

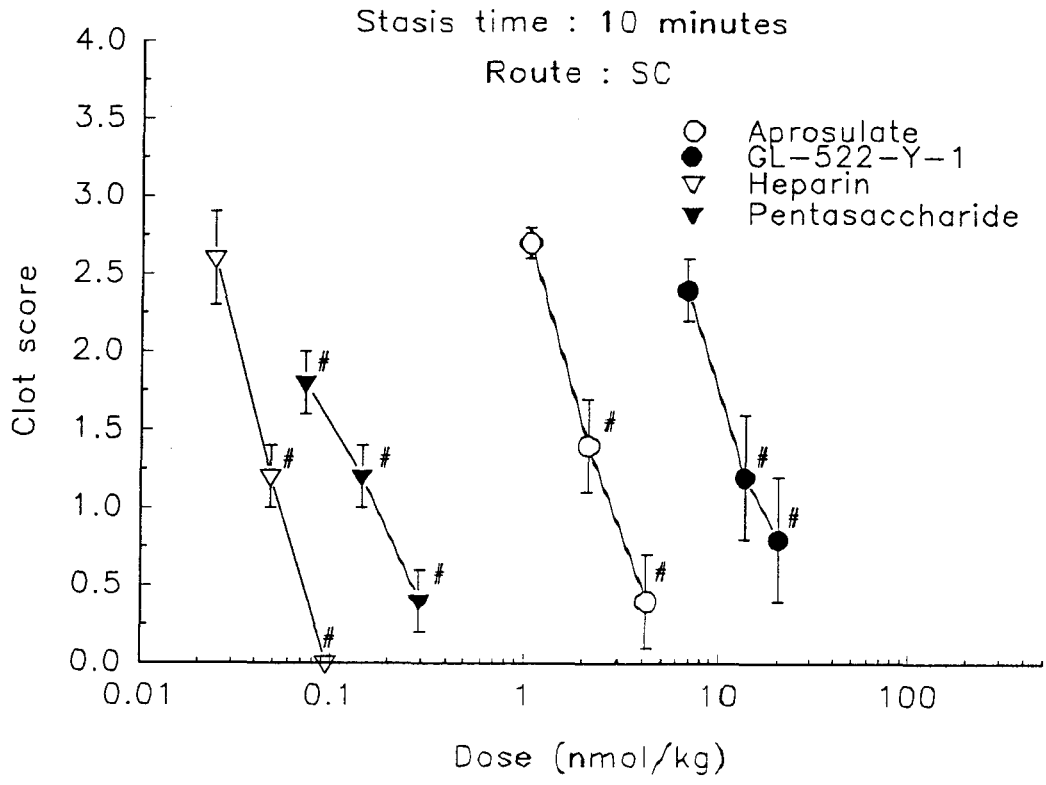


Figure 51. Comparison of the antithrombotic actions of heparin analogues in a rabbit model of stasis thrombosis. The antithrombotic activity of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were determined using the rabbit stasis thrombosis model. Dose-response curves of the clot scores obtained following a stasis time of 10 minutes after subcutaneous administration are depicted. An absorption time of 2 hours was utilized prior to administration of the thrombogenic challenge. 7.5 U/kg FEIBA was used as a thrombogenic challenge. The results represent the mean ± S.E.M. of 5 rabbits per treatment group. Saline treatment resulted in a mean clot score of 2.9 ± 0.1. All agents produced a dose-dependent decrease in clot score. The rank order potency of antithrombotic activity was observed to be heparin > pentasaccharide > aprosulate > GL-522-Y-1. Statistical comparisons were made using the Kruskal-Wallis non-parametric analysis of variance following by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 48.

measured two hours post- administration of the test agent. Each agent was able to prevent thrombus formation in a dose-dependent manner. Individual clot scores for the various agents studied are presented in Table 48. Potency was evaluated as described above. The same rank order potency was observed as in the intravenous study. Heparin was the most potent with an ED_{50} calculated to be 0.043 μmol/kg. Doses greater than 0.048 μmol/kg resulted in significant reductions of clot scores ($p < 0.01$). The ED_{50} for pentasaccharide was approximately three fold higher at 0.12 μmol/kg. All doses of pentasaccharide tested produced significant reductions in clot score. Aprosulate and GL-522-Y-1 were considerably weaker with ED_{50} values of 2.2 and 12.3 μmol/kg, respectively.

After 20 minutes stasis time following subcutaneous administration, heparin, aprosulate and pentasaccharide exhibited a progressive, dose-dependent decrease in clot score with increasing dose as shown in Figure 52. Such an effect was not observed in GL-522-Y-1 treated rabbits as all clot scores remained greater than 3 regardless of dose ($p = 0.408$; Kruskal-Wallis). ED_{50} values for heparin, aprosulate, and pentasaccharide were determined to be 0.058, 2.74, and 0.17 μmol/kg, respectively based on a clot score of 3.6 for the saline treated control animals. Doses greater than 2 μmol/kg aprosulate significantly reduced clot scores compared to control. Only the highest doses of heparin and pentasaccharide produced a significant reduction in clot score.

3. Time Dependent Antithrombotic Effects in the Rabbit Stasis Thrombosis Model Following Intravenous Administration

The time dependence on the antithrombotic activity of heparin analogues was

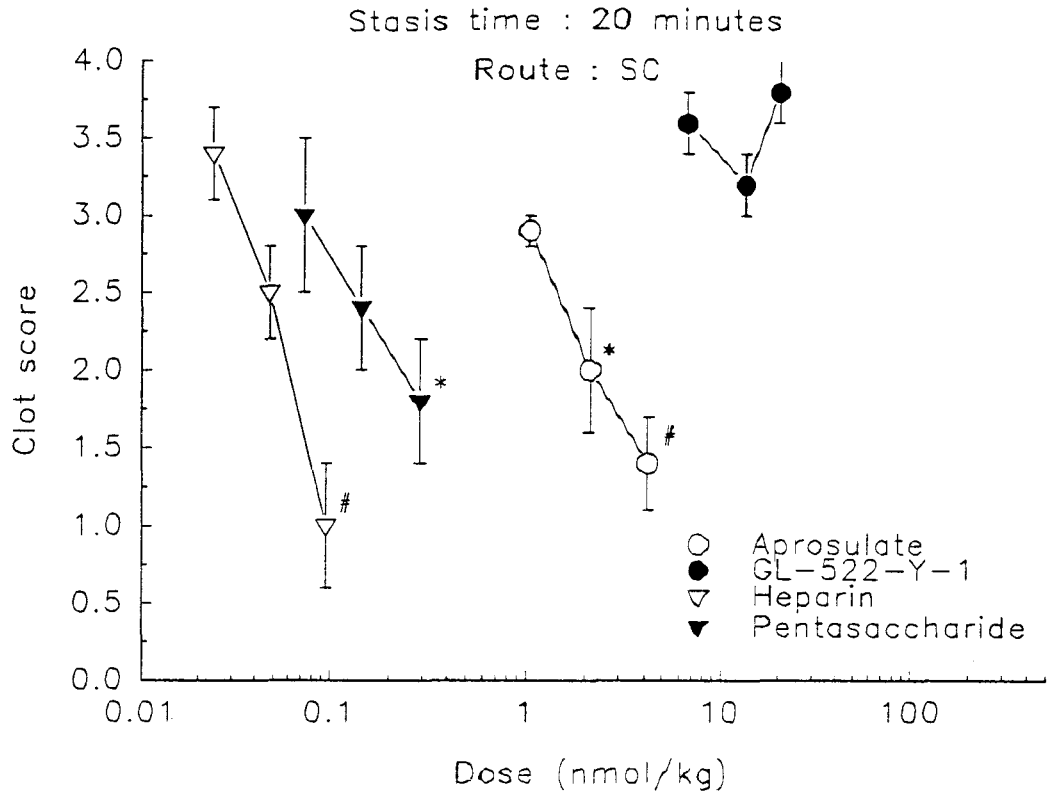


Figure 52. Comparison of the antithrombotic actions of heparin analogues in a rabbit model of stasis thrombosis. The antithrombotic activity of aprosulate, GL-522-Y-1, heparin, and pentasaccharide were determined using the rabbit stasis thrombosis model. Dose-response curves of the clot scores obtained following a stasis time of 20 minutes after subcutaneous administration are depicted. An absorption time of 2 hours was utilized prior to administration of the thrombogenic challenge. 7.5 U/kg FEIBA was used as a thrombogenic challenge. The results represent the mean ± S.E.M. of 5 rabbits per treatment group. Saline treatment resulted in a mean clot score of 3.6 ± 0.3. Clot scores were decreased in a dose-dependent manner by heparin, pentasaccharide and aprosulate. GL-522-Y-1 administration did not effect the clot score. The rank order potency of antithrombotic activity was observed to be heparin > pentasaccharide > aprosulate > GL-522-Y-1. Statistical comparisons were made using the Kruskal-Wallis non-parametric analysis of variance following by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 48.

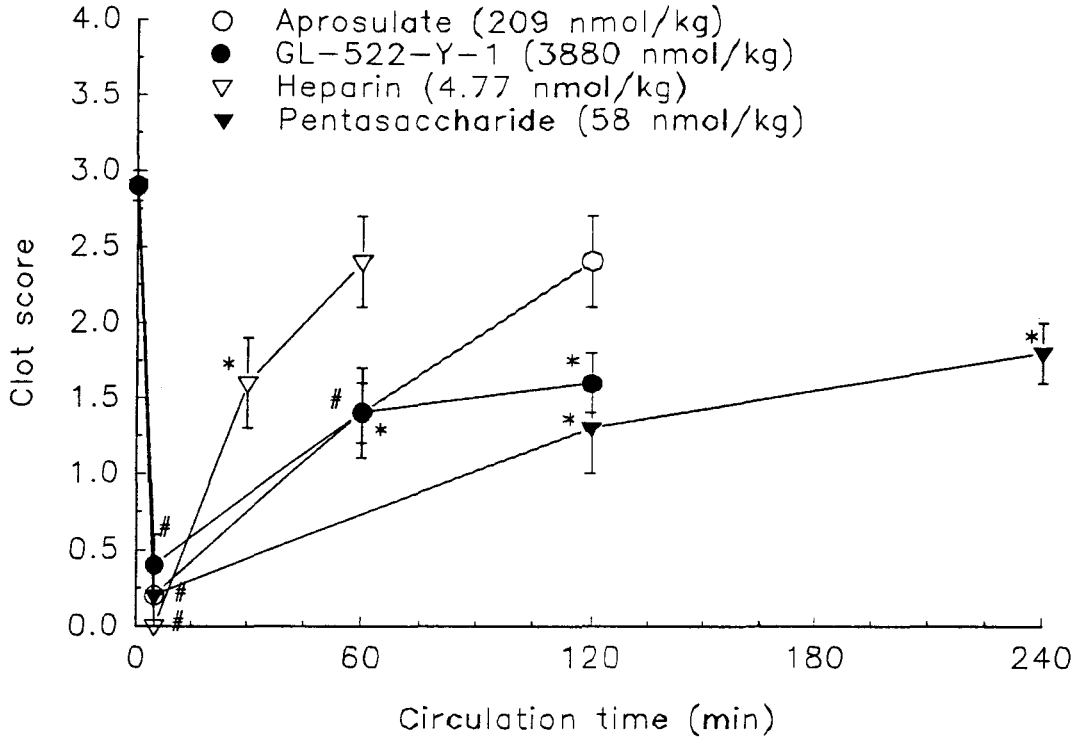


Figure 53. Comparison of the antithrombotic activity of heparin analogues as determined using the rabbit stasis thrombosis model. The time-dependence on the antithrombotic activity of the synthetic heparin analogues was determined by administering an equally antithrombotic dose of each agent intravenously and determining clot scores at various time points post-administration. 10 minutes stasis time was utilized. Each point represents the mean ± SEM of 5 rabbits. The duration of antithrombotic activity was shortest for heparin. Pentasaccharide produced a statistically significant antithrombotic effect at time points out to 240 minutes post-administration. Statistical comparisons were made using the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. No statistical differences were noted between clot scores at 5 minutes circulation time. Data are compiled in Table 49.

determined by varying the circulation time after administration of a selected antithrombotic dose of each agent. Individual clot scores are tabulated in Table 49. The time-response curves following intravenous administration are depicted in Figure 53. No statistical differences were noted in the clot scores determined after 5 minutes circulation time for the various agents ($p = 0.680$). The antithrombotic activity of heparin was the shortest lived of the agents tested. By 60 minutes, a statistically significant antithrombotic effect was no longer obtained. Aprosulate did not exhibit any antithrombotic actions 2 hours after administration. Both GL-522-Y-1 and pentasaccharide exhibited a significant antithrombotic effect at 2 hours ($p < 0.05$, pentasaccharide *vs.* control and GL-522-Y-1 *vs.* control). Pentasaccharide maintained its antithrombotic effect at 4 hours. GL-522-Y-1 was not tested at this time point.

After 20 minutes stasis time, the antithrombotic activity of these agents was rapidly diminished. By 60 minutes, all agents failed to produce significant antithrombotic effects relative to control. These time-response curves are depicted in Figure 54.

4. Time Dependent Antithrombotic Effects in a Rabbit Stasis Thrombosis Model Following Subcutaneous Administration

The time-response curves for aprosulate, GL-522-Y-1, heparin, and pentasaccharide following subcutaneous administration are depicted in Figure 55. Individual clot scores are tabulated in Table 50. At 2 hours post-administration, all agents exhibited equivalent antithrombotic activity ($p = 0.184$). The antithrombotic activities of heparin and GL-522-Y-1 were rapidly diminished. By 4 hours post-administration, an

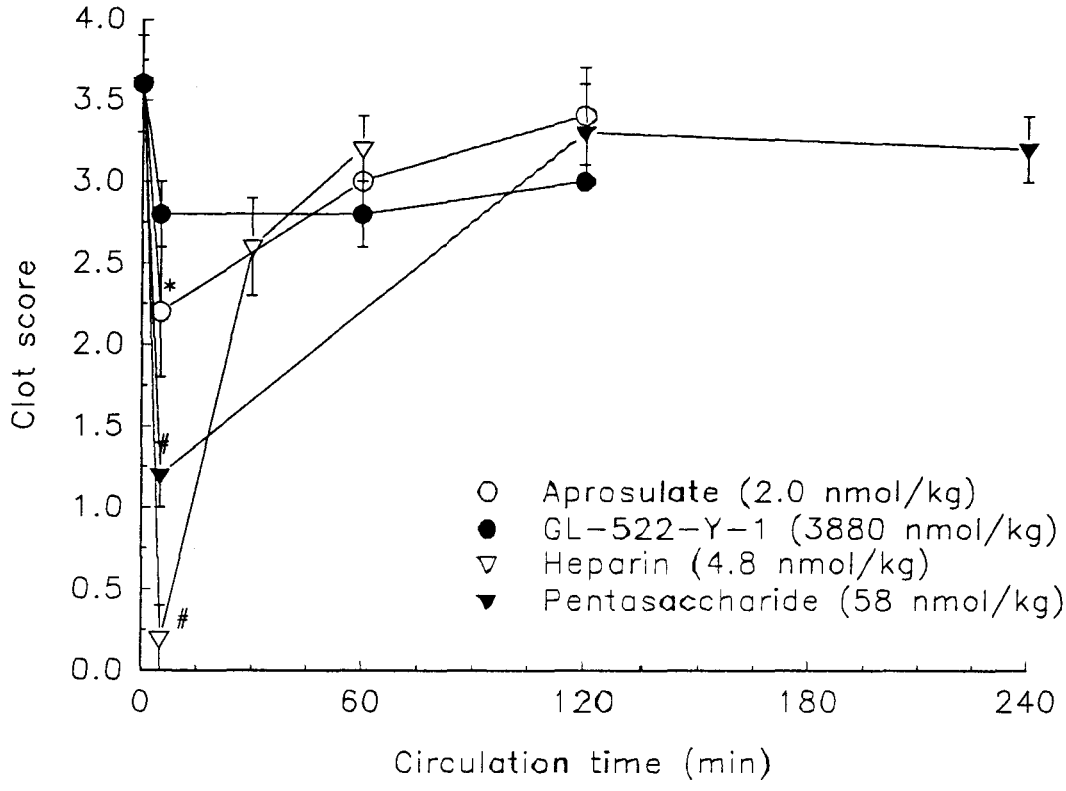


Figure 54. Comparison of the antithrombotic activity of heparin analogues as determined using the rabbit stasis thrombosis model. The time-dependence on the antithrombotic activity of the synthetic heparin analogues was determined by administering an antithrombotically effective dose of each agent intravenously and determining clot scores a various time points post-administration. 20 minutes stasis time was utilized. Each point represents the mean ± SEM of 5 rabbits. Antithrombotic activity was rapidly diminished at circulation times greater than 5 minutes. Statistical comparisons were made using the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 49.

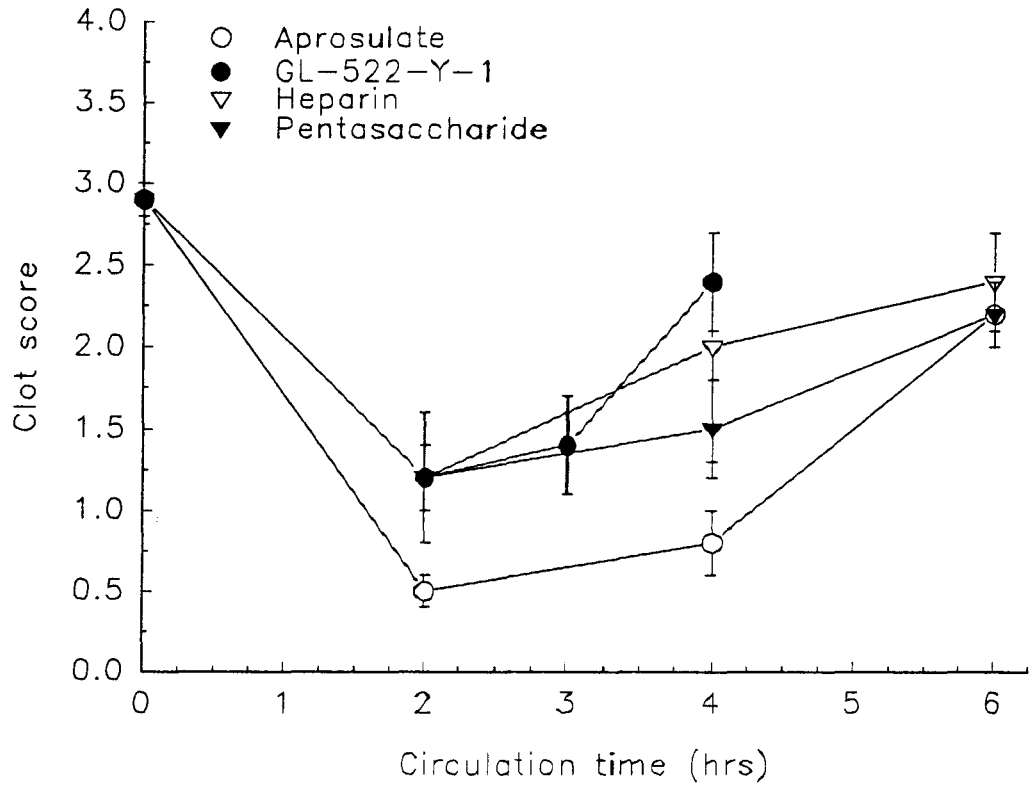


Figure 55. Comparison of the antithrombotic activity of heparin analogues as determined using the rabbit stasis thrombosis model. The time-dependence on the antithrombotic activity of the synthetic heparin analogues was determined by administering an antithrombotically effective dose of each agent subcutaneously and determining clot scores a various time points post-administration. 10 minutes stasis time was utilized. Each point represents the mean ± SEM of 5 rabbits. The antithrombotic activity of GL-522-Y-1 and heparin was diminished after a 4 hour circulation time whereas aprosulate and pentasaccharide continued to produce a significant antithrombotic effect. Statistical comparisons were made using the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. No statistical differences were noted between clot scores at 5 minutes circulation time. Data are compiled in Table 50.

average clot score of 2.4 was observed in GL-522-Y-1 treated rabbits. This clot score was not significantly different from control. The antithrombotic activity of heparin was no longer present after a four hour circulation time. The antithrombotic activities of aprosulate and pentasaccharide decreased at a slower rate than for heparin or GL-522-Y-1. After a four hour circulation time, both agents produced a significant antithrombotic effect ($p < 0.01$ for aprosulate and $p < 0.05$ for pentasaccharide). By 6 hours post-administration, the antithrombotic activity of aprosulate and pentasaccharide was no longer significantly different from control.

The relative thrombogenic effects in this model at 20 minutes stasis time were stronger than the ones observed at 10 minutes stasis. Time-response curves are depicted in Figure 56. At 2 and 4 hours post administration of aprosulate, significant reductions in clot score were achieved ($p < 0.01$ and $p < 0.05$, respectively). Clot scores in these animals were less than 2. At all time points tested following GL-522-Y-1, heparin, and pentasacharide administration, a significant reduction in clot score was not observed.

The potencies of each agent following intravenous and subcutaneous administration are compared in Table 7. Potency was evaluated by extrapolating the dose which produced a clot score one half that observed in saline treated rabbits from the dose response curves for each agent. Following intravenous administration, heparin exhibited the highest potency. The potencies of pentasaccharide, aprosulate and GL-522-Y-1 were observed to be 12, 55, and 390 fold higher than heparin, respectively. Following subcutaneous administration, the potency of pentasaccharide was only 3 fold higher than that of heparin. Aprosulate and GL-522-Y-1 were considerably weaker, exhibiting

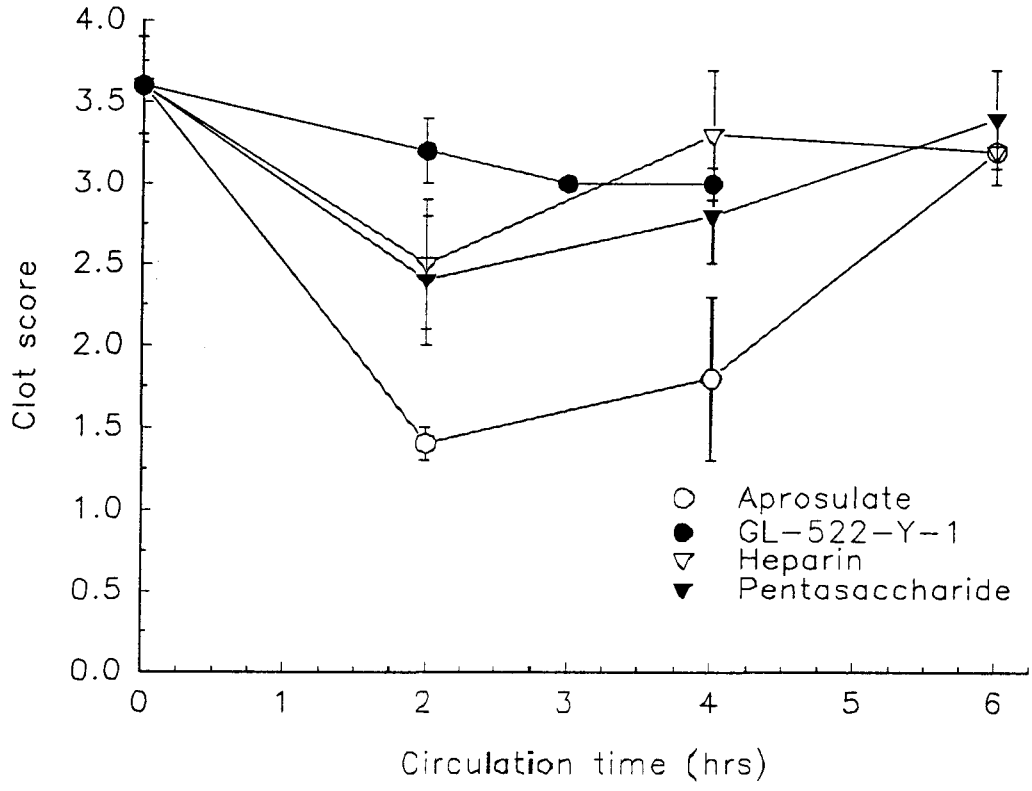


Figure 56. Comparison of the antithrombotic activity of heparin analogues as determined using the rabbit stasis thrombosis model. The time-dependence on the antithrombotic activity of the synthetic heparin analogues was determined by administering an antithrombotically effective dose of each agent subcutaneously and determining clot scores a various time points post-administration. 20 minutes stasis time was utilized. Each point represents the mean ± SEM of 5 rabbits. Statistical comparisons were made using the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control. Data are compiled in Table 50.

TABLE 7

ED_{50} VALUES FOR SYNTHETIC HEPARIN ANALOGUES FOLLOWING INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION IN A RABBIT STASIS THROMBOSIS MODEL

	IV (nmol/kg)	SC (μmol/kg)
Aprosulate	93.4	2.20
GL-522-Y-1	662.4	12.3
Heparin	1.7	0.043
Pentasaccharide	20.6	0.12

Potency of each agent is compared by determining the dose which inhibits 50 % clot formation. This was achieved by fitting the individual concentration - clot score points to a straight line. Doses providing 50 % inhibition were extrapolated from the curves based on a clot score of 2.9 $\pm$ 0.1 for saline treated animals after 10 minutes stasis time.

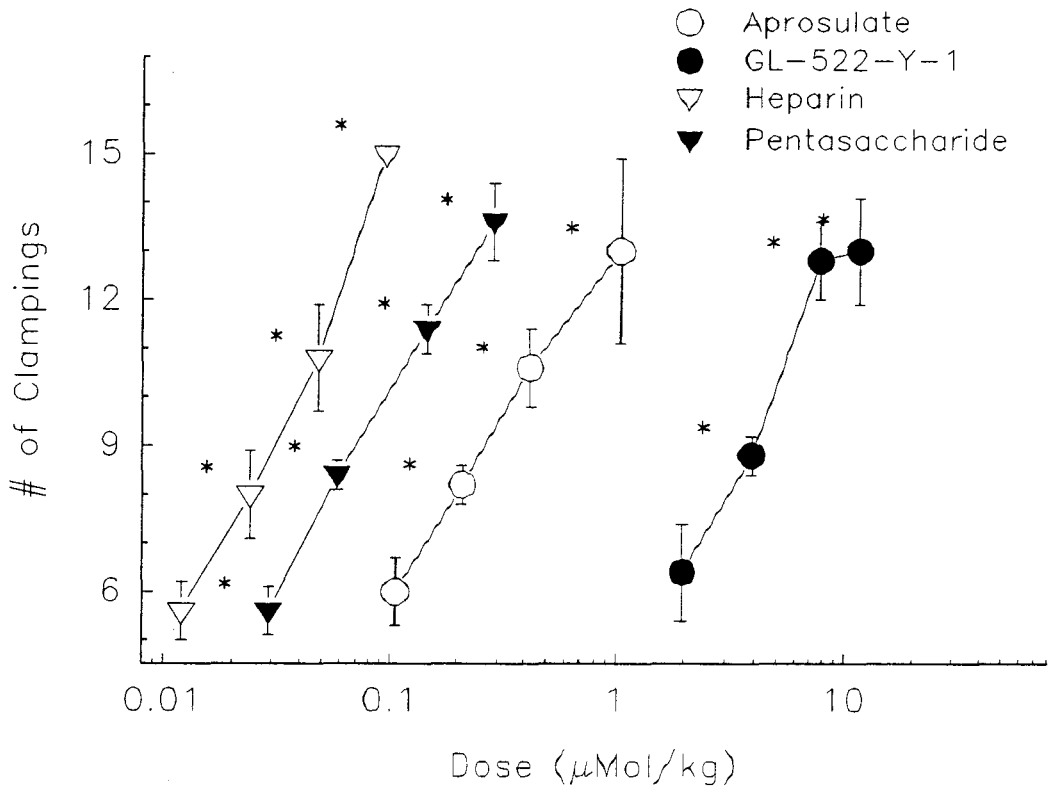


Figure 57. Comparison of the antithrombotic activity of heparin analogues in a rat model of thrombosis. Jugular patency was assessed by Doppler flow measurement. Data presented represents the mean ± SEM for 5 rats in each treatment group. Saline treated control rats required 4.4 ± 0.3 clampings on average for jugular occlusion. All agents were administered via the left femoral vein 5 minutes prior to the initiation of clamping. Each agent produced a dose-dependent increase in clamping number. The rank order potency of antithrombotic activity in this model was observed to be heparin > pentasaccharide > aprosulate > GL-522-Y-1. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 51.

potencies of 2.2 and 12.3 μmol/kg, respectively.

5. Dose-response in the Rat Jugular Vein Clamping Model Following Intravenous Administration

Figure 57 depicts the dose-response curves for heparin, aprosulate, GL-522-Y-1 and pentasaccharide in a rat jugular vein clamping model of thrombosis following intravenous administration. An increasing number of clampings is reflective of the antithrombotic activity of an agent. Saline treated control rats required 4.4 ± 0.3 clampings on average to cause jugular occlusion. Clamping numbers for each intravenous administration are tabulated in Table 51.

Increasing aprosulate dosages from 0.1 to 1.0 μmol/kg dose-dependently increased the number of clampings required for occlusion compared to control. Statistical analysis of this data using one way ANOVA followed by the Newman-Keuls multiple comparison test indicated that the clamping numbers for the three highest doses were significantly higher than control ($p < 0.05$). GL-522-Y-1 was antithrombotically active at approximately 10 fold higher doses than aprosulate. A dose-dependent increase in clamping number was observed over a dose range of 1.9 to 7.8 μmol/kg. Increasing the dose beyond 7.8 μmol/kg did not significantly increase the number of clampings required for occlusion (7.8 μmol/kg *vs.* 11.6 μmol/kg, $p = 0.751$). Heparin also increased the number of clampings for occlusion in a dose-dependent manner over a dose range of 12 to 95 nmol/kg. At a dose of 95 nmol/kg, the number of clampings exceeded 15. Pentasaccharide dose-dependently increased the number of clampings needed to occlude

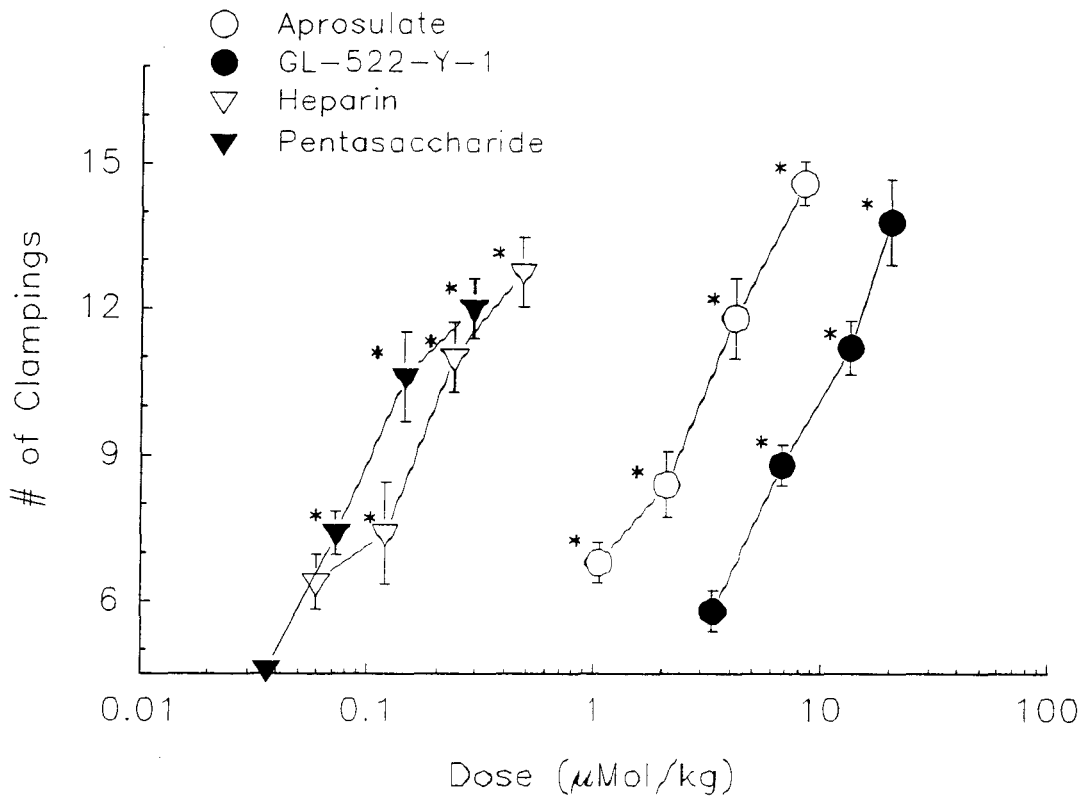


Figure 58. Comparison of the antithrombotic activity of heparin analogues in a rat model of thrombosis. Jugular patency was assessed by Doppler flow measurement. Data presented represents the mean ± SEM for 5 rats in each treatment group. Saline treated control rats required 4.4 ± 0.3 clampings on average for jugular occlusion. All agents were administered subcutaneously 2 hours prior to the initiation of clamping. Each agent produced a dose-dependent increase in clamping number. The rank order potency of antithrombotic activity in this model was observed to be pentasaccharide > heparin > aprosulate > GL-522-Y-1. Statistical comparisons were made by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 52.

the vessel. At all doses tested the number of clampings in pentasaccharide treated rats was significantly higher than in control treated rats ($p < 0.05$).

To compare the potency of each agent, linear regression was performed on the log dose *vs.* clamping number curves. 5 points were used at each dose. The clamping numbers for the highest dose of GL-522-Y-1 were not used in this analysis as they did not fit a straight line. Correlation coefficients greater than 0.80 were obtained for each set of data. The dose which doubled the baseline number of clampings was extrapolated from these curves and used as an index of potency. By this analysis, heparin was observed to be the most potent agent, requiring a dose of 25 nmol/kg to achieve a level of 8.8 clampings. Pentasaccharide was approximately 3 fold less potent, and aprosulate required approximately 10 fold more drug to achieve the same effect. GL-522-Y-1 was the least potent agent, requiring 3.8 μmol/kg to double the baseline number of clampings. These potency values are compared in Table 8.

6. Dose-response in the Rat Jugular Vein Clamping Model Following Subcutaneous Administration

Figure 57 depicts the dose-response curves for heparin, aprosulate, GL-522-Y-1, and pentasaccharide in a rat jugular vein clamping model 2 hours following subcutaneous administration. All agents produced a dose-dependent increase in clamping number, though the rank order potency was somewhat different than following intravenous administration. The clamping numbers for all subcutaneous treatments are tabulated in Table 52. All doses of aprosulate produced a statistically significant increase

TABLE 8

DOSES OF SYNTHETIC HEPARIN ANALOGUES REQUIRED TO DOUBLE BASELINE CLAMPING NUMBERS

	IV (μM)	SC (μM)
Aprosulate	0.28	2.09
GL-522-Y-1	3.84	7.07
Heparin	0.025	0.145
Pentasaccharide	0.071	0.106

Data points for each agent were fitted to a straight line by linear regression. Treatments producing clamping numbers which did not fit a straight line (0.012 μmol/kg heparin and 13.88 μmol/kg GL-522-Y-1) were excluded from this analysis. Doses which doubled the baseline clamping number of 4.4 clampings were extrapolated from each curve. Correlation coefficients greater than 0.80 were achieved for each regression curve.

in clamping number relative to control ($p < 0.05$; ANOVA followed by Newman-Keuls). A dose of 2.09 μmol/kg was determined to double the baseline clamping number. GL-522-Y-1 was again the weakest of the agents tested, requiring 7.1 μmol/kg to double the baseline clamping number. Doses above 6.7 μmol/kg significantly increased the number of clampings relative to baseline. Heparin and pentasaccharide were notably more potent than aprosulate or GL-522-Y-1. Heparin and pentasaccharide exhibited nearly equal potency for doubling baseline clamping number (0.145 and 0.106 μmol/kg, respectively). For this analysis, the data obtained with the lowest dose of heparin, 0.012 μmol/kg, was not included as it did not fit a straight line.

When comparing the doses required for doubling the clamping number following intravenous and subcutaneous adminstration, it was observed that both pentasaccharide and GL-522-Y-1 are relatively well absorbed, exhibiting SC/IV ratios of 1.49 and 1.84, respectively. Aprosulate and heparin were absorbed to a lesser degree and therefore required higher doses subcutaneously to achieve a comparable effect (SC/IV ratios of 7.5 and 5.8, respectively).

7. Dose-response in the Rabbit Ear Bleeding Model Following Intravenous Administration

The hemorrhagic potential of aprosulate, GL-522-Y-1, heparin, and pentasaccharide was determined using a rabbit ear bleeding model. In this model, five standardized incisions were made in the ear following drug administration, and blood cells lost from these incisions were collected for ten minutes. The blood cells collected

were quantitated by hemocytometer and used as an index of bleeding potential. Control treated animals were administered a dose of 0.1 mL/kg saline. Statistical comparisons of red blood cell counts were made by one way ANOVA followed by the Newman-Keuls test. $p < 0.05$ was considered to be statistically significant. Individual data are presented in Tables 53 to 56.

Figure 59 depicts the dose-response curves for aprosulate, GL-522-Y-1, heparin, and pentasaccharide in the rabbit ear bleeding model following intravenous administration. The five standardized incisions were made 5 minutes following administration of the test agent. Both heparin and GL-522-Y-1 were observed to linearly increase blood loss with increasing dose, though over a different concentration range. Heparin increased blood loss over a concentration range of 0.02 to 0.10 μmol/kg. At doses of at least 0.10 μmol/kg, blood loss significantly higher than control was observed ($p < 0.05$). GL-522-Y-1 produced more blood loss over the concentration range tested. Significantly higher bleeding compared to control was achieved with doses above 3.36 μmol/kg. Aprosulate produced a significant increase in blood loss compared to control at all doses tested. This increase was approximately 225 % of control over a dose range of 0.4 to 2.1 μmol/kg. No dose-dependency was observed. Pentasaccharide did not increase blood loss at doses as high as 2.9 μmol/kg.

To assess the potency of each agent in producing a bleeding effect, a bleeding index was calculated. This index was calculated as the dose required to elicit a blood cell loss 3 times that measured in control treated animals. For heparin and GL-522-Y-1, the dose response curves were fitted to a straight line by linear regression. The dose eliciting

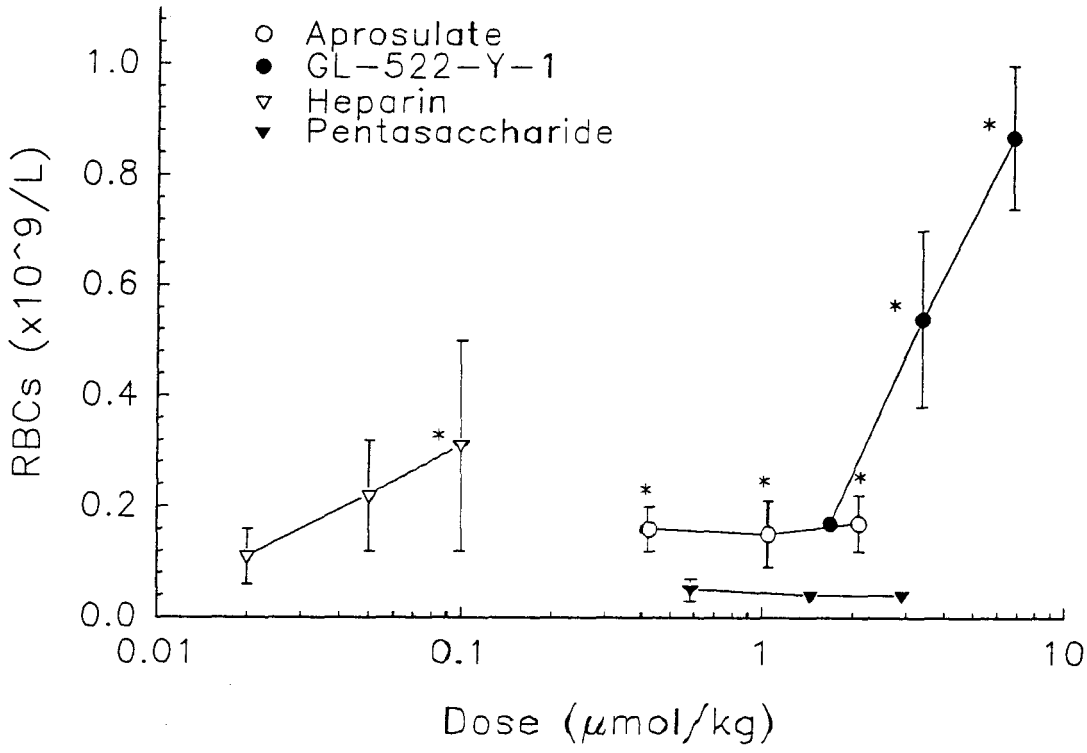


Figure 59. Hemorrhagic effects of heparin analogues following intravenous administration in a rabbit ear bleeding model. All results represent the mean ± SEM of 5 rabbits per treatment group. Heparin and GL-522-Y-1 exhibited dose-dependent increases in blood loss. Neither aprosulate nor pentasaccharide treatment resulted in significant increases in blood loss. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls test. A p-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 53.

a loss of blood cells equal to 0.21 x 10^9 / liter was extrapolated from the curve. For heparin, this dose was 0.045 μmol/kg. The dose of GL-522-Y-1 was nearly 40 times higher at 1.79 μmol/kg. Neither aprosulate nor pentasaccharide reached this level of blood loss at the highest dose tested. The bleeding indices for each agent following intravenous and subcutaneous administration are listed in Table 9.

At 15 minutes post-administration, only GL-522-Y-1 produced a significant bleeding effect. The dose-response at 15 minutes was similar to that at 5 minutes for this agent. Neither aprosulate, heparin, nor pentasaccharide produced a significant increase in bleeding 15 minutes post administration.

8. Dose-response in the Rabbit Ear Bleeding Model Following Subcutaneous Administration

Hemorrhagic potential was also studied two hours following subcutaneous administration. In this study, aprosulate, heparin, and GL-522-Y-1 produced significant dose-dependent increases in bleeding. In aprosulate treated animals, doses above 4 μmol/kg produced significant blood loss compared to control. The dose producing a three fold increase in blood loss was higher than the highest dose tested (8.4 μmol/kg). GL-522-Y-1 produced significant increases in blood loss at doses above 3.4 μmol/kg. The dose producing a three fold increase in blood loss was lower than the lowest dose tested (1.7 μmol/kg). Heparin produced a dose-dependent increase in blood loss at doses above 0.5 μmol/kg. Statistically significant blood loss was observed at heparin doses above 0.95 μmol/kg. The dose which caused a three fold increase in blood loss was extrapolated to

be 0.64 μmol/kg. Pentasaccharide did not cause significant bleeding at doses below 12 μmol/kg. These results are depicted in Figure 60.

9. Time Dependent Effects in the Rabbit Ear Bleeding Model Following Intravenous Administration

Time dependence on the bleeding effect was measured at various time points following intravenous administration. This data is depicted in Figure 61. Aprosulate treatment resulted in a small increase in blood loss (150 %) compared to control treated animals. This effect was statistically significant at 5 and 30 minutes post-administration. Blood loss returned to control levels by 60 minutes. GL-522-Y-1 exhibited significant bleeding at all time points measured. The blood loss was higher at 60 minutes than at 5 minutes post-administration. Heparin administration produced a strong bleeding effect at 5 minutes post administration. This effect decreased with time to levels which were not significant at 60 minutes. Pentasaccharide administration did not produce significant increases in blood loss.

10. Time Dependent Effects in the Rabbit Ear Bleeding Model Following Subcutaneous Administration

Time dependence on the hemorrhagic effects of heparin, aprosulate, and GL-522-Y-1 were examined following subcutaneous administration. This data is depicted in Figure 62. Treatment with 2.1 μmol/kg aprosulate resulted in a statistically significant increase in blood loss at 1, 2, and 4 hours post-administration. By 6 hours, control levels

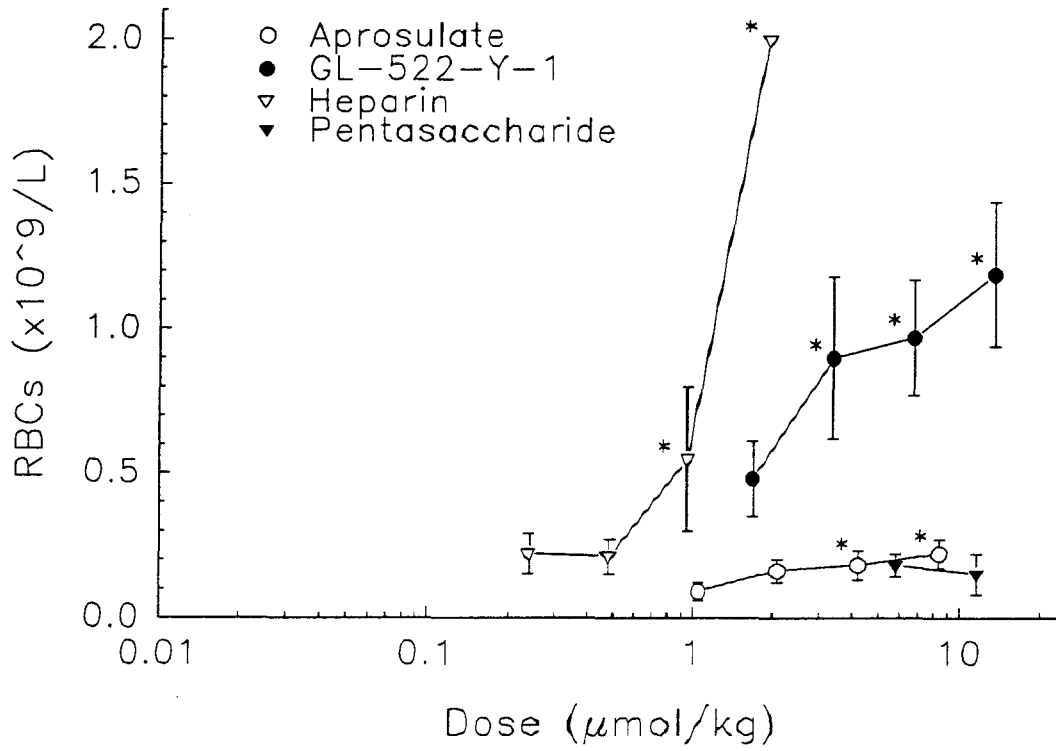


Figure 60. Hemorrhagic effects of heparin analogues following subcutaneous administration in a rabbit ear bleeding model. All results represent the mean ± SEM of 5 rabbits per treatment group. Heparin and GL-522-Y-1 exhibited dose-dependent increases in blood loss. Neither aprosulate nor pentasaccharide treatment resulted in significant increases in blood loss. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls test. A p-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 54.

TABLE 9

BLEEDING INDEX FOR APROSULATE, GL-522-Y-1, HEPARIN, AND PENTASACCHARIDE IN A RABBIT EAR BLEEDING MODEL

	IV (μmol/kg)	SC (μmol/kg)
Aprosulate	> 2.09	> 8.38
GL-522-Y-1	1.79	< 1.68
Heparin	0.04	0.64
Pentasaccharide	> 2.90	> 11.60

Bleeding index was calculated as the dose of a given agent which caused a three fold increase in the number of RBC's lost relative to control. Values were extrapolated from the best fit curve of the data. The regression curves exhibited correlation coefficients greater than 0.94. The highest dose tested is tabulated where an agent did not significantly increase the amount of bleeding relative to control.

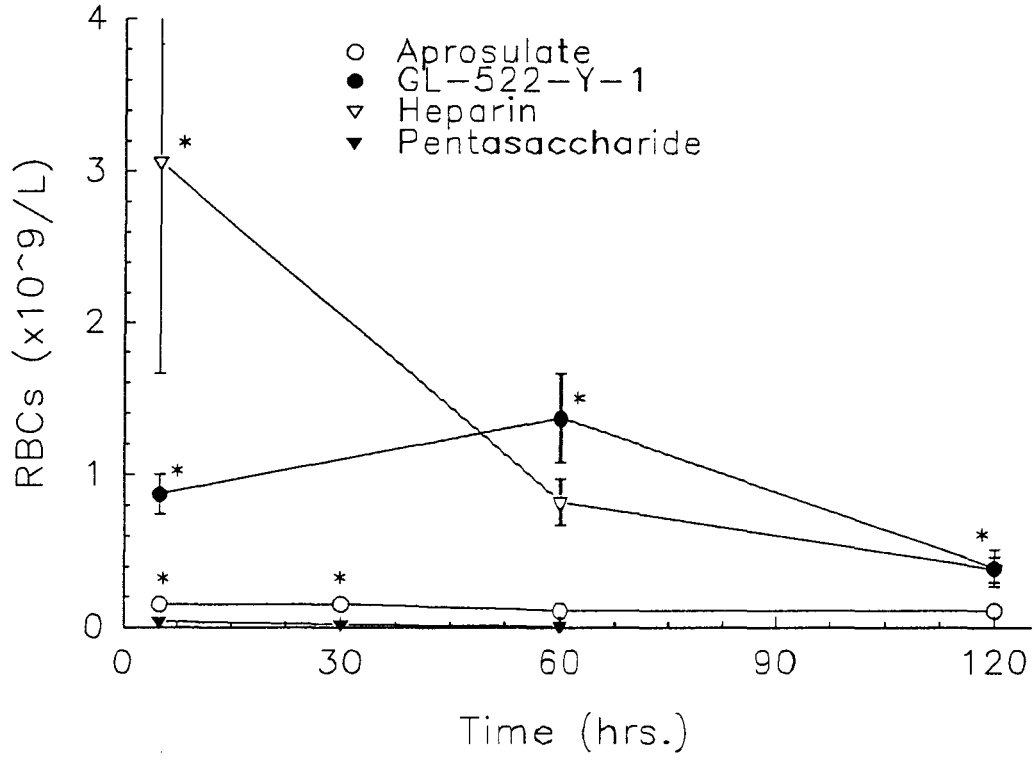


Figure 61. Time dependence on the hemorrhagic effect of heparin analogues following intravenous administration in a rabbit ear bleeding model. Each time point represents the mean ± SEM of 5 rabbits. Heparin exhibited a time-dependent decrease in blood loss. Neither aprosulate nor pentasaccharide treatment resulted in significant increases in blood loss. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls test. A *p*-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 55.

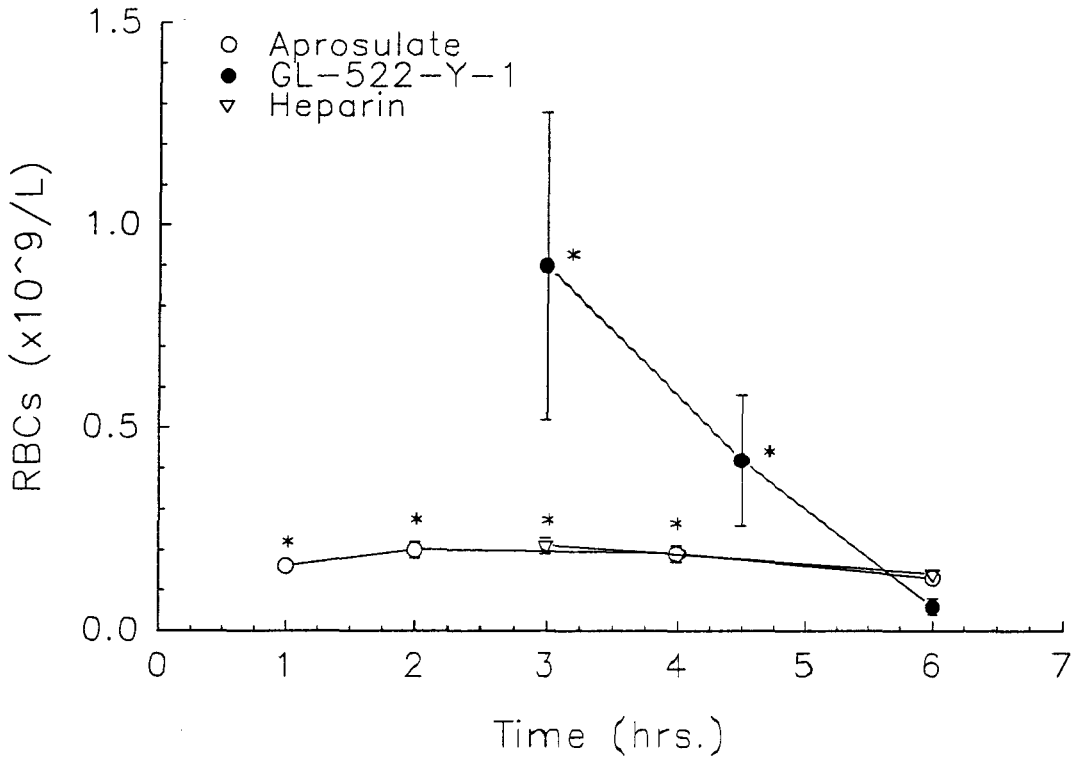


Figure 62. Time dependence on the hemorrhagic effect of heparin analogues following subcutaneous administration in a rabbit ear bleeding model. Each time point represents the mean ± SEM of 5 rabbits. Heparin, aprosulate and GL-522-Y-1 exhibited time-dependent decreases in blood loss. Pentasaccharide treatment was not examined. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls test. A p-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* control. Data are compiled in Table 56.

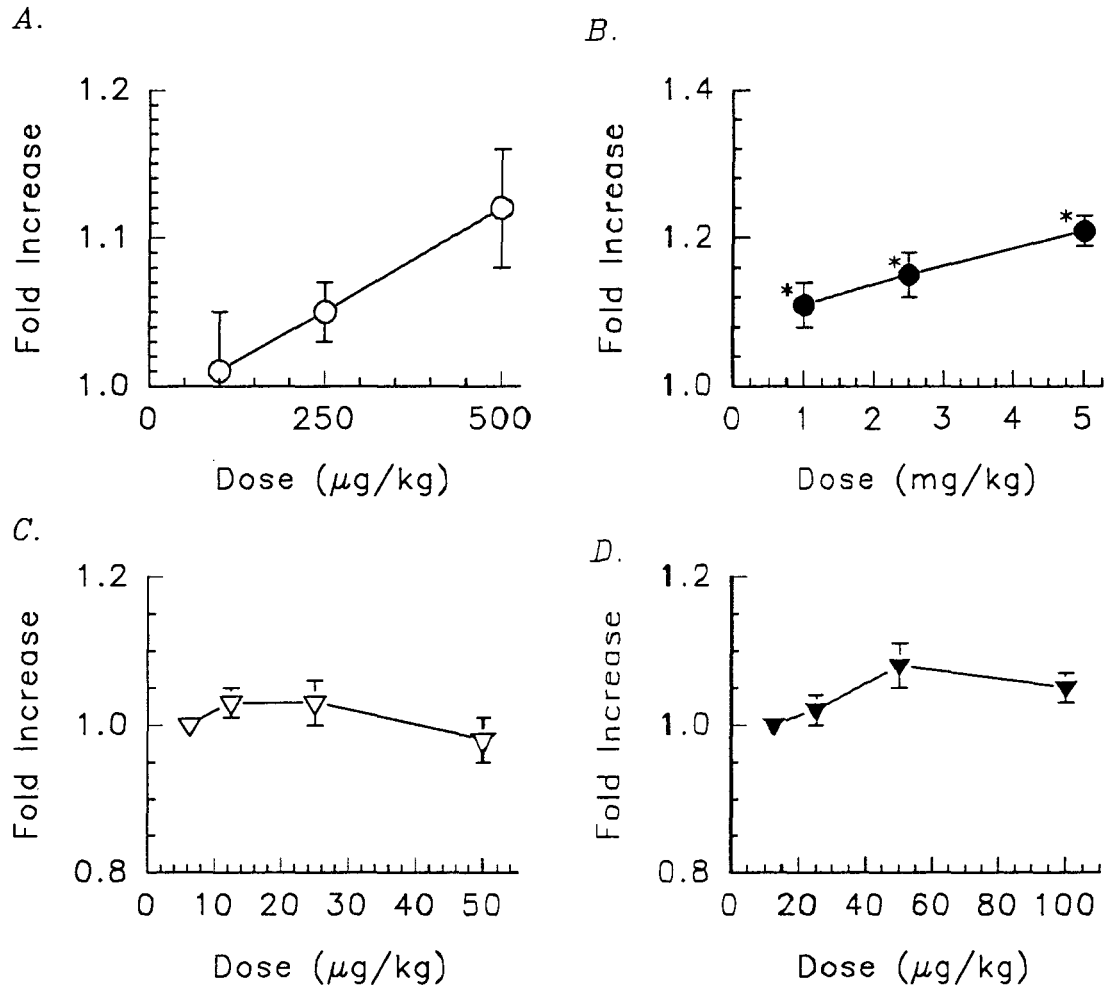


Figure 63. Effect of intravenous administration of heparin analogues on the celite ACT. Dose-response curves for aprosulate (panel A), GL-522-Y-1 (panel B), heparin (panel C), and pentasaccharide (panel D) in the celite ACT assay on rabbit blood are presented. Blood was drawn at baseline and 5 minutes post intravenous administration of the test agent. Fold increase is calculated relative to each individual baseline. Data represents the mean ± SEM of 3 to 5 rabbits. Both aprosulate and GL-522-Y-1 were observed to prolong the ACT in a dose-dependent fashion. Heparin and pentasaccharide did not prolong the ACT over the dose range tested. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. $^{*}p < 0.05$ *vs.* saline treatment is considered statistically significant. Data are compiled in Table 57.

of blood loss had been attained. At a dose of 3.4 μmol/kg, GL-522-Y-1 produced a strong bleeding effect at 3 and 4.5 hours post-administration. By 6 hours, the blood loss had returned to control levels. The bleeding effect induced by heparin administration was also lost by 6 hours post-administration.

11. *Ex Vivo* Anticoagulant Responses

a. celite ACT

Blood samples were drawn from rabbits at baseline and at 5 minutes post-adminstration of the test agent in the intravenous dose-response studies for determination of the celite activated clotting time. Dose-response curves are depicted in Figure 63. In panel A it is shown that aprosulate produces a weak dose dependent prolongation of the activated clotting time over the range of 100 to 500 μg/kg. At 500 μg/kg, the ACT was prolonged 1.12 fold relative to baseline. None of these increases were statistically significant compared to saline treatment. The dose response curve for GL-522-Y-1 is shown in panel B. A dose dependent increase in clotting time was observed over a dose range of 1.0 to 5.0 mg/kg with the clotting time reaching 1.2 fold baseline at a dose of 5.0 mg/kg. All increases in clotting time induced by GL-522-Y-1 administration were statistically significant relative to saline treatment ($p < 0.05$). In panel C, the dose-response curve for heparin is depicted. No significant changes in the ACT were observed at heparin dosages which were antithrombotically effective ($p > 0.05$). Panel D depicts the dose-response curve for pentasaccharide. A concentration dependent increase in clotting time was observed with increasing dose from 12.5 to 50 μg/kg. These increases

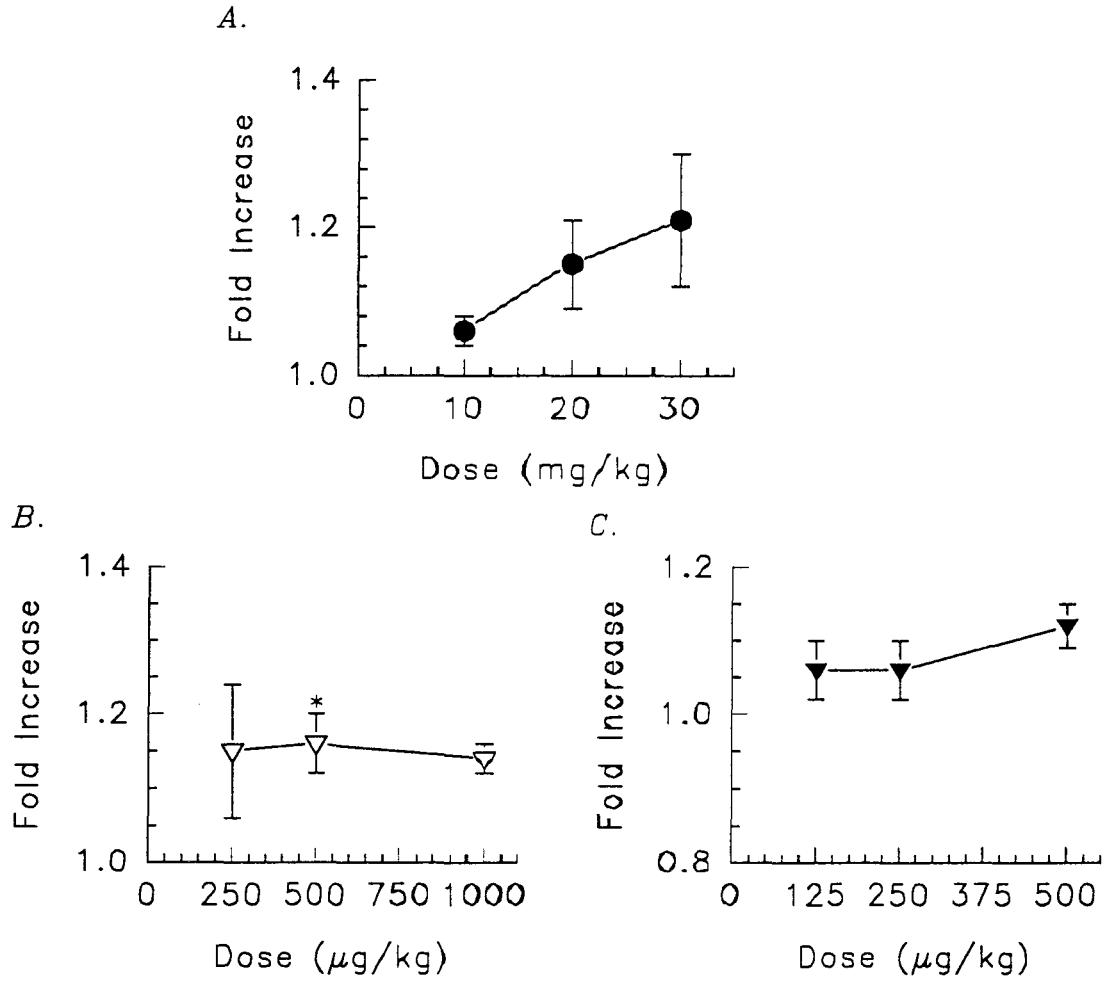


Figure 64. Effect of intravenous administration of heparin analogues on the celite ACT. Dose-response curves for GL-522-Y-1 (panel A), heparin (panel B), and pentasaccharide (panel C) in the celite ACT assay on rabbit blood are presented. Blood was drawn at baseline and 2 hours post subcutaneous administration of the test agent. Fold increase is calculated relative to each individual baseline. Data represents the mean ± SEM of 3 to 5 rabbits. Only GL-522-Y-1 was observed to dose-dependently prolong the ACT following subcutaneous administration. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. $^{*}p < 0.05$ *vs.* saline treatment is considered statistically significant. Data are compiled in Table 58.

were not statistically significant ($p > 0.05$).

The dose-response curves for heparin, GL-522-Y-1, and pentasaccharide in the celite ACT following subcutaneous administration are shown in Figure 64. GL-522-Y-1 was observed to increase the ACT in a dose-dependent fashion over the dose range of 10 to 30 mg/kg. At a dose of 30 mg/kg, the ACT was increased to 1.2 fold baseline. None of these increases was determined to be statistically significant ($p > 0.05$). Heparin dosages from 250 to 1000 μg/kg produced a dose-independent increase in the ACT. Only the clotting time at a dose of 500 μg/kg was statistically significant compared to saline treated controls. Pentasaccharide did not significantly increase activated clotting times ($p > 0.05$). Individual celite ACT clotting times are tabulated in Tables 57 and 58.

b. Thrombelastographic Analysis

Figure 65 depicts the effect of intravenous administration of aprosulate, GL-522-Y-1, heparin, and pentasaccharide on the TEG in whole rabbit blood. Data for the R-time, or time to clot formation, are presented. All results are presented as fold increase relative to baseline. Fold increase was determined using each individual rabbits baseline. In panel A, the effect of aprosulate on R-time is shown. While a dose-dependent prolongation of the time to clot was observed, statistical significance was not achieved due to the high variability, particularly at the 500 μg/kg dosage. Following a 500 μg/kg dosage, a mean fold increase of 4.0 was determined. In panel B it is observed that GL-522-Y-1 also dose-dependently prolonged the R-time. As with aprosulate, wide variations prevented these increases from being statistically significant. At a dose of 5.0 mg/kg, the

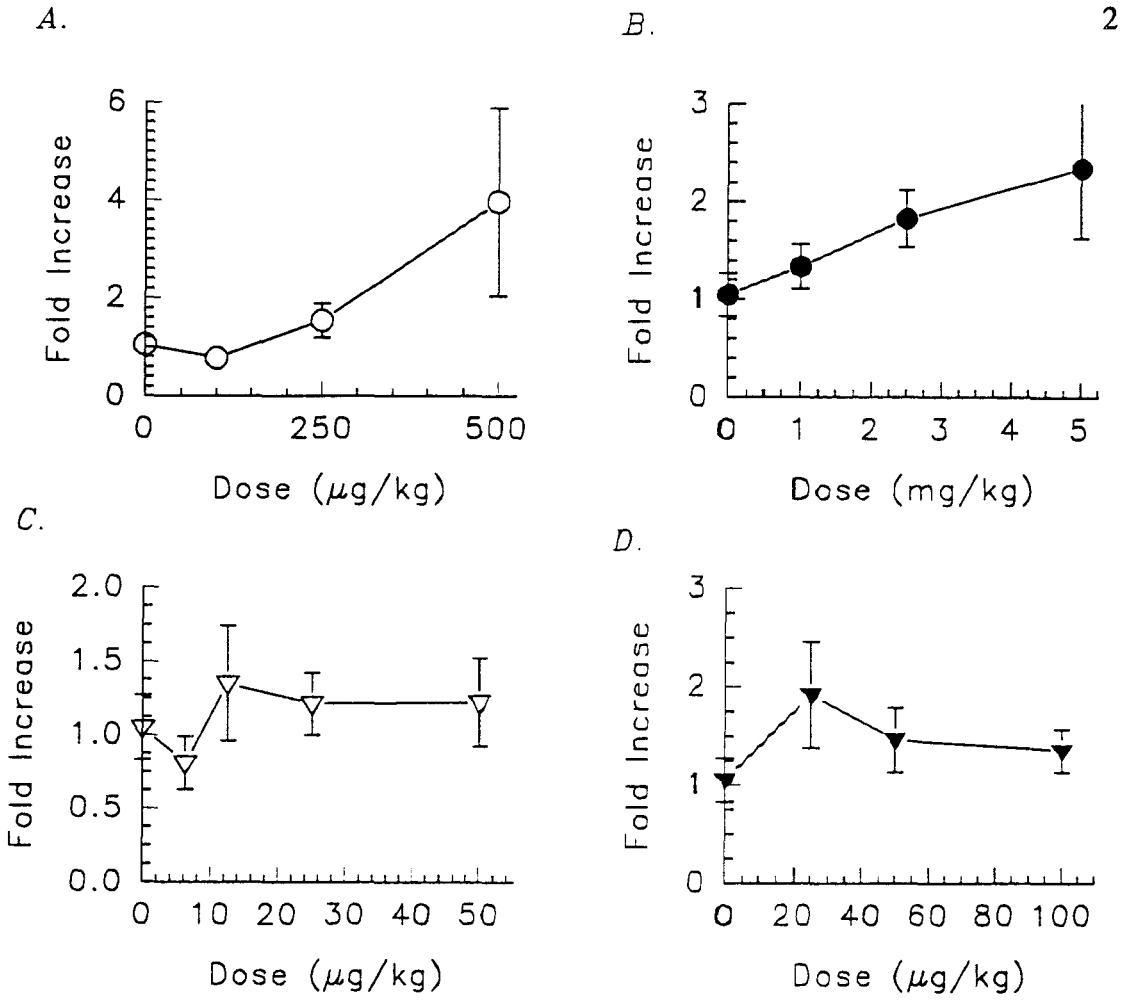


Figure 65. Effect of intravenous administration of heparin analogues on the R-time of the thrombelastograph. Both aprosulate and GL-522-Y-1 dose-dependently prolonged the R-time (panels A and B, respectively). Heparin and pentasaccharide elevated the R-time in a dose-independent manner (panels C and D, respectively). Results represent the mean ± SEM of 3 to 5 observations. Statistical significance was assessed for each agent using one way ANOVA followed by the Newman-Keuls multiple comparison test. All p values are for treatment versus saline treated control animals. Data are compiled in Table 59.

Aprosulate : $p = 0.146$

GL-52-Y-1 : $p = 0.168$

Heparin : $p = 0.636$

Pentasaccharide : p 0.388.

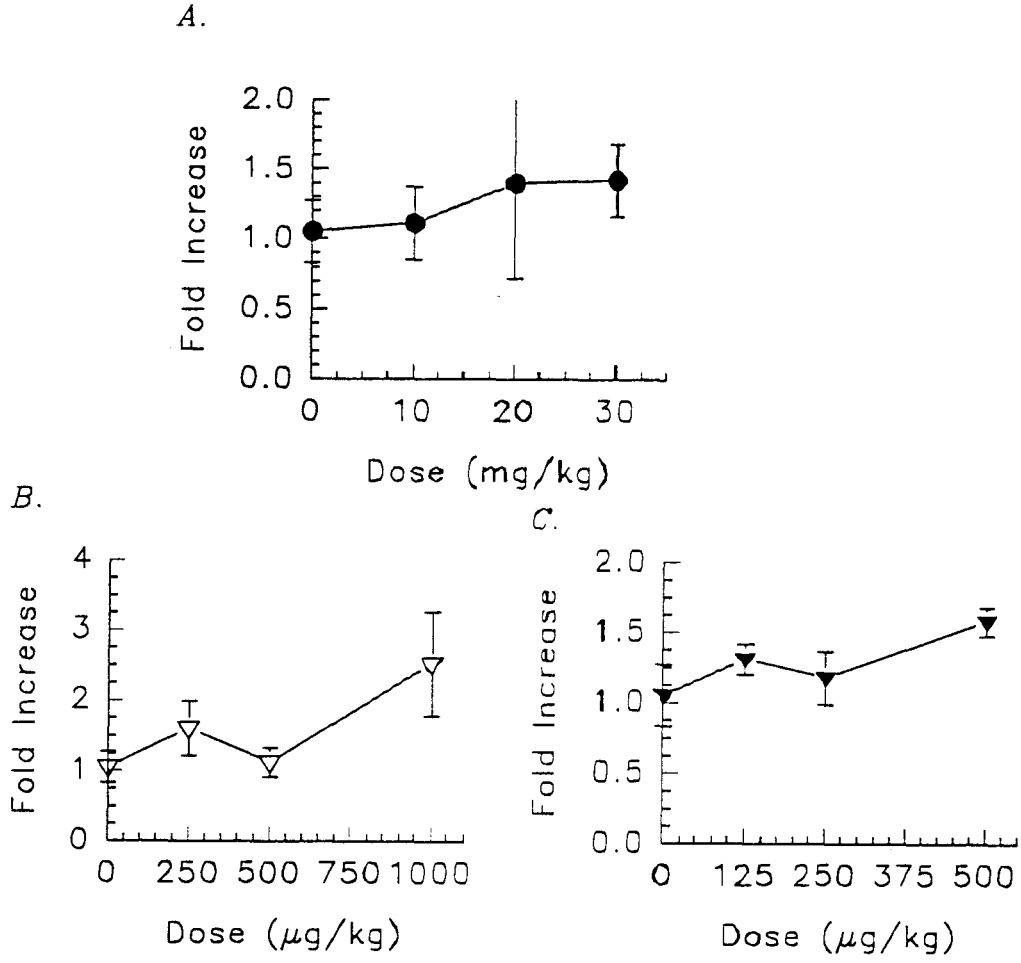


Figure 66. Effect of subcutaneous administration of heparin analogues (GL-522-Y-1 (A), heparin (B), and pentasaccharide (C)) on the R-time of the thrombelastograph. Results represent the mean ± SEM of 3 to 5 observations. None of the agents were observed to prolong the R-time following subcutaneous administration. Statistical significance was assessed for each agent using one way ANOVA followed by the Newman-Keuls multiple comparison test. All p values are for treatment versus saline treated control animals. Data are compiled in Table 60.

GL-52-Y-1 : $p = 0.842$

Heparin : $p = 0.113$

Pentasaccharide : $p = 0.251$

R-time was prolonged 2.3 fold baseline. The effect of heparin administration on the R-time is shown in panel C. The R-time was prolonged at doses above 12.5 μg/kg in a dose-independent manner. There was no difference in the prolongations produced by 12.5, 25, or 50 μg/kg. Pentasaccharide also elevated the R-time in a dose-independent manner. These results are depicted in panel D. Individual R-time values are presented in Table 59.

Figure 66 depicts the effects of subcutaneous administration of GL-522-Y-1, heparin and pentasaccharide on the R-time as measured by thrombelastography. The dose-response curve for GL-522-Y-1 is pictured in panel A. Small changes in the R-time were evident with high doses of GL-522-Y-1. Wide variation, particularly at a dose of 20 mg/kg prevented any statistically significant differences. Heparin increased the R-time at all doses tested, though not in a dose-dependent manner (panel B). The smallest increase in R-time was observed at a dose of 500 μg/kg. At a dose of 1 mg/kg, heparin prolonged the R-time to 2.5 fold baseline. As with heparin, a dose-independent increase in R-time was observed following pentasaccharide administration. In panel C in is observed that at the highest dose, the R-time was prolonged 1.6 fold over baseline. R-time values are presented in Table 60.

c. *Ex Vivo* Anticoagulant Effects as Measured by the Global Clotting Assays

Blood samples were drawn at baseline and immediately prior to administration of the thrombogenic challenge for the purpose of determining anticoagulant activity at

these time points. PT, APTT, Heptest®, and 2.5 U thrombin times were performed on plasma derived from these samples. Due to the wide variation of the clotting parameters among the rabbits, anticoagulant activity was expressed as fold increase over baseline. This anticoagulant activity was plotted against clot score and a correlation coefficient for each agent was determined. The clotting time data is presented in Tables 61 to 68.

Following intravenous administration, none of the agents dose-dependently prolonged the prothrombin time. The APTT was dose-dependently increased by aprosulate administration. At the highest dose tested, 500 μg/kg, the APTT was increased 1.5 fold above baseline values. A correlation coefficient of 0.85 was observed between clotting times in the APTT assay and clot scores following 10 minutes stasis time. Administration of the other agents did not produce a dose-dependent increase clotting time in this assay.

In the Heptest® assay, aprosulate was observed to increase the clotting time in a dose-independent manner. Heparin and GL-522-Y-1 administration resulted in elevation of the clotting time only at the highest dose tested. Both agents increased clotting times to approximately 1.4 fold baseline. Pentasaccharide produced a dose-dependent elevation of the Heptest® clotting time which demonstrated a strong correlation with the observed antithrombotic activity ($r = 0.97$). At a pentasaccharide dose of 100 μg/kg, the Heptest® clotting time was 2.7 fold baseline.

Pentasaccharide was the only agent which did not prolong the 2.5 U thrombin time following administration. Aprosulate, GL-522-Y-1, and heparin all increased the clotting time in this assay in a dose-dependent fashion. Correlation coefficients between

clotting time and clot scores were 0.87, 0.89 and 0.94 for aprosulate, GL-522-Y-1, and heparin, respectively. Heparin exhibited the highest potency in the thrombin time assay, prolonging the clotting time 3.2 fold baseline following a dose of 50 μg/kg. Aprosulate increased clotting time 2.6 fold following a dose of 500 μg/kg. GL-522-Y-1 administration produced the least potent increase in thrombin time, elevating the clotting time 1.9 fold at a dose of 5000 μg/kg.

As following intravenous administration, none of the agents produced a dose-dependent increase in prothrombin time following subcutaneous administration. In the APTT assay, heparin was the only agent to dose-dependently prolong the clotting time. This increase in clotting time correlated well with the clot scores determined after 10 minutes stasis ($r = 0.98$). The APTT was increased 1.3 fold after a dose of 1 mg/kg heparin.

In the Heptest® assay, both heparin and pentasaccharide were observed to dose-dependently prolong the clotting time. For a given gravimetric dose of each agent, pentasaccharide produced a larger increase in the clotting time. Following a dose of 1 mg/kg, heparin produced a 2 fold increase in clotting time relative to baseline whereas administration of pentasaccharide at a dose of 250 μg/kg produced a similar prolongation. In the case of both agents, the prolongation of the clotting time was highly correlated with the antithrombotic activity in the stasis thrombosis model. Correlation coefficients of 0.98 and 0.90 were determined for heparin and pentasaccharide, respectively.

In the 2.5 U thrombin time, heparin was the only agent observed to increase the clotting time in a dose-dependent fashion. Following a dose of 1 mg/kg, the clotting time

was increased more than 5 fold baseline. This prolongation of the clotting time correlated well with heparin's antithrombotic activity ($r = 0.99$).

12. *Ex Vivo* Functional TFPI Levels

TFPI release was measured in rabbit plasma samples obtained during the rabbit stasis thrombosis model. TFPI was measured using a modified functional assay on baseline and post-drug samples. The post-drug samples chosen were those from rabbits treated with the highest intravenous dosage of each agent. These treatments included 50 μg/kg heparin, 100 μg/kg pentasaccharide, 500 μg/kg aprosulate, and 5 mg/kg GL-522-Y-1. At these doses, there was equivalent antithrombotic activity in the stasis thrombosis model. TFPI levels expressed as U/mL were determined in each sample relative to a calibration curve made by diluting normal rabbit pool plasma. NRP is designated as having 1 U/mL TFPI. Fold increase over baseline was determined for each post-drug sample. Figure 67 depicts the results of this analysis. The largest increase in functional TFPI levels were observed following heparin administration. Levels in the post-drug samples were 180 % those in the baseline samples of the same rabbits. This increase in TFPI by heparin was significantly larger than that observed in saline, aprosulate, or pentasaccharide treated rabbits ($p < 0.05$). The synthetic analogues increased plasma TFPI levels to varying degrees. Pentasaccharide administration produced the smallest increase in TFPI levels. None of the increases produced by the synthetic analogues were significantly higher than in the saline treated control rabbits.

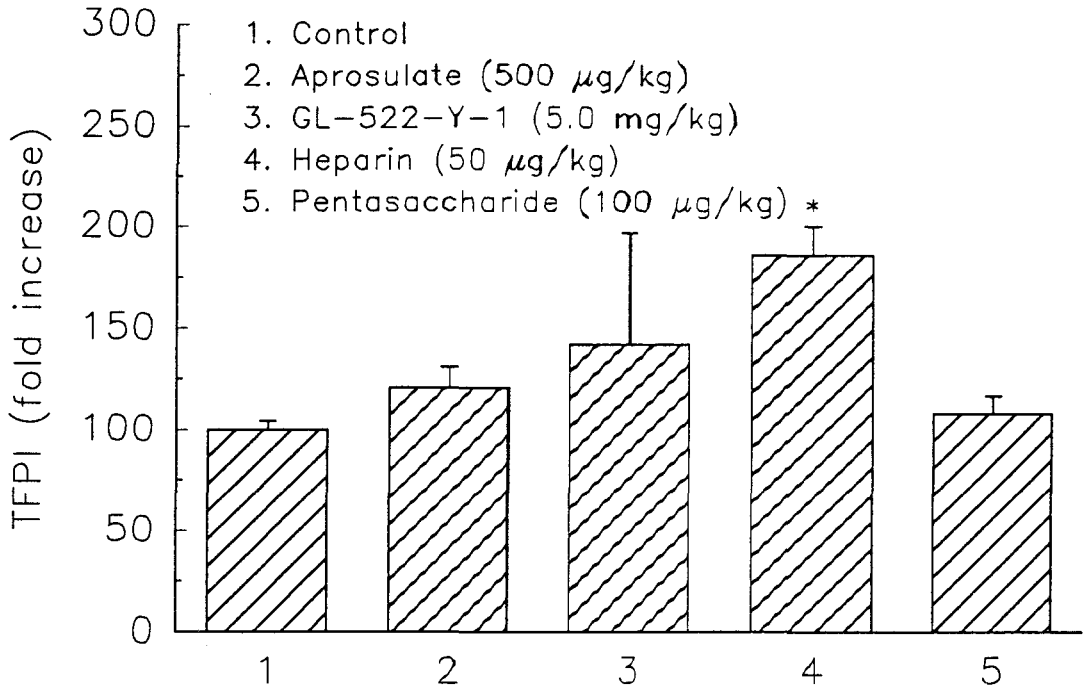


Figure 67. TFPI release by heparin analogues in rabbits following intravenous administration. TFPI levels were determined using a modified functional assay. All results are expressed as the mean ± SEM fold increase relative to baseline of n = 5 rabbits. Doses represent those producing maximal antithrombotic activity in the stasis thrombosis model. Only heparin administration was observed to significantly increase the functional TFPI levels measured in plasma. Statistical comparisons were made by one way ANOVA ($p = 0.008$) followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

Heparin *vs.* saline; $p < 0.05$
Heparin *vs.* aprosulate; $p < 0.05$
Heparin *vs.* GL-522-Y-1; $p < 0.05$
Heparin *vs.* pentasaccharide; $p < 0.05$

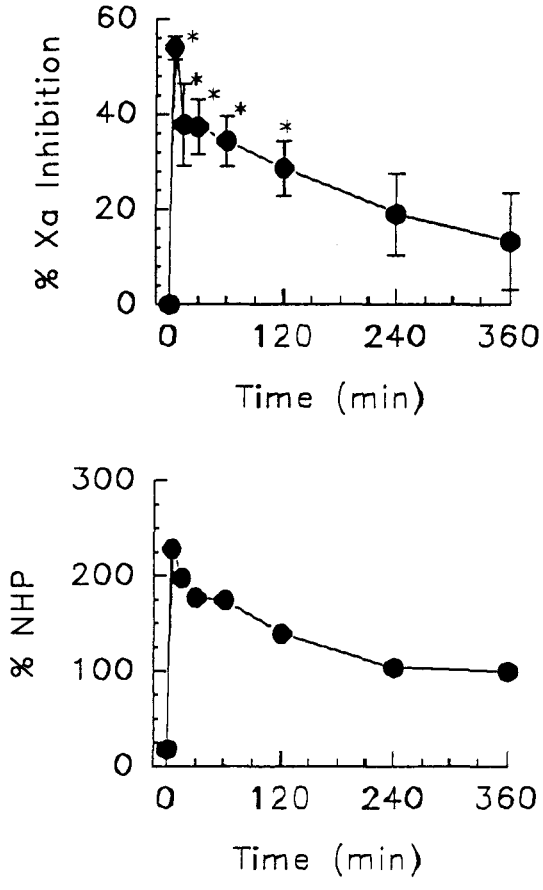


Figure 68. TFPI levels following intravenous administration of 10 mg/kg GL-522-Y-1 to monkeys. TFPI levels were measured using an amidolytic functional assay (panel A) and an immunologic assay (panel B). The results in panel A represent the mean ± SEM of 3 monkeys. TFPI levels were compared using one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant. The results in panel B represent the TFPI levels in one of the monkeys analyzed with the functional assay. Both functional and immunologic TFPI levels were observed to peak 5 minutes post-administration of GL-522-Y-1. Functional TFPI levels were no longer observed to be significantly elevated form control at 240 minutes post-administration.

D. Other *In Vivo* Studies in Various Animal Models

Non-human primates offer a unique opportunity to determine the modulation of endogenous mediators such as TFPI after heparin administration. To determine the relative release of TFPI by some of these analogues, non-human primates were used. Newly developed immunologic methods along with functional assays for TPFI were employed.

1. Effect of GL-522-Y-1 on TFPI Levels in Non-human Primates

The release of TFPI following intravenous administration of GL-522-Y-1 to *Macaca fascicularis* was determined using both the functional and immunologic assays. Three monkeys received an intravenous dose of 10 mg/kg GL-522-Y-1. Blood samples were drawn at baseline and at 5, 15, 30, 60, 120, 240, and 360 minutes post-administration. Figure 68 depicts the time response curves of the plasma TFPI levels following GL-522-Y-1 administration. In panel A, the functional TFPI levels expressed as % Xa inhibition at various time points are shown. At 5 minutes post-administration, a peak of 54 $\pm$ 2.5 % Xa inhibition was observed. The TFPI level decreased to a level below 40 % inhibition by 15 minutes and then progressively declined with time through 360 minutes. TFPI levels were significantly elevated from 5 to 120 minutes post-administration ($p < 0.05$). A wide variation in the inhibition of functional TFPI activity was observed in these monkeys. In panel B, a similar pattern of immunologic TFPI levels was observed. Using the immunologic assay, TFPI levels were observed to peak 2.25 fold in relation to a human TFPI based standard at 5 minutes post-administration. TFPI

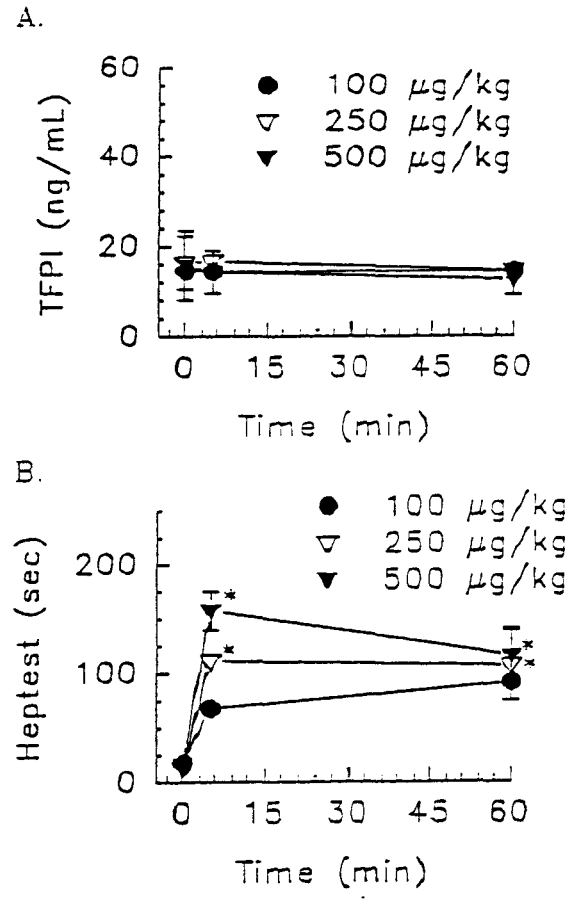


Figure 69. TFPI levels following intravenous adminstration of pentasaccaharide to monkeys. TFPI levels (panel A) were determined using an immunologic assay. The results represent the mean ± SEM of 1 to 3 monkeys per treatment group. Post-drug results were compared to baseline using ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant. TFPI antigen levels were not effected by pentasaccharide administration. The Heptest® clotting time was dose-dependently elevated at 5 and 60 minutes post-pentasaccharide administration.

levels reached 100 % normal human plasma by 240 minutes post-administration. It is unknown why the TFPI level in the baseline sample was unusually low.

2. Immunologic TFPI Levels in Primates Treated with Pentasaccharide

The release of TFPI following intravenous administration of pentasaccharide to *Macaca mulatta* was determined by immunologic assay. Individual groups of monkeys received an intravenous injection of pentasaccharide at a dose of either 100, 250, or 500 μg/kg. Blood samples were drawn at baseline and at 5 and 60 minutes post-administration of pentasaccharide. These results are presented in Figure 69. In panel A, the effect of increasing pentasaccharide dosage on plasma TFPI levels is shown. Baseline TFPI antigen levels in monkey plasma were determined to be 16 ng/mL. No increase in TFPI levels was observed at any of the pentasaccharide dosages studied despite a dose-dependent increase in the Heptest® clotting time following administration. The effect of the pentasaccharide dosages on the Heptest® are shown in panel B. Significant, dose-dependent prolongations of the clotting time were observed at 5 and 60 minutes post-administration of 250 and 500 μg/kg pentasaccharide ($p < 0.05$). Following administration of 500 μg/kg pentasaccharide, Heptest® clotting times were increased up to 9 fold baseline ($p < 0.05$ *vs.* control).

E. Human Trials with Aprosulate

To further validate the preclinical data obtained with aprosulate, TFPI levels were measured with a newly developed immunologic assay in plasma samples obtained

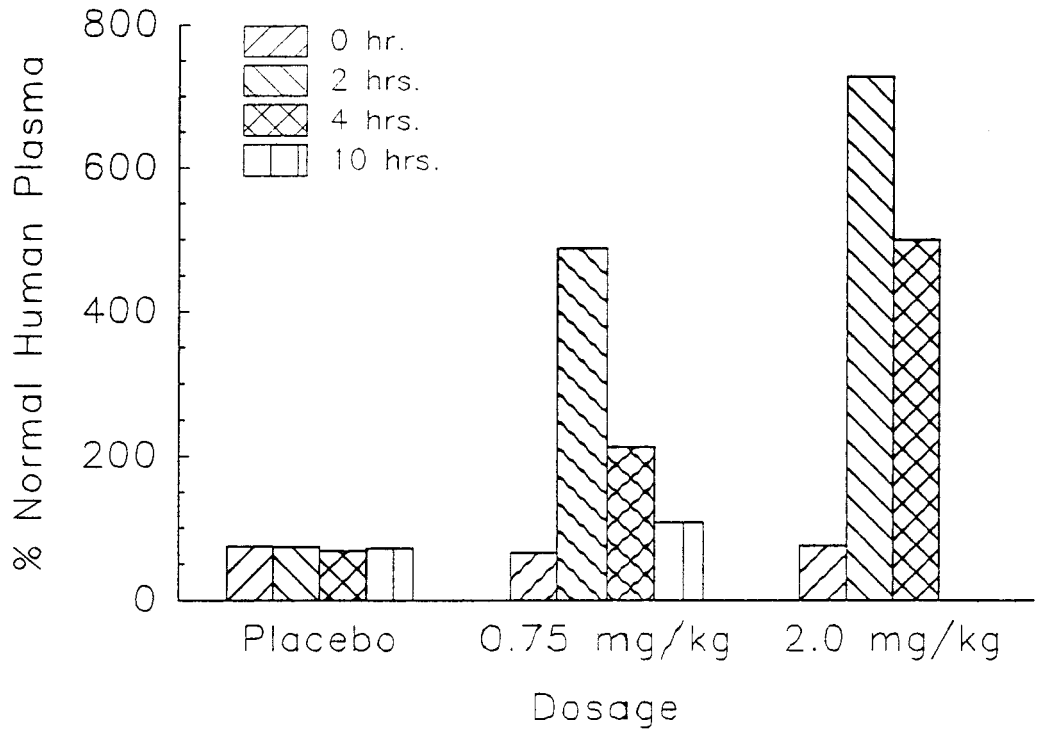


Figure 70. TFPI levels in human volunteers treated with ascending doses of aprosulate. Volunteers received placebo, 0.75 mg/kg and 2.0 mg/kg aprosulate subcutaneously on days 1, 6, and 12, respectively, of the trial. TFPI levels 2 hours post-administration of aprosulate were increased in a dose-dependent manner. TFPI antigen levels were observed to decrease in samples drawn subsequent to 2 hours.

from human trials. Two well developed studies carried out in human volunteers were included. The anticoagulant effects were also investigated utilizing global clotting assays to explain the contribution of TFPI for the mediation of these effects.

1. DELPHI Study

TFPI levels were measured in DELPHI trial samples using an ELISA based assay for the quantitation of TFPI antigen. In this trial, volunteers received ascending dosages of aprosulate subcutaneously for a period of 12 days. Blood samples were drawn at baseline and at 2, 4, and 10 hours post-administration. As seen in Figure 70, on day 1 where volunteers were treated with placebo, no fluctuation in TFPI levels was observed. TFPI levels were approximately 75 % that of a normal human plasma pool which was used as a standard. On day 6, volunteers received 0.75 mg/kg aprosulate subcutaneously. At the two hour point, the TFPI levels were approximately 7 fold higher than baseline. The TFPI levels gradually declined as the time post administration increased. By 10 hours, TFPI levels had nearly returned to baseline. On day 12 of the study where volunteers received 2.0 mg/kg aprosulate subcutaneously, TFPI levels were 9.5 fold that of baseline. At four hours, TFPI levels remained 6 fold higher than baseline.

2. PALLAS Study

The ability of aprosulate administration to increase plasma TFPI levels was examined in plasma samples obtained from a phase I clinical trial of this agent in healthy,

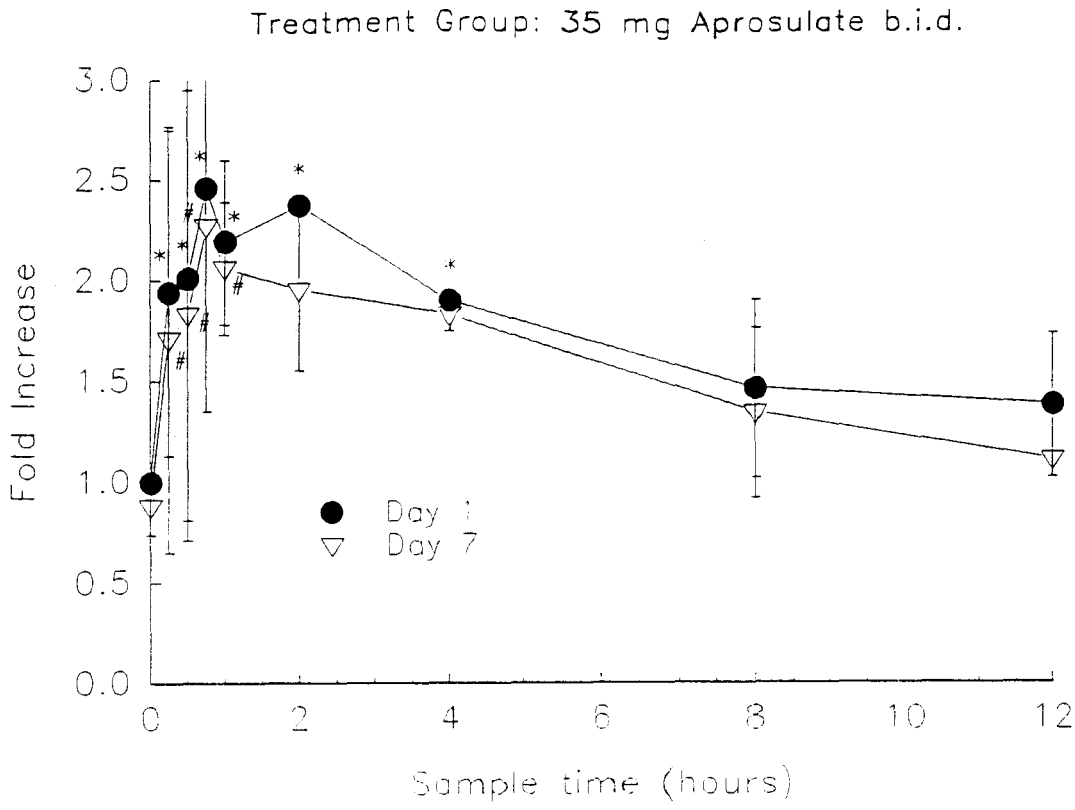


Figure 71. Comparison of TFPI release following subcutaneous administration of 35 mg b.i.d. aprosulate on days 1 and 7 of the PALLAS study. Blood samples were obtained at time points as described in "Materials and Methods". The results represent the mean ± SEM of six human volunteers. Fold increase was calculated relative to the pretreatment control level of each individual volunteer. TFPI antigen levels were observed to peak 45 to 60 minutes after administration of aprosulate. Significant differences between days 1 and 7 were not observed. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. A p-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* baseline on Day 1. $^{\#}p < 0.05$ *vs.* baseline on Day 7.

male volunteers. TFPI levels were determined using an ELISA assay. Statistical comparisons between timepoints on the same day were made by one way ANOVA followed by the Newman-Keuls test. Comparison between Day 1 and Day 7 were made by t-test.

Figure 71 illustrates a comparison of the TFPI antigen (TFPI:Ag) levels measured on Day 1 of treatment with those measured on Day 7 of treatment at the same time points. On Day 1, TFPI levels were observed to rapidly increase following subcutaneous administration of aprosulate. Peak TFPI:Ag levels, 2.5 fold baseline, were measured at 45 minutes post-injection, with the TFPI:Ag levels declining over the next 12 hours. TFPI:Ag levels were significantly elevated over baseline up to the 4 hour time point ($p < 0.05$ *vs.* baseline). At 12 hours, TFPI:Ag levels remained elevated approximately 40 % over baseline. A similar increase in TFPI:Ag levels was observed on Day 7 of the study. While the mean TFPI:Ag were slightly lower on Day 7, the levels on Day 1 and Day 7 were not statistically different. Heptest® clotting times were also determined on these samples. The anticoagulant activity measured by the Heptest® exhibited a strong correlation with the TFPI antigen levels. Regression analysis of these parameters indicated a correlation coefficient of 0.92.

Figure 72 depicts the data on the comparison of the TFPI:Ag measured on Day 1 of treatment with those measured on Day 7 of treatment at the same time points in the group of volunteers receiving 70 mg aprosulate once daily. On Day 1, the peak TFPI:Ag level was achieved 60 minutes post-injection and was 2.6 fold baseline ($p = 0.001$ *vs.* control). TFPI:Ag levels declined less rapidly following the peak in the 70 mg o.d. group

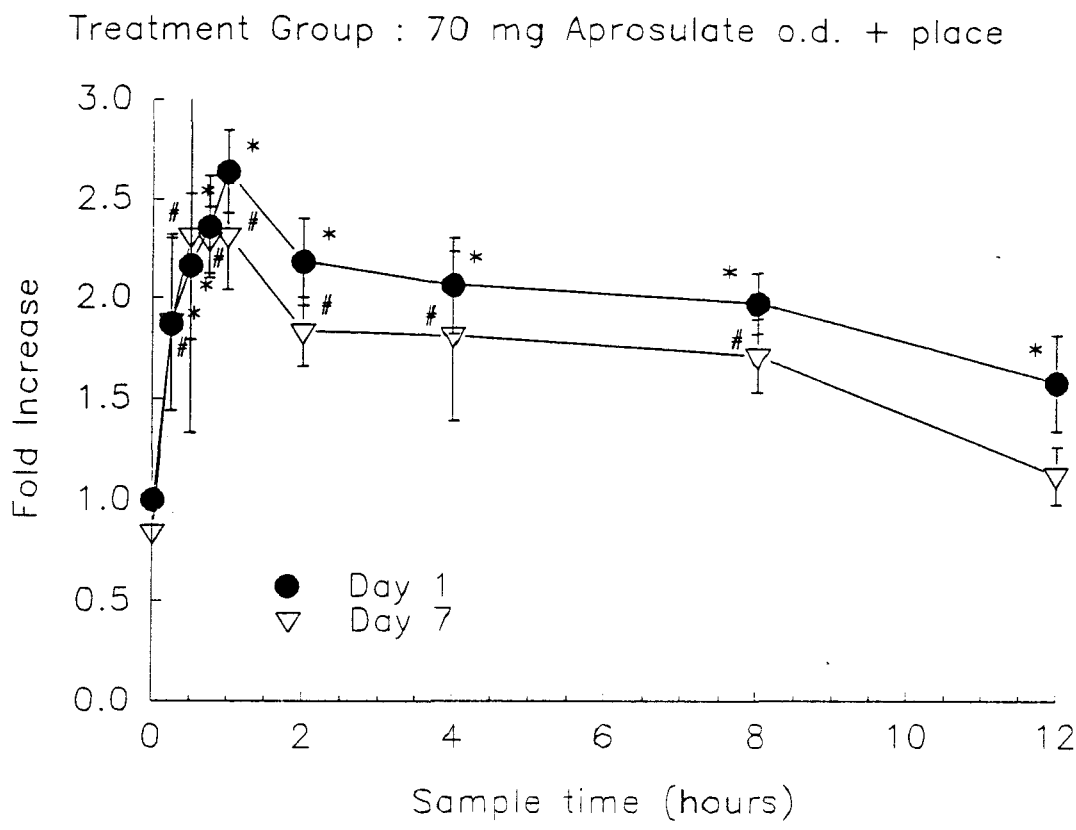


Figure 72. Comparison of TFPI release following subcutaneous administration of 70 mg o.d. aprosulate on days 1 and 7 of the PALLAS study. Blood samples were obtained at time points as described in "Materials and Methods". The results represent the mean ± SEM of six human volunteers. Fold increase was calculated relative to the pretreatment control level of each individual volunteer. TFPI antigen levels were observed to peak 45 to 60 minutes after administration of aprosulate. Significant differences between the TFPI levels on days 1 and 7 were not observed. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. A *p*-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* baseline on Day 1. $^{\#}p < 0.05$ *vs.* baseline on Day 7.

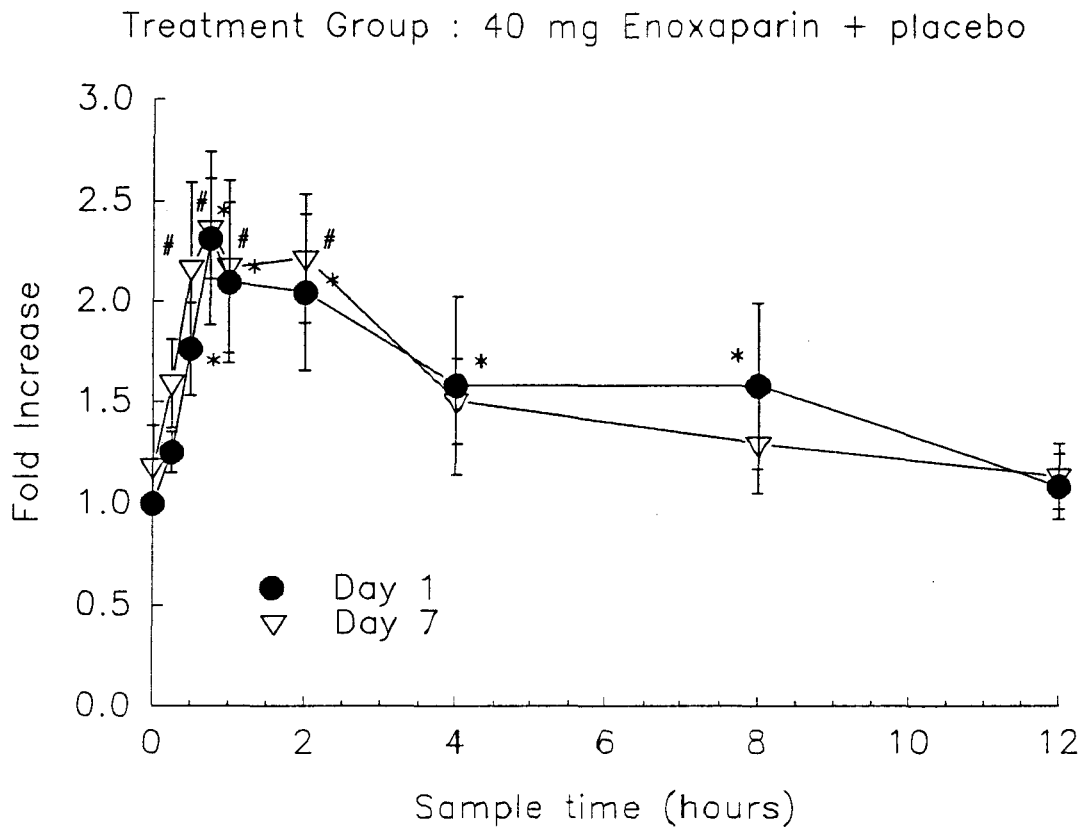


Figure 73. Comparison of TFPI release following subcutaneous administration of 40 mg o.d. Enoxaparin® on days 1 and 7 of the PALLAS study. Blood samples were obtained at time points as described in "Materials and Methods". The results represent the mean ± SEM of six human volunteers. Fold increase was calculated relative to the pretreatment control level of each individual volunteer. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. A *p*-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$ *vs.* baseline on Day 1. $^{\#}p < 0.05$ *vs.* baseline on Day 7.

than in the 35 mg b.i.d. group. At 8 hours, TFPI:Ag levels remained nearly 2 fold baseline in the 70 mg o.d. group. All time points post-administration had TFPI:Ag levels significantly elevated relative to baseline. A difference between Day 7 and Day 1 TFPI:Ag levels was more evident in this treatment group than in those volunteers treated with the lower dose of aprosulate. The peak TFPI:Ag level on Day 7 was approximately 15 % lower on Day 7 than on Day 1. This difference, evident at all subsequent time points, was not statistically significant. A good correlation between Heptest® clotting times and TFPI:Ag levels was again observed ($r = 0.79$).

Figure 73 illustrates the TFPI:Ag levels in the Enoxaparin® treated group of volunteers on Days 1 and 7. Peak TFPI:Ag levels were observed at 45 minutes post-administration. Maximal levels were determined to be approximately 2.4 fold over baseline on both Days 1 and 7. TFPI levels were significantly elevated compared to baseline from 45 minutes to 8 hours post-administration. The TFPI:Ag levels measured on Days 1 and 7 were nearly identical. By 12 hours, the TFPI:Ag levels returned to baseline values. The correlation between anticoagulant activity and TFPI levels was 0.90.

Figure 74 depicts the results of the anticoagulant activity as measured by the APTT in the plasma samples from volunteers treated with 35 mg b.i.d. aprosulate, 70 mg o.d aprosulate, and 40 mg o.d. Enoxaparin®. Enoxaparin® administration resulted in a weak effect on the APTT which peaked 4 hours post-administration ($p < 0.05$). Aprosulate was observed to prolong the APTT to a greater degree than Enoxaparin® and did so in a dose-dependent manner. Peak APTTs in the aprosulate treated groups occurrred at 45 minutes (35 mg b.i.d. aprosulate) and 60 minutes (70 mg o.d. aprosulate)

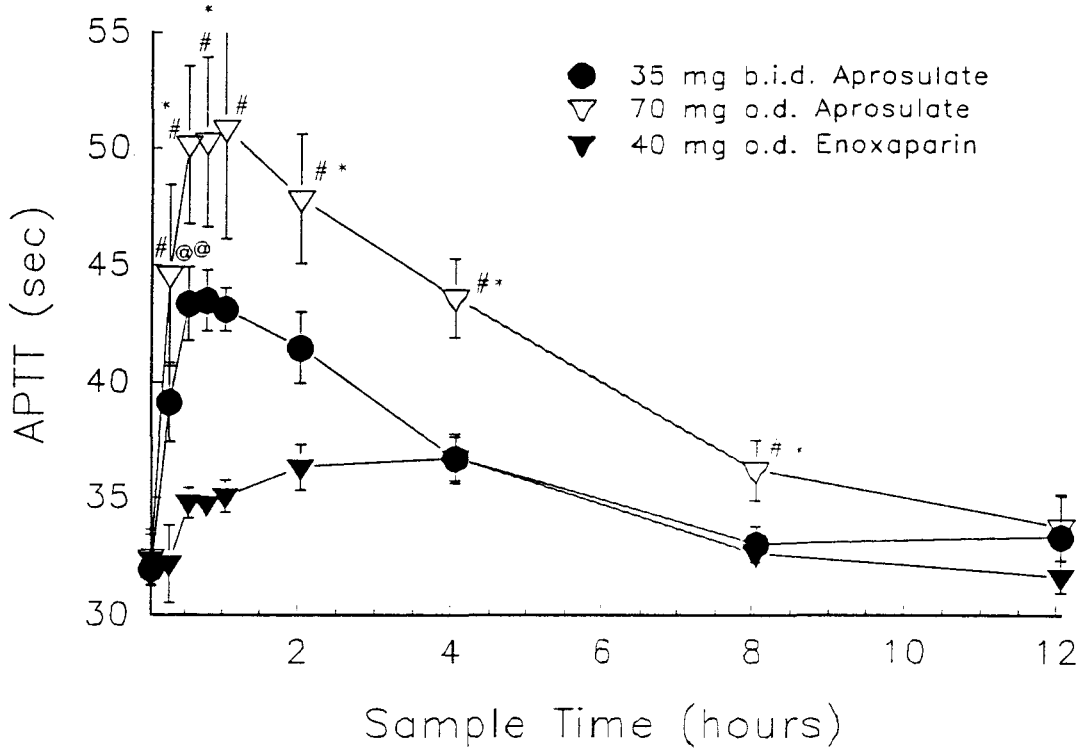


Figure 74. Comparison of anticoagulant activity as measured by the APTT in PALLAS study samples. Blood samples were obtained at time points as described in "Materials and Methods". The results represent the mean ± SEM of six human volunteers. Statistical differences were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. A p-value less than 0.05 was considered statistically significant. $^{*}p < 0.05$; aprosulate 70 mg *vs.* aprosulate 35 mg. $^{\#}p < 0.05$; aprosulate 70 mg *vs.* Enoxaparin®. $^{@}p < 0.05$; aprosulate 35 mg *vs.* Enoxaparin®.

post-administration. Administration of 70 mg aprosulate produced significantly elevated clotting times compared to Enoxaparin® treatment at times up to eight hours post-administration. The lower dose of aprosulate significantly elevated clotting times relative to those in the Enoxaparin® treatment group only at 30 and 45 minutes post-administration. 70 mg aprosulate significantly prolonged APTT clotting times relative to a 35 mg aprosulate treatment up to eight hours post-administration. Baseline APTT values were reachieved by 12 hours in each of the treatment groups.

CHAPTER V

DISCUSSION

Despite an incomplete understanding of its mechanism of action, heparin has been used effectively as an antithrombotic agent for nearly 60 years. The study of heparin's mechanism of action is complicated by the polycomponent and polyfunctional nature of this agent. Heparin preparations which are used clinically contain components which vary in molecular weight from 1,500 to 30,000 daltons. In addition to the gradient in molecular weight, heparin chains of a similar molecular weight can exhibit a microheterogeneity in chemical structure in that the sulfation pattern of the polysaccharide chains is not consistent. The specific sequence of heparin required for high affinity binding to antithrombin III is present in approximately 20 % of the chains (Casu, 1989).

Heparin's primary mechanism of action involves the indirect inhibition of coagulation proteases mediated by binding to the endogenous plasma protein antithrombin III. Heparin acts as a catalyst in this reaction, increasing the rate of inhibition of several coagulation enzymes more than 1,000 fold (Jordan et al., 1980). The primary targets of the heparin-antithrombin III complex are factors Xa and IIa (thrombin). The inhibition of these enzymes has been utilized both for the monitoring of heparin therapy and for the

designation of potency of heparin preparations.

The potency of heparin is typically designated in relation to its anticoagulant or antiprotease activity. Heparins are usually dosed based on their anti-Xa or antithrombin units per milligram potency. Due to amplification reactions in the coagulation cascade, the anti-Xa and antithrombin potencies of a given material are not necessarily equivalent. Furthermore, the in vitro potency of heparin does not truly reflect this endogenous actions.

Heparin therapy has traditionally been monitored using the activated partial thromboplastin time (APTT) (Noureddine, 1995). In this assay, a patient's plasma is incubated with an activator of the contact system. The inhibition of each intrinsic pathway enzyme by the heparin-antithrombin III complex contributes to the overall anticoagulant activity observed. More specific assays are also used to monitor heparin. In clotting assays such as the Heptest®, activated factor X is added to the test plasma so as to more specifically measure the common pathway of coagulation. Amidolytic assays for specific coagulation factors are also employed. While these assays allow for the monitoring of a specific activity of heparin, they are considered somewhat less physiologic than the clotting assays as the test plasma is diluted and the substrates used only mimic the enzyme's natural substrate.

Higher doses of heparin used in cardiopulmonary bypass, angioplasty and other indications are monitored using the activated clotting time. These tests are usually performed on native whole blood in the presence of an activator. Heparin levels up to 5 U/mL can be measured using this test. This test also measures the effect of TFPI

which is released from the vascular sites.

Heparin produces several distinct effects on the coagulation system. The anticoagulant effect of heparin is related to its ability to inhibit preformed serine proteases through binding with endogenous plasma cofactors such as antithrombin III and heparin cofactor II. By inhibiting the proteolytic actions of the coagulation factors, heparin also acts to limit further generation of these enzymes. The anticoagulant properties of heparin can be measured *in vitro*. The antithrombotic activity of heparin refers to the *in vivo* inhibition of thrombus formation. This property of heparin relates not only to the SERPIN mediated inhibition of coagulation proteases but also to heparin's ability to modulate endothelial function (heparan sulfate synthesis, TFPI release) and interact with other cellular components of the vascular system. The inhibition of hemostatic function by heparin following higher doses may be the result of heparin's interactions with platelets and result in the hemorrhagic side effects observed with heparin therapy.

The low molecular weight heparins are depolymerized derivatives of porcine and bovine mucosal heparins (Fareed et al., 1995). These agents usually exhibit a molecular weight in the range of 4000 to 6000 Da. A large proportion of the molecular components of a given low molecular weight heparin is below 7500 Da. Because of this difference in molecular weight composition, low molecular weight heparins exhibit a relatively weaker antithrombin activity. This is primarily due to the proportionately lower chain lengths of these agents. Barrowcliffe et al. described the effect of chain length on the relative antithrombin and anti-factor Xa potencies of heparins (Barrowcliffe et al., 1979).

Consistent with this hypothesis, pentasaccharide only exhibits anti-Xa properties. While aprosulate is also a low molecular weight analogue of heparin, it does have antithrombin activity. The data in this dissertation clearly demonstrate that this antithrombin activity was primarily mediated by HCII and not by ATIII. Because of endogenous pharmacodynamic interactions, heparin and its analogues produce their effects by modulating endogenous serpins in a differential manner.

Thus, while molecular weight dependence on the antithrombin/anti-factor Xa activity in a given molecular species of heparin may be valid for the high ATIII affinity components, it does not hold true for the non-ATIII affinity components. Charge density also plays an important role in the mediation of the antithrombin actions. As depicted in Figure 75, the smaller oligosaccharide components in heparin are present in relatively lower proportions whereas low molecular weight heparin and ultralow molecular weight heparin contain a higher proportion of these components. Pentasaccharide is a high affinity, low molecular weight fragment of heparin. Aprosulate mimics heparin fragments with almost no ATIII affinity. The data presented in this study provides strong evidence on the different role of ATIII and charge density independent of molecular weight. It is likely that if pentasaccharide is hypersulfated, its ability to interact with HC-II would be enhanced.

Synthetic antithrombotic agents offer several potential advantages over the classic heparin-type anticoagulant agents. Whereas heparin is polycomponent in nature, a synthetic agent can be made with a high degree of purity. Product homogeneity would be beneficial in two respects. First, the agent could be administered on a gravimetric or

to determine the chemical structure of a given agent. For this study, mass spectral analysis was only made for aprosulate and GL-522-Y-1. As these agents possess symmetrical structures, their fragmentation patterns are more easily understood. Practical considerations limit the usefulness of this technique for the structural determination of heparin. Fragmentation patterns of heparin would be difficult to interpret as multiple species are present in the initial preparation.

The molecular heterogeneity of heparin contributes significantly to its pharmacologic effects. The polycomponent nature of this product is largely due to components of varying chain length which also exhibit differing degrees of sulfation and binding to antithrombin III. The synthetic analogues used in these investigations are highly pure compounds which do not exhibit structural heterogeneity. The three physicochemical methods used to characterize the molecular and structural profile of heparin provide information on the molecular weight, molecular mass distribution, degree of sulfation, and the absolute molecular mass. The results obtained using gel permeation chromatography, NMR, and mass spectral analysis are consistent with the proposed structure of each agent. The purity of each agent was also consistent with their specifications. By utilizing these three different methods, reliable information of the molecular and structural characteristics of heparin and its synthetic analogues was obtained.

B. Comparative Studies on SERPIN Modulation

Heparin is known to promote both antithrombin III and heparin cofactor II mediated antiprotease activities. In the amidolytic assay systems used here, it was possible

molar basis. Heparin must be dosed on the basis of activity units as it is a mixture of many components whose biologic activity varies with molecular size. Second, the range of biologic activity may be more limited than that observed with heparin, potentially providing a more specific approach to treat various disorders.

In this study, the relative role of SERPIN activation to clarify the mechanisms of action of heparin was investigated by studying the biologic effects of synthetic heparin analogues in a variety of *in vitro* assays and *in vivo* animal models. The *in vitro* systems were chosen so that the effect of these agents on various steps of the coagulation cascade could be determined. Two models of thrombosis were chosen so that the effect of these agents on thrombogenesis induced by varying triggers could be studied. The hemostatic compromising effects of these agents were compared in a rabbit ear bleeding model. By performing these studies in a integrated fashion, the importance of SERPIN activation to the anticoagulant and antithrombotic actions of heparin was determined. While this study does not address the vascular effects of heparin and its analogues, an attempt has been made to measure the pharmacodynamic effects in terms of the endogenous release of vascular markers such as TFPI. Such data may provide information on the vascular modulation by heparin and its analogues. Dedicated studies on tissue culture on their expression of various antithrombotic mediators such as TFPI and heparan sulfate may also be useful to support the data presented in this dissertation.

A. Physical Characterization of Heparin and Various Analogues

As aprosulate, GL-522-Y-1, and pentasaccharide are homogeneous compounds,

their molecular weights can be calculated based on their chemical structure. Since heparin is polycomponent, however, its average molecular weight was determined using gel permeation chromatography. This technique, which separates the components of the heparin mixture based on molecular size, has previously been used to characterize the molecular weight profiles of heparins, low molecular weight heparins and other glycosaminoglycans (Ahsan et al., 1994; Ahsan et al., 1995; Nieduszynski, 1989). By using the appropriate buffers, the effect of charge density on chromatographic separation can be neutralized and separations are made based on hydrodynamic size. This analysis was important so that the activities of all agents could be compared on a molar basis.

A number of methods have been used to characterize the molecular weight of heparins. These include viscometry (Mathews et al., 1971), low angle laser light scatter (Patat et al., 1959), NMR (Desai et al., 1995), ultracentrifugation (Lasker et al., 1966), and gel permeation chromatography (Ahsan et al., 1994). Gel permeation chromatography offers the advantages of minimal sample preparation and handling and a rapid turn around time. In order to obtain molecular weight data from the HPLC elution profiles, however, the columns used require calibration. These calibrators are optimally derived from a similar material as that being analyzed. Three different calibrations were used in these studies. The first consisted of nineteen fractions of heparin of varying molecular weight. These calibrators were produced by fractionation of heparin. The homogeneity of these samples is increased relative to unfractionated heparin by a series of chromatographic steps. The molecular weights of these calibrators have previously been reported (Ahsan et al., 1994).

The elution profile of heparin was observed to take the form of a Gaussian distribution, indicating the presence of multiple species. Dispersity, a measure of heterogeneity, was observed to be higher for heparin than for the other agents. The molecular weight of heparin was determined to be 10.5 kDa by this analysis. The weight average molecular weight value for GL-522-Y-1 was the most discrepant with its known formula weight. It was determined that due to its chemical nature, GL-522-Y-1 was being bound by the packing material in the HPLC column thereby skewing the elution profile. For this reason, the elution profile of GL-522-Y-1 was not analyzed using the other calibrants. The dispersities determined for aprosulate and pentasaccharide were closer to 1.0.

Each of these two analogues represented homogenous and chemically pure compounds that can be used to study the SAR relationship in heparin. Since aprosulate is highly sulfated in comparison to heparin and pentasaccharide, it's retention on the chromatographic column was different than that of a comparably sized heparin chain. This resulted in a falsely lowered molecular weight value. With both the HMC and the 19 narrow range calibrators, the molecular weight was lower than the calculated formula weight. Since both methods utilized heparin derived products of a lower charge density, these structural differences contributed to the altered molecular mass distribution. Regardless of these differences, the dispersity of aprosulate was noted to be near 1.

The molecular weights of these agents were also determined based on two other calibrations which have been developed for profiling low molecular weight heparins. One of these calibrators, F913B, was proposed to be the European Pharmacopoeial calibration

standard for low molecular weight heparins (van Dedem et al., 1991). Each of these calibrants is a mixture of partially degraded heparins. It has previously been shown that the effectiveness of these calibrators was dependent on the weight range of the sample (Ahsan et al., 1994). With both calibrators, coded HMC and F913B, the molecular weights determined for aprosulate and pentasaccharide were much lower than their true formula weights. This was likely due to the fact that these agents fall at the extreme low end of the calibration curve. The molecular weight of heparin determined with the two calibrations varied from 9.9 to 12.8 kDa. The difference in molecular weights calculated using these two methods is due to the difference in composition of the calibrants (Ahsan et al., 1994). The HMC calibrant contains a larger fraction of higher molecular weight material. This difference in molecular weights using these two methods is consistent with that published for several heparins and low molecular weight heparins (Ahsan et al., 1994).

To calculate molar concentrations and dosages, the known formula weights of the synthetic analogues were used along with the weight average molecular weight of heparin as determined by the 19 calibrator method. This method is of major value in the study of the molecular mass distribution profile of heparin and low molecular weight heparin as the distribution profile and various other parameters can be calculated.

NMR spectroscopy is a valuable technique for the analysis of heparins. This technique provides both information describing the primary structure of heparin as well as its solution conformation. With regard to the primary structure, major components of the heparin chain such as glucosamine and iduronic acid residues can be identified,

sulfate content at various positions can be ascertained, and the configuration of the disaccharide linkage (α or ß) can be determined. NMR analysis is also a powerful tool for determining the solution conformation of the heparin chain. The measured chemical shifts are dependent on the molecular environment of the nucleus and the size of interproton coupling constants is dependent upon the dihedral angle formed between adjacent C-H bonds. Thus, it can be determined whether adjacent hydrogen moieties are positioned cis or trans to each other. NMR spectra can provide information on the purity of the sample. As the intensity of a given peak is related to the amount of substance present, purity can be readily assessed. Recently, NMR spectroscopy has been used to determine the calibration free molecular weight of heparin (Desai et al., 1995).

Because of the polycomponent nature of heparin, peaks in the NMR spectra tend to overlap. In order to make specific assignments for various peaks, the peaks must be clearly resolved. The spectra of the agents used in this dissertation were obtained using 300 and 500 MHz instruments so as to obtain high resolution spectra.

The degree of sulfation of the heparin used in these studies was characterized by integrating certain peak areas of the ^{13}C spectra. By this analysis, it was shown that in this heparin, 76 % of the 6-OH groups were sulfated and that 88 % of the amino groups were sulfated. More importantly, it is possible to estimate the amount of high affinity ATIII binding sites present in the sample by determining the percentage of 3-O sulfate. For this heparin, it was estimated that 7 % of the chains contained ATIII binding sites. This is in contrast to the pentasaccharide which has an ATIII binding site on every molecule. No peaks related to other glycosaminoglycans such as dermatan sulfate or

heparan sulfate were present in these spectra, indicating a pure heparin sample. This is important as dermatans and heparans exhibit variable effects on the coagulation system.

The spectra of aprosulate and GL-522-Y-1 are much simpler than those of heparin or pentasaccharide. Each structure is symmetrical, and contains no microheterogeneities in sulfation as does heparin. In each of these spectra, all peaks could be assigned. The purity of each agent was confirmed by the lack of extraneous peaks.

^{1}H NMR spectra are complementary to the ^{13}C spectra obtained on a given sample. ^{1}H spectra are not as easily used for the rapid characterization of the purity of polysaccharides such as heparin due to the overlap of signals and a poorer resolution compared to that observed in ^{13}C spectra. The ^{1}H spectra are beneficial, however, for the determination of the secondary structure of such agents (Casu, 1989). Based upon coupling constants and nuclear Overhauser enhancements, conformation data can be obtained.

Mass spectral analysis provides information on the molecular weight and structural features of tested agents. By examining the fragmentation pattern, it is possible to determine the chemical structure of a given agent. For this study, mass spectral analysis was only made for aprosulate and GL-522-Y-1. As these agents possess symmetrical structures, their fragmentation patterns are more easily understood. Practical considerations limit the usefulness of this technique for the structural determination of heparin. Fragmentation patterns of heparin would be difficult to interpret as multiple species are present in the initial preparation.

The molecular heterogeneity of heparin contributes significantly to its pharmacologic effects. The polycomponent nature of this product is largely due to components of varying chain length which also exhibit differing degrees of sulfation and binding to antithrombin III. The synthetic analogues used in these investigations are highly pure compounds which do not exhibit structural heterogeneity. The three physicochemical methods used to characterize the molecular and structural profile of heparin provide information on the molecular weight, molecular mass distribution, degree of sulfation, and the absolute molecular mass. The results obtained using gel permeation chromatography, NMR, and mass spectral analysis are consistent with the proposed structure of each agent. The purity of each agent was also consistent with their specifications. By utilizing these three different methods, reliable information of the molecular and structural characteristics of heparin and its synthetic analogues was obtained.

B. Comparative Studies on SERPIN Modulation

Heparin is known to promote both antithrombin III and heparin cofactor II mediated antiprotease activities. In the amidolytic assay systems used here, it was possible to examine the effect of each analogue on a distinct SERPIN mediated event. It was observed that heparin more strongly inhibited thrombin via antithrombin III than by heparin cofactor II. The IC_{50} value determined for ATIII mediated thrombin inhibition was 60 fold lower than the value determined for the HCII system. This is consistent with the data of Griffith which demonstrates an apparent K_m of 150 nM for the thrombin-HCII

reaction compared with 35 nM for the thrombin-ATIII reaction (Griffith et al., 1983) and data which demonstrates a 5 fold higher NaCl concentration required to elute ATIII from heparin agarose compared with that needed for HCII (Yamagishi et al., 1984; Griffith et al., 1985; Tran et al., 1986). In the ATIII mediated antithrombin assay, maximal inhibition of approximately 95 % relative to unsupplemented control was observed. In the HCII assay, however, a maximal inhibition of only 80 % was observed. Several assays were performed in which either the concentration of HCII, the concentration of heparin or the concentrations of both were increased two fold relative to the standard assay. In these systems, the maximal inhibition remained at 80 %. This can be explained by taking into account the reported V_{max} values of 0.9 and 3.8 nM/min/ng heparin for the HCII-thrombin and ATIII-thrombin reactions, respectively (Griffith et al., 1983). As the kinetics of thrombin interaction with HCII are slower than with ATIII, it is possible that some portion of the thrombin in the assay can interact with the substrate before being inhibited by HCII.

The synthetic analogues exhibited a more selective SERPIN inhibitory profile than heparin. Pentasaccharide was the only analogue to mediate significant antiprotease actions via ATIII. Due to its molecular size, pentasaccharide only inhibits factor Xa. It has been shown previously that a heparin chain length of 18 monosaccharides is minimally required to catalyze the inhibition of thrombin by ATIII (Laurent et al., 1978; Oosta et al., 1981; Holmer et al., 1981; Lane et al., 1984; Danielsson et al., 1986). Pentasaccharide only promotes small increases in the HCII mediated antithrombin activity. It does so at relatively high concentrations compared to those required for ATIII

mediated Xa inhibition. While data is not currently available, a pentasaccharide with higher charge density may exhibit appreciable activity via HCII. The activation of HCII by glycosaminoglycans has been shown to be dependent on the charge density of the GAG rather than on a specific sequence as with the case of ATIII (Hurst et al. 1983). Neither aprosulate nor GL-522-Y-1 produced significant antiprotease actions via antithrombin III as they lack the proper 3-O sulfate conformation needed for high affinity binding. Despite differences in their chemical structure and the functional groups providing the negative charge, GL-522-Y-1 and aprosulate inhibited thrombin via HCII with similar potency. This potency was approximately 20 fold lower than that observed with heparin. Kinetics models of the inhibition of thrombin by HCII suggest that it is necessary for heparin to bind both HCII and thrombin to achieve a catalytic effect (Tollefsen, 1989). Studies with thrombin mutants suggest that an allosteric model rather than a template model better describes the inhibition of thrombin by heparin cofactor II (Sheehan et al., 1994). In the case of ATIII mediated inhibition of thrombin, a minimal chain length of 18 saccharide units is required for heparin to inhibit thrombin. It is possible that the weaker potency of aprosulate and GL-522-Y-1 in inhibiting thrombin via HCII is due to their smaller molecular size.

Although both aprosulate and GL-522-Y-1 produced comparable inhibitory effects mediated by HCII, there was a clear dissociation between the anticoagulant and antithrombotic actions of these two agents. This suggests that aprosulate may have additional functional properties in addition to its interaction with HCII. This data also suggests that HCII alone may have a relatively minor role in the production of

anticoagulant and antithrombotic responses. Such properties as the non-specific binding of these analogues to fibrinogen and other coagulation factors and direct effects on the generation of various protease during activation may be contributory to their actions.

C. Endogenous Release of TFPI by Heparin and Heparin Analogues

In addition to antithrombin III and heparin cofactor II, other endogenous modulators of the coagulation system are known to exist. These include protein C which acts to limit procoagulant activity by inactivating factors Va and VIIIa, protease nexins which have been shown to inhibit thrombin, activated protein C, and factor XIa, and tissue factor pathway inhibitor, a multi-Kunitz inhibitor of factors VIIa and Xa.

Tissue factor pathway inhibitor is a recently recharacterized Kunitz-type protease inhibitor which may be an important mediator of heparin's actions. It is known that heparin administration causes an increase in plasma TFPI levels (Ariens et al., 1994; Warn-Cramer et al., 1993). Additionally, it has been reported that heparin and related glycosaminoglycans are capable of binding to TFPI (Valentin et al., 1994) and can promote a synergistic anticoagulant action (Wun, 1992). Based on these studies, it has been suggested that TFPI may be just as important as ATIII and HCII for the anticoagulant actions of heparin (Valentin et al., 1992) and may significantly contribute to the antithrombotic actions of heparin and heparin analogues (Ostergaard et al., 1993).

In phase I clinical trials using human volunteers, the effect of the synthetic analogues on TFPI release was investigated. In addition, because of the molecular homology of TFPI in non-human primates, additional experimental studies were carried

out in non-human primates. TFPI levels were measured using functional and immunologic assays. The functional and immunologic assays for TFPI can potentially give different results. In the ELISA assay, TFPI is detected by a monoclonal antibody directed against the first Kunitz domain. This assay, therefore, measures the total TFPI content of the sample. Carboxy truncation of the TFPI molecule is known to result in a reduced antiprotease activity (Nordfang et al., 1991). The functional assay, therefore, may only measure a fraction of the TFPI present in plasma, that which is not truncated.

Intravenous administration of GL-522-Y-1 to non-human primates was observed to rapidly increase the levels of functional TFPI activity measured as Xa inhibition. The antigenic TFPI levels measured in the samples of one of these treated monkeys correlated highly with the functional TFPI levels ($r = 0.976$) indicating that full-length, functional TFPI is released upon GL-522-Y-1 administration. Administration of pentasaccharide at doses up to 500 μg/kg did not elevate the plasma TFPI antigen levels in non-human primates. Despite this, a dose-dependent anticoagulant effect was observed suggesting that the release of TFPI does not contribute to the anti-Xa effects of pentasaccharide. It has been suggested that glycosaminoglycan binding to TFPI may be dependent not only on the total sulfate content, but also on the localization of the charged groups (Valentin et al., 1994). Calixarenes such as GL-522-Y-1 have been shown to be in a folded conformation in solution which may act to form a region of high negative charge (Atwood et al., 1992; Gutsche et al., 1981). Based on charge density alone, pentasaccharide would be expected to release TFPI. Both unfractionated heparin and various low molecular weight heparins have been shown to increase plasma TFPI levels

following intravenous and subcutaneous administration (Ariens et al., 1994; Warn-Cramer et al., 1993; Bara et al., 1993; Holst et al., 1993; Vogel, 1995). The number of sulfate groups per saccharide in heparin is not markedly different from that in pentasaccharide. The size of the pentasaccharide molecule appears to play a role in its inability to release TFPI.

The effects of aprosulate on plasma TFPI levels were investigated as part on two phase I clinical trials. In the PALLAS study, aprosulate was administered daily for a period of seven days. It was observed that aprosulate increased plasma TFPI antigen levels within 15 minutes after subcutaneous administration and peak levels were achieved 45 to 60 minutes post-administration. The TFPI antigen levels were observed to correlate with anticoagulant activity measured by the APTT and plasma drug levels determined using the Heptest®. Repeated administration of aprosulate did not deplete endogenous TFPI stores following seven days of treatment (Jeske et al., 1995). The time course of TFPI levels and the time to peak levels were not significantly different on days 1 and 7 of the study. Limited data from the DELPHI dose-escalation study indicates that aprosulate dose-dependently increases the plasma TFPI antigen levels. As in the PALLAS trial, dosing of aprosulate on alternate days for a period of 12 days did not deplete the TFPI stores.

The significance of the heparin releasable TFPI to the pharmacologic effects of heparin remains unknown at this time. To date, no TFPI deficient individuals have been identified. Since small amounts of tissue factor generation on the cell surface is sufficient to initiate thrombogenesis, circulating levels of TFPI may be sufficient to blunt the

thrombogenic effects of tissue factor. It is also unknown as to whether any of the synthetic analogues are capable of interacting with and potentiating the activity of TFPI. Studies presented in the literature have indicated that in *in vitro* systems, heparin potentiates the anticoagulant activity of TFPI. The mechanism for this effect has not been fully elucidated. Heparin and its analogues may also directly interact with circulating and released TFPI and alter its pharmacodynamic or pharmacokinetic behavior. These interactions may be charge dependent and result in certain modifications of TFPI molecules resulting in increased functionality.

D. Comparative Anticoagulant Profile

The anticoagulant potency of the heparin analogues was compared in human and rabbit plasma using global clotting tests such as the prothrombin time, activated partial thromboplastin time, Heptest® and the thrombin time. In these assays, different triggers are used to activate the coagulation system in distinct locations. In the prothrombin time, a rabbit brain tissue thromboplastin is used to activate factor VII and the extrinsic pathway of coagulation. This assay is routinely used to monitor oral anticoagulant therapy as it is sensitive to low levels or inhibition of factor VIIa (Hirsh et al., 1994). In the APTT, a micronized silica solution is used to activate the contact system. This assay is used clinically to monitor heparin therapy as the heparin-ATIII complex inhibits most of the intrinsic pathway enzymes (Noureddine, 1995). The Heptest® and the thrombin time are more specific clot based assays. The Heptest® is designed to measure the inhibition of the conversion of prothrombin to thrombin. This is achieved by using

bovine factor Xa as an activator of coagulation. As thrombin is ultimately formed prior to reaching the assay endpoint, this assay is also sensitive to the antithrombin actions of different agents. The thrombin time measures the conversion of fibrinogen to fibrin following the addition of a known amount of thrombin to plasma. Thus, each of these assays provide a distinct point in the coagulation cascade for the evaluation of the anticoagulant effects of various drugs.

The anticoagulant potency of heparin and related drugs is dependent on several factors. Due to the differing and more limited mechanisms of action of the heparin analogues, the procoagulant trigger will influence the systems in which these agents are active. In addition, the dilution of the plasma with the assay reagents will affect the anticoagulant activity observed. In the thrombin time assay, 200 μL of plasma is used to determine the clotting time, whereas in the other clotting assays, 100 μL of plasma is used. In the thrombin time, therefore, twice as much anticoagulant is present in the system as is present in the other assays. Lastly, the anticoagulant potency of each agent was compared based on agent concentrations required to increase the clotting time to 100 seconds. In the various assays, baseline clotting times in normal human plasma ranged from 12.0 $\pm$ 0.2 seconds in the PT to 37.3 $\pm$ 4.0 seconds in the APTT. A clotting time of 100 seconds is therefore, a three fold increase over baseline in the APTT, but more than 8 fold in the PT. This approach to comparing the potencies of each agent was used due to the fact that a maximal anticoagulant activity was not observed. Clotting times were artificially limited to a maximum of 300 seconds based on the linear range of the assays.

Heparin exhibited the broadest anticoagulant effect, prolonging clotting times in all assays. This is consistent with the known range of activities of heparin. The primary targets of the heparin-ATIII complex are Xa and thrombin. Heparin is also a potent inhibitor of factor IXa (Barrow et al., 1994; Beguin et al., 1991) and recent reports indicate that heparin-ATIII can also inhibit factor VIIa (Rao et al., 1995; Rao et al., 1993; Lawson et al., 1993). The observed anticoagulant potencies range from 0.08 $\pm$ 0.05 μM in the 5 U thrombin time to 6.2 $\pm$ 2.1 μM in the PT. The strongest effects are observed in the assays in which intrinsic pathway enzymes are involved. Identical potencies are noted in the Heptest® and APTT where the observed anticoagulant activity of heparin is dependent on its ability to inhibit both factors Xa and thrombin. The thrombin time assay was the most sensitive to the anticoagulant actions of heparin. This is due to the biochemical nature of the assay systems. In the thrombin time assay, active enzyme is added to plasma containing the test agent. In the APTT and Heptest®, active thrombin is generated following an activation process further upstream in the coagulation cascade.

Aprosulate exhibited a weaker anticoagulant activity consistent with its more limited SERPIN mediated antiprotease activity. The potency of aprosulate in the APTT and the Heptest® was 10 to 25 times weaker than that of heparin. Whereas in the APTT heparin would be expected to limit thrombin formation by inhibiting the upstream intrinsic pathway enzymes as well as inhibit any thrombin that is formed, aprosulate can only promote the inhibition of thrombin once it has been formed. Similarly in the Heptest® assay, heparin-ATIII is capable of inhibiting the supplemented bovine Xa as

well as any thrombin which may be formed. Aprosulate which only activates HCII may not inhibit the added Xa and thereby limit the amount of thrombin formed. In addition, the inhibition of thrombin by HCII is kinetically slower than thrombin inhibition by ATIII. The free thrombin can therefore promote feedback activation reactions of other coagulation enzymes such as factor VIII. This is also observed in the weaker potency of aprosulate in the thrombin time assay.

Pentasaccharide which has been shown to inhibit Xa via ATIII but not thrombin was active only in the Heptest®. Single targeting of Xa results in pentasaccharide's lower potency compared to heparin in this assay. Inhibition of Xa would be expected to prolong the APTT as activation of the intrinsic pathway results in Xa generation. The lack of effect of pentasaccharide in the APTT may be related to the inability of the pentasaccharide-ATIII complex to inhibit prothrombinase bound Xa as other ATIII independent Xa inhibitors such as antistasin and DX-9065a concentration dependently prolong the APTT (Vlasuk et al., 1991; Hara et al., 1994).

Based on the concentration response of GL-522-Y-1 in the amidolytic HCII assay, it would be expected that GL-522-Y-1 would also demonstrate appreciable anticoagulant activity similar to aprosulate. GL-522-Y-1, however, demonstrated almost no *in vitro* anticoagulant activity. The APTT was the only assay which showed a weak prolongation of the clotting time. Thus, it appears that the direct inhibition of thrombin by GL-522-Y-1 observed in the HCII assay may not be relevant to the anticoagulant effects as measured with the APTT assay. This observation further reinforces the concept that thrombin generation inhibition is more important in the mediation of the

anticoagulant activity of heparins than the direct inhibition of proteases. The plasma based systems using the thrombin generation process to determine the inhibitory actions of various agents therefore provide a polycomponent assay system where different actions of various agents can be differentiated.

Amidolytic antiprotease assays were used to examine the effect of these heparin analogues at two specific stages of coagulation. These results were largely consistent with those obtained in the amidolytic SERPIN activity assays. The difference between these systems and the SERPIN activation systems is the presence of plasma. In these systems, each test agent was supplemented to plasma. The activities measured can, therefore, be the result of that agent's interaction with multiple cofactors. Both heparin and aprosulate were observed to inhibit the amidolytic activity of thrombin. The potency of aprosulate was lower than that of heparin consistent with the ability of heparin to inhibit thrombin via both ATIII and HCII. Interestingly, GL-522-Y-1 did not demonstrate any antithrombin effects in this assay. If in fact the inhibition of thrombin which was seen in the HCII activity assay is not SERPIN related, this finding in the anti-IIa assay may be partially explained by the lower concentrations of GL-522-Y-1 in this assay. In the HCII assay, the highest concentration of GL-522-Y-1 studied was 26.5 μM. Due to plasma dilution in the anti-Iia assay, however, the highest GL-522-Y-1 assay concentration tested was 3.4 μM. An alternate explanation for the lack of antiprotease activity in the anti-IIa assay by GL-522-Y-1 may be related to the protein binding ability of this agent. While the protein binding profile of GL-522-Y-1 was not determined, other sulfonate containing polymers have been shown to bind to a variety of plasma proteins

(Santerre et al., 1992).

Both heparin and pentasaccharide were observed to inhibit Xa amidolytic activity in this assay. The potency of pentasaccharide was only 2 fold lower than that of heparin in the plasma based assay. In the plasma-free system, heparin was observed to be more than 30 fold stronger than pentasaccharide. The IC_{50} values for both agents were higher in the plasma based assays than in the plasma-free systems. The difference in the relative potency of pentasaccharide is largely due to a marked increase in heparin levels required to inhibit Xa activity in the plasma based systems. This may be related to endogenous protein binding of heparin. Low molecular weight heparins are suggested to have a lesser affinity for heparin binding proteins (Young et al., 1994). It would be expected that pentasaccharide, which is even smaller than the low molecular weight heparins, would exhibit even less protein binding.

The anticoagulant activity of the heparin analogues was also examined following supplementation to normal rabbit pool plasma. In the rabbit plasma, the baseline clotting times were significantly lower in human plasma in the APTT, Heptest®, and the thrombin time assays. Baseline PT's were significantly lower in rabbit plasma than in human plasma. Differences in relative potencies observed with the different agents were minor when compared to the results obtained in the NHP systems. The most notable difference in potency was observed in the Heptest®, where all agents required higher concentrations to prolong the clotting time to 100 seconds. For both aprosulate and pentasaccharide, three fold more agent was required to prolong the clotting time to 100 seconds despite a higher baseline clotting time. GL-522-Y-1 exhibited less anticoagulant activity in rabbit

plasma than in human plasma. In the amidolytic antiprotease assays, heparin was equally potent in rabbit and human plasma with respect to both thrombin and factor Xa inhibition. In these assays, pentasaccharide exhibited a 2.5 fold lower potency in the anti-Xa assay in rabbit plasma compared to its potency in human plasma. This corresponds to the three fold difference in potency observed in the Heptest®. Whereas aprosulate exhibited a potency of 1.05 $\pm$ 0.05 μM in the anti-IIa assay in human plasma, no inhibition of thrombin was observed in the rabbit plasma system. While this may explain the weaker effects of aprosulate in the Heptest®, it does not explain why the potency of aprosulate is higher in rabbit plasma in the thrombin time assay. These differences in clotting time may be attributable to differences in coagulation factor levels in rabbit plasma compared with human plasma. Most clotting factor levels in rabbit plasma are elevated relative to human plasma levels, particularly factors VII, IX, XI and V. In addition to the differences in the coagulation process, vascular lining and cellular factors also contribute to the differences in the hemostatic responses in different species.

As discussed previously, pentasaccharide and aprosulate exhibit differential specificity to ATIII and HCII. These differences, together with the compositional differences between rabbit and human plasma, may account for the observed potency differences noted in this study. In addition, species dependent differences in the function of TFPI have been documented (Warn-Cramer et al., 1992). Such differences may also exist in the functional properties of other SERPINs and contribute to the observed differences in the anticoagulant actions of various heparin analogues.

The anticoagulant effect of a given agent can be markedly different in whole

blood compared to the effect in plasma. For coagulation assays, platelet poor plasma prepared from citrated whole blood is generally used. Addition of citrate removes calcium ions from solution, thereby preventing coagulation as the formation of activated factors VII, IX, X, and thrombin are calcium dependent (Davie et al., 1991). Recalcification of the plasma in global clotting assays results in a dilution of plasmatic coagulation factors. Additionally, platelet poor plasma does not contain the platelets, red cells or white cells normally found in whole blood. Platelets are known to contain both procoagulant material and heparin neutralizing agents in their granules as well to provide a procoagulant surface upon activation (Majerus et al., 1987; Davie et al., 1991). Macrophages and monocytes also may provide procoagulant surfaces (Altieri, 1993; Edwards et al., 1992) and be involved in complex interactions with platelets.

To study the anticoagulant effects of heparin analogues in whole blood, celite activated clotting times and thrombelastography were used on supplemented, freshly drawn human blood. Celite is an activator of the intrinsic pathway. When the analogues were tested on an equimolar basis, the activated clotting time was markedly prolonged by aprosulate, appearing to indicate that the potential to inhibit thrombin is more important than inhibition of factor Xa to produce this effect. Pentasaccharide produced a stronger anticoagulant effect that aprosulate in the TEG analysis. This may be due to pentasaccharide's ability to limit the generation of thrombin by inhibiting factor Xa.

Although unfractionated heparin and pentasaccharide exhibit very strong anti-Xa activities which are mediated by ATIII, the relative prolongation of the whole blood ACT was not proportional to the respective anti-Xa potency of those agents. Aprosulate

produced the strongest effect in this assay, whereas GL-522-Y-1 produced a comparable response to pentasaccharide. This observation is suggestive that in whole blood, factor Xa inhibition has a relatively minor role in the mediation of whole blood clot formation. Aprosulate may have additional cellular mediated effects which can partially account for its stronger effect.

Pentasaccharide exhibited stronger anticoagulant effects in comparison to aprosulate and GL-522-Y-1 in the TEG analysis. It appears that ATIII mediated Xa inhibition may be more important in the mediation of the anticoagulant activity of this agent. It is somewhat paradoxical that there is a different potency profile of these analogues in each whole blood assay. While heparin is a strong anticoagulant, pentasaccharide and aprosulate exhibited a differential behavior. This observation points to the fact that assay dependent variations are observed and may depend on several factors such as the trigger mechanisms involved in such assays. These observations clearly suggest that activation mechanisms are the primary determinant of the relative anticoagulant effects of heparin and its analogues. In contrast to heparin, the analogues certainly alter the coagulation process at specific sites.

E. Protease Generation Assays

For a complete understanding of the anticoagulant and antithrombotic mechanisms of action of various drugs, it is necessary to analyze the actions of these agents at various steps in the coagulation process. In addition to direct antiprotease activities, a given agent can also modulate the formation of active proteases. To identify

these activities, protease generation was measured continuously in a fibrinogen deficient plasma system. By using fibrinogen deficient plasma, it was possible to monitor the formation of active factor X or thrombin using amidolytic substrates. The absence of fibrinogen renders the plasma unclottable. In fibrinogen clotting systems fibrin polymerization would result in aberrant changes in optical density. Methods to defibrinate normal human plasma are known (Dupouy, et al. 1988; Prentice et al., 1993). Use of these defibrinations, however, may result in factor level alterations. It is also possible to use ELISA technology to measure levels of F1.2 and factor X activation peptide as indices of protease generation.

In the fibrinogen deficient plasma systems used here, heparin is expected to exhibit a potent inhibitory effect. Not only is the heparin-ATIII complex capable of preventing thrombin generation by inhibiting intrinsic pathway enzymes, but by directly inhibiting thrombin, heparin limits thrombin feedback activation reactions. Heparin was observed to be a potent inhibitor of Xa and thrombin generation following thromboplastin induced activation, with IC_{50} values of 0.6 and 2.1 μM determined, respectively. The four fold higher amount of heparin needed for thrombin generation inhibition is consistent with the additional amplification step present in its generation. In both systems, GL-522-Y-1 also inhibited protease generation, but exhibited a lower potency than heparin. This may be related to the more limited antiprotease actions of this agent. Neither aprosulate nor pentasaccharide promoted substantial inhibition in these assay systems. The lack of effect by aprosulate suggests that the inhibition by GL-522-Y-1 is not related to HCII mediated thrombin inhibition. The lack of effect by pentasaccharide

is unexpected in light of the data from Lormeau et al. which indicates that pentasaccharide is a potent inhibitor of protease generation (Lormeau et al., 1993), particularly following activation of the extrinsic pathway. Plasma dilution in this assay may be an explanation for this discrepancy. The relatively high plasma dilution (1:24 final dilution) used in this assay results in a lack of ATIII through which pentasaccharide can act. In the work of Lormeau, protease generation was measured in undiluted difibrinated plasma where the relative proportions of various plasma proteins were significantly different from the system used in these studies.

Following activation of the intrinsic pathway, GL-522-Y-1 was observed to inhibit both thrombin and factor Xa generation. Inhibition following intrinsic activation was observed to be more potent than following extrinsic activation. Aprosulate inhibited thrombin and factor Xa generation with similar potencies. This is in contrast to the lack of effect observed with this agent following extrinsic activation. Pentasaccharide dose-dependently inhibited Xa generation following intrinsic activation. The pentasaccharide-ATIII complex has not been shown to inhibit proteases other than Xa, indicating that this effect may reflect direct Xa inhibition. Neither aprosulate nor GL-522-Y-1 has been shown to produce direct inhibition of Xa however these agents inhibited activities, yet inhibited Xa generation with a potency approximately 20 fold higher than that of pentasaccharide. These results indicate that these agents may exhibit other non-SERPIN mediated activities as these agents do not activate ATIII and HCII does not inhibit coagulation proteases beside thrombin (Tollefsen, 1989).

To further define the protease generation modulatory actions of these agents,

non-plasmatic assay systems were utilized. In these systems, prothrombin complex concentrates were used to provide the necessary coagulation factors. These concentrates contain factors II, VII, IX, and X (Konyne®) or their activated forms (FEIBA®). These systems allow the modulatory actions of these agents to be assessed in the absence of endogenous cofactors such as ATIII and HCII and other plasma components. Also missing from these systems are cofactors factors V and VIII which enhance "tenase" and prothrombinase activity. Both GL-522-Y-1 and heparin inhibit thrombin generation in the FEIBA® based system, indicating a non-SERPIN mediated inhibition of protease activity. Heparin did not inhibit Xa generation in the same system, however. Smaller levels of inhibition were noted in the Konyne® based systems. Previous studies with the Konyne® based systems have shown an increased effect by heparin with ATIII supplementation (Kaiser et al., 1994). The difference in heparin's activity may also be related to differences in ATIII content of the prothrombin complex concentrates (Kohler et al., 1990). Some of these concentrates are supplemented with heparin to reduce their thrombogenicity. If HCII contamination is present in the Konyne®, this may also explain the inhibitory effects of aprosulate in this system.

To examine the modulation of the intrinsic pathway in the absence of plasma, a factor VIII:C mediated Xa generation system was used. In this system, the ability of an agent to inhibit conversion of X to Xa by factor IXa in the presence of factor VIII is determined. Previous work has indicated that heparin is capable of directly inhibiting IXa activity at concentrations lower than those normally achieved during antithrombotic therapy (Barrow et al., 1994). This study also demonstrated that this inhibition of IXa

activity was ATIII independent as low and high affinity heparins exhibited similar k_i values. It is also known that the activity of factor IXa is inhibited by the heparin-ATIII complex (Jordan et al., 1980). It is not known what effect the addition of ATIII to the system of Barrow et al. would have on Xa generation. Data obtained with the heparin analogues suggests the presence of non-HCII mediated actions of aprosulate and GL-522-Y-1. Both agents were observed to dose-dependently inhibit Xa amidolytic activity in this system in the absence of plasma derived cofactors. Studies by Sugidachi et al. have confirmed the direct inhibitory effects of aprosulate on the IXa/VIIIa complex (Sugidachi et al., 1994).

The direct inhibition of the IXa/FVIIIa complex by heparin and like agents may also explain the inhibitory effect observed in the FEIBA® and the Konyne® based systems. In both systems, factor IX is activated by the tissue factor/VIIa complex. This IXa could potentially be inhibited by heparin or a synthetic heparin analogue. Alternately, a FVIII contaminant of the prothrombin complex may be the site of inhibition of these agents. Levels of IXa and VIII in each prothrombin complex may explain the differing effects of the synthetic analogues in each different system.

It is interesting to note that heparin produces measurable anticoagulant effects, whereas the heparin analogues produce relatively weaker effects in the global tests. However, the relative inhibitory effects of these agents are stronger in the protease generation tests. This indicates that protease generation inhibition actions of heparin analogues may depend on their direct interactions with the component of the coagulation network resulting in an alteration of their function.

F. Cellular Modulation

The endothelium is important for the maintenance of hemostasis. Endothelial cells produce several antithrombotic materials including PGI_2, TFPI and tPA (Davie et al., 1992). The majority of the vascular system's TFPI is thought to be stored bound to endothelial glycosaminoglycans (Werling et al., 1993). Endothelial cells are known to constitutively produce several glycosaminoglycans. A heparan sulfate has been isolated which exhibits anticoagulant activity. Heparin is known to modulate the production of heparan sulfate by endothelial cells (Nader et al., 1989). To study the effects of these heparin analogues on the modulation of glycosaminoglycan synthesis, each agent was supplemented to the culture media of rabbit aortic endothelial cells. Incorporation of ^{35}S into the glycosaminoglycan was measured by scintillation counting and used as an index of glycosaminoglycan modulation. Glycosaminoglycan production was measured in two areas, the culture media and associated with the cells.

Heparin, as had been previously been published, concentration dependently increased the amount of heparan sulfate in the culture media. At heparin concentrations above 1 μM, the heparan sulfate levels were significantly elevated relative to control treated cultures. Both aprosulate and GL-522-Y-1 were also able to concentration dependently increase the amount of heparan sulfate in the media, though statistical significance was achieved at higher concentrations than with heparin. Heparin's modulation of glycosaminoglycan synthesis by endothelial cells was limited to heparan sulfate measured in the media. Heparan sulfate associated with the cells was not effected by heparin supplementation. The synthesis of chondroitin sulfate was also not effected

by heparin. The actions of aprosulate and GL-522-Y-1 were not as specific as those of heparin. Cell associated chondroitin sulfate was increased following incubation with aprosulate at concentrations above 10 μM. GL-522-Y-1 significantly increased cell associated chondroitin and heparan sulfates. Due to a wide variation among cultures and its lower molecular weight and lesser charge density, pentasaccharide did not significantly increase glycosaminoglycan synthesis. The mean level of cell associated chondroitin sulfate was elevated by pentasaccharide ($p > 0.05$).

To compare the potency of each agent for inducing heparan sulfate synthesis, the concentration required to increase the number of C.P.M. 2 fold over control was determined for each agent. Heparin and GL-522-Y-1 exhibited a higher potency than aprosulate or pentasaccharide for promoting heparan sulfate synthesis. Heparin and GL-522-Y-1 supplementation resulted in a doubling of the C.P.M. at concentrations of 3.3 and 4.5 μM, respectively. Aprosulate was relatively ineffective at promoting heparan sulfate synthesis, with a doubling concentration of 41 μM. Supplementation of pentasaccharide did not result in an increase in heparan sulfate production.

TFPI is known to be produced by and bound to endothelial cells (Osterud et al., 1995). For this reason, attempts were made to measure TFPI levels in aliquots of the culture media. Due to limitations in specificity of the anti-TFPI antibodies, TFPI levels could not be measured in these samples. The anti-TFPI antibodies used in the ELISA TFPI assay are directed against human TFPI. Cross reactivity of these antibodies with rabbit TFPI could not be demonstrated using the standard assay conditions. Additional studies were also performed using rabbit aortic smooth muscle cell cultures to determine

the specificity of this effect. No significant increases in heparan or chondroitin sulfate production by smooth muscle cells were observed following supplementation of any of the agents.

Heparin analogues were found to produce variable effects on the synthesis of various glycosaminoglycans by different cell cultures. The observed rank order for heparan sulfate synthesis was found to be heparin > GL-522-Y-1 > aprosulate > pentasaccharide. The physiologic effects of these effects remain to be determined. Studies with phorbol esters suggest that the release of heparan sulfate may be one of the responses of the cell to a mitogenic stimulus (Porcionatto et al., 1994).

The consequences of cellular modulation produced by heparin and its analogues are largely dependent on the route and duration of their use. In the acute settings, these effects may be rather limited. However, in chronic and sub-chronic settings, such effects may be amplified at different levels. Thus, both the efficacy and safety of these agents may be dependent on the duration of therapy.

G. Platelet Studies

Platelets play an important role in the hemostatic process. Platelets adhere to damaged areas of the blood vessel wall forming the first line of defense against blood loss. These platelets become activated in this process through interaction with vessel wall collagen. A large number of other agonists have also been shown to activate platelets (Packham et al., 1994). Heparin's effect on this process has not definitively been elucidated. Studies have shown heparin to both promote and inhibit platelet activation

responses. This may be related to the concentration of heparin used in these studies. To determine the effect of the synthetic analogues on platelet function, several agonists were used to promote aggregation in platelet rich plasma supplemented with each agent. To make platelet rich plasma, citrated whole blood is gently centrifuged to remove red and white blood cells. In this assay, platelet aggregation is monitored by measuring light transmittance through the plasma sample. Prior to agonist addition, platelet rich plasma is relatively opaque due to platelets in suspension. As platelets aggregate, the larger aggregates fall out of solution and light transmittance is increased. While this assay allows for the easy determination of the effect of agents on the final aggregation response, the system is somewhat unphysiologic. Different components of the aggregation response such as platelet activation, receptor expression, or granule release can not be studied. Additionally, platelet interaction with white cells during the activation process does not occur.

In these studies, platelet rich plasma was supplemented with 10 μg/mL of the heparin analogues. Aggregation was induced by a number of known platelet agonists including ADP, epinephrine, collagen, arachidonic acid, and thrombin. Addition of optimal concentrations of these agonists resulted in strong aggregation responses. Heparin and aprosulate supplementation resulted in an attenuation of the proaggregatory response to thrombin. Heparin produced a stronger inhibition than aprosulate despite a lower molar concentration. Neither pentasaccharide nor GL-522-Y-1 were able to inhibit thrombin induced aggregation. The effects of the other agonists were not modulated by any of the agents at the concentration tested.

In these studies, heparin and its analogues were used at a relatively high concentration of 10 μg/mL. At concentrations below 2.5 μg/mL, heparin is known to augment platelet aggregation induced by such agonists as low concentrations of ADP (Brace et al., 1986). Similarly, the effects of other agonists can also be augmented. Thus, the platelet modulation was only observed in terms of the inhibitory responses.

Heparin induced thrombocytopenia is an increasingly common side-effect of heparin therapy. In patients with HIT, heparin administration results in a dramatic decrease in platelet number due to consumption. HIT patients rarely bleed, but rather present with arterial thrombi which can lead to loss of limb or life. The mechanism for this pathology is largely unknown. Current hypotheses suggest that upon administration, heparin combines with endogenous platelet factor 4 to form a neoantigen (Chong et al., 1982). The antibody to heparin-PF4 activates platelets via the FcIIa receptor (Chong et al., 1993). The endothelium is also postulated to play a role in this process. Quantities of this epitope can be measured using a highly sensitive ELISA method. The initial results with this methodology, however, indicate a lack of correlation between clinically observed HIT and the generation of antibodies (Raible et al., 1995).

Several assays are used to help make the clinical diagnosis of HIT. These include ^{14}C-serotonin release assays, platelet aggregation, immunoblots, and ELISA assays (Walenga et al., 1996). To identify whether any of the heparin analogues were capable of generating a HIT response, an aggregation assay was set up in which platelet rich plasma from normal donors is mixed with serum collected from known HIT positive individuals and the heparin analogues. An aggregation response indicates the potential

to cause HIT. The platelet aggregation assays for HIT are known to be effected by a number of variables. One is the source of the platelet rich plasma. Plasmas from all volunteers do not react in the same manner to HIT serum. The reason for this is not known, though it may be related to individual differences in platelet Fc receptor genotype (Warkentin et al., 1995). In a study performed on five individuals using 17 different HIT sera, the least aggregable PRP reacted with 29 % of the test sera while the most aggregable reacted with 82 % of the sera (Chong et al., 1993). In the studies with the heparin analogues, 5 to 10 volunteers were used for each concentration of heparin analogue. To account for individual variations, each analogue was tested on each donors plasma. Using an inappropriate heparin concentration has also been shown to reduce the accuracy of the assay.

In this assay system, heparin produced a concentration dependent increase in the level of platelet aggregation at concentrations from 5.5 to 22 μg/mL. This corresponds to a heparin level of 0.9 to 3.5 U/mL. The only heparin analogue observed to cause a HIT response was aprosulate. As with heparin, aprosulate produced a statistically significant increase in the aggregation level at concentrations above 11 μg/mL. On a molar basis, this concentration of aprosulate is 4.5 fold higher than the concentration of heparin. Neither GL-522-Y-1 nor pentasaccharide promoted a HIT response. Reports in the literature have indicated that the size of the heparin chain is an important factor in determining whether a HIT response will be generated (Greinacher et al., 1995). While pentasaccharide has a similar composition to heparin, it is below the critical size to illicit an antigenic response. The other critical factor for the production of a HIT response by

heparin-like agents is the degree of sulfation. Agents with a higher degree of sulfation tend to produce higher amounts of HIT responses. Aprosulate contains four sulfates per saccharide unit, twice as many as pentasaccharide and GL-522-Y-1 on a similarly sized backbone. Pentasaccharide provides a suitable alternative antithrombotic agent which may be completely free of any heparin induced thrombocytopenic effects. This agent has already been compared in a large number of people using the HIT aggregation assay. This data indicates that it may be used as a substitute for heparin.

The fact that pentasaccharide is devoid of any thrombocytopenic potential makes this analogue especially attractive for use in patients who exhibit this syndrome. However, because of the lower antiplatelet effects, its use may be limited for prophylactic indications.

H. Antithrombotic Effects

To study the antithrombotic activity of the heparin analogues and to determine the relative role of SERPIN modulation on this activity, two animal models of thrombosis were utilized. In the rabbit stasis thrombosis model, a hypercoagulable state is mimicked by administration of an activated prothrombin complex concentrate. This administration serves to increase plasma levels of coagulation factors II, IX and X. Additionally, it provides activated factor VII to initiate clot formation. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. This thrombogenic environment simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a

thrombus.

The rat jugular vein clamping model was also used to characterize the antithrombotic activity of each agent. In this model, endothelial damage and subsequent exposure of the underlying tissue leads to the formation of a prothrombotic locus. Tissue factor present subendothelially is exposed to the flowing blood where it can complex with FVII and initiate coagulation. Additionally, collagen from the vessel wall can activate platelets. Blood flow through the damaged area is partially maintained in this model.

With both models, studies were performed during the light cycles of the animals. Several studies have examined the effect of circadian rhythm on the hemostatic system. Studies in man have indicated that the time of venepuncture does not influence the plasma levels of factor VII or fibrinogen (Miller et al., 1995). Additionally, it has been shown that the level of platelet aggregation and blood coagulation are increased during the morning hours, whereas fibrinolytic activity was observed to be decreased at this time of day (Decousus et al., 1991). In a study performed in rats, levels of factors II, VII and X were observed to be higher during the light cycle whereas factor IX levels were not influenced by the time of day (Soulban et al., 1989). The levels of factor V and ATIII in humans have also shown no circadian variations (Haus et al., 1990). The effect of heparin administered by constant infusion has been shown to peak in the early morning and to have its minimal effect around noon (Krulder et al., 1994). It has been suggested that the extent of circadian change in the hemostatic system is not of sufficient magnitude to cause diagnostic problems (Haus et al., 1990) and that further studies are need to ascertain the clinical significance of such variations (Labrecque et al., 1991). During the

course of the studies performed here, significant differences in antithrombotic activity in animals treated in the morning or afternoon were not observed.

Ever since its introduction by Wessler, the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents (Wessler et al., 1959). The development of low molecular weight heparins was facilitated by the use of this model. The pharmacodynamic effects of antithrombotic agents have also been investigated using this model and by analyzing blood samples post-administration. While several different thrombogenic triggers have been used (Fareed et al., 1985), in this investigation, a commercially available activated prothrombin complex concentrate (FEIBA®) was employed. These complexes provide a uniform activation of the coagulation process in which the inhibitory effects of heparin and its analogues can be readily assessed.

The antithrombotic activity of each agent was determined in the rabbit stasis thrombosis model following a 10 and 20 minute stasis period. The prothrombotic environment was much stronger following 20 minutes stasis than after only 10 minutes as evidenced by the mean clot score of 3.6 after 20 minutes versus 2.9 after 10 minutes. The longer period of stasis led to a larger generation of activated coagulation factors. All agents were tested following intravenous administration and a 5 minute circulation time. By using this route of administration and short circulation time, little clearance or metabolism of each of the heparin analogues is likely to have occurred. In this system, each agent produced a dose-dependent reduction in thrombus formation. Despite differing mechanisms of action, each agent is able to completely suppress thrombogenesis when

administered at the appropriate dose. The most potent agents were those which were capable of mediating protease inhibition via antithrombin III. Both heparin and pentasaccharide were notably more potent than either aprosulate or GL-522-Y-1. While the sole inhibition of factor Xa was observed to be effective for limiting thrombogenesis, concurrent inhibition of thrombin was seen to enhance antithrombotic activity. The apparent ED_{50} following intravenous administration for pentasaccharide was 20.6 nmol/kg, while that for heparin was 1.7 nmol/kg.

The relative antithrombotic actions of aprosulate and GL-522-Y-1 were considerably weaker than that of heparin. On a molar basis, the antithrombotic potencies of aprosulate and GL-522-Y-1 were observed to be 55 and 390 fold lower than the potency of heparin, respectively. The most likely explanation for this finding is the multiple sites at which the heparin-ATIII complex is capable of producing its action. While the heparin-ATIII complex can inhibit various serine proteases to differing degrees, thrombin is the only coagulation protease known to be inhibited by HCII (Tollefsen, 1989). A certain degree of this difference may also be due to the kinetic differences in protease inhibition by the different SERPINs. To elucidate the role of kinetics, an agent with sole ability to inhibit thrombin via antithrombin III would be required. No such agent is currently available. In light of the similar SERPIN profile of GL-522-Y-1 and aprosulate, it was surprising to observe the 7 fold difference in antithrombotic potency of these agents. The lower potency antithrombotic activity of GL-522-Y-1 is understandable when considering the *in vitro* anticoagulant activity of these agents. When a maximal intravenous dose of 5 mg/kg GL-522-Y-1 was administered to

the rabbits, a plasma concentration no more than 100 μg/mL would be expected. At these concentrations, GL-522-Y-1 produced no plasmatic anticoagulant effect after *in vitro* supplementation to rabbit pool plasma. It is evident from these observations that the *in vitro* antiprotease activity of these agents may not be the sole mediator of the antithrombotic effect.

The stronger prothrombotic environment was evident after 20 minutes stasis time. A weaker antithrombotic activity was observed with each agent though the same rank order potency was observed. Only with the highest doses of each agent was a significant antithrombotic action observed. For heparin, a weak progressive antithrombotic activity was observed at doses ranging from 0.6 to 2.4 nmol/kg. At doses higher than 2.4 nmol/kg was a significant antithrombotic activity observed. No effect on clot score was observed with pentasaccharide doses up to 29 nmol/kg. ED_{50} values extrapolated from the dose-response curves for heparin and pentasaccharide were approximately 2 fold higher following 20 minutes stasis time than were calculated following 10 minutes stasis. ED_{50} values for aprosulate and GL-522-Y-1 could not be calculated from this data due to the relative ineffectiveness of these agents. GL-522-Y-1 was particularly ineffective in preventing thrombus formation as clot scores below +3 were not observed.

It is of interest to note that the relative antithrombotic effects of heparin did differ at 10 and 20 minutes stasis time and the ED_{50}s were in close proximity. In the case of pentasaccharide, a lag in the antithrombotic response at the lower doses was noted. Similarly, the antithrombotic actions of aprosulate and GL-52-Y-1 were considerably

lower when the 20 minute endpoint was used. This data clearly suggests that in contrast to heparin, which is a polycomponent drug, the heparin analogues did not exhibit a buffering capacity for the stronger thrombogenic environment. Thus, additional factors which may play a role in a prolonged thrombogenesis may not be inhibited by ATIII or HCII.

When an antithrombotic agent is administered subcutaneously, pharmacokinetic and bioavailability considerations become important in assessing the observed antithrombotic activity. Plasma drug levels are influenced by the extent of drug absorption as well as the time needed for the absorption to occur. Drug absorption and drug metabolism may occur simultaneously following subcutaneous administration. In these studies, each agent was administered subcutaneously two hours prior to the initiation of thrombus formation. As with the intravenous studies, all agents produced dose-dependent but weaker antithrombotic effects. The dose-range where each agent was effective was higher following subcutaneous administration than following intravenous administration. The subcutaneous absorption of each synthetic analogue was better than that of heparin. Ratios of the ED_{50}s following subcutaneous and intravenous administration were calculated to compare the absorption of each agent. This ratio was lowest for pentasaccharide, where less than 6 fold higher doses were required subcutaneously compared to intravenous administration. Ratios of 18.6, 23.6 and 25.3 were determined for GL-522-Y-1, aprosulate, and heparin, respectively. This was most dramatically seen with the pentasaccharide whose potency was less than three fold lower than heparin following subcutaneous administration, but 12 fold lower following

intravenous administration.

When the ratios of the subcutaneous and the intravenous antithrombotic effects at a single time point (5 minutes for IV and 120 minutes for SC) were calculated, heparin, aprosulate, and GL-522-Y-1 exhibited a similar behavior. Pentasaccharide exhibited a considerably smaller ratio. While these studies do not predict bioavailability, they suggest that pentasaccharide's behavior in producing an antithrombotic response was distinct from the other analogues. This data also suggests that at the time points of the observation, pentasaccharide was absorbed to a greater degree than the other agents studied.

As with the intravenous studies, a twenty minute stasis period produced a stronger thrombogenic challenge. Aprosulate, heparin and pentasaccharide produced dose-dependent antithrombotic effects following subcutaneous administration and 20 minutes stasis time. An ED_{50} value for GL-522-Y-1 was not determined as it failed to prevent thrombus formation after 20 minutes stasis. Pentasaccharide and heparin were relatively stronger in inhibiting clot formation, with ED_{50} values of 0.067 and 0.289 μmol/kg, respectively. Aprosulate was at least 10 fold less potent with an ED_{50} value of 2.6 μmol/kg. For aprosulate and heparin, the ED_{50} value calculated from the dose-response curves following 20 minutes stasis were nearly equal to those determined following 10 minutes stasis. The ED_{50} value for pentasaccharide was more than two fold higher following 20 minutes stasis compared to 10 minutes stasis.

Heparin is conventionally standardized in terms of United States Pharmacopoeial (USP) units per milligram (van Dedem et al., 1981). No standardization methods for any

of the analogues studied here have been made available. All of these agents are known to produce specific effects but their potency relative to heparin can not be measured using the USP assay. Most of these agents exhibit less than 5 USP units/mg potency. Thus, the results of the inhibitory actions can not be compared in biologically standardized manner.

On a gravimetric basis, pentasaccharide and GL-522-Y-1 exhibit comparable mass whereas that of aprosulate was nearly two fold higher. On the other hand, heparin has a 4 to 5 fold higher molecular mass. While a direct comparison of the analogues on a molar basis is valid, in the case of heparin, due to the presence of different molecular species ranging in molecular mass for 1,000 to 50,000 Da, and varying in their proportions, this comparison may not be entirely valid. Since the interaction of heparin with ATIII and HCII is known to occur stoichiometrically, the comparison of these agents on a molar basis may be relevant. The antithrombotic activities of these agents are also depicted in gravimetric amounts in the individual tables. It should be noted that potency ratios markedly change when the results are calculated in terms of gravimetric doses. For pharmacologic comparisons, the molar doses of each agent appear ideal. For clinical applications, a gravimetric dosage is more conventionally accepted.

SERPIN affinity did not predict the duration of antithrombotic activity. In a time-dependent study, doses of each agent which exhibited equal antithrombotic activity were administered intravenously to rabbits and stasis was induced at various time points following administration. In interpreting the data from this study, it is important to note that the doses used for each agent were different. More than 10 fold more pentasaccharide on a molar basis was used to achieve the equivalent antithrombotic effect

of heparin. While pentasaccharide has been shown to exhibit a prolonged elimination half-life in humans (Boneu et al., 1995), a molar equivalent dosage of heparin administered to rabbits would also be expected to exhibit a prolonged antithrombotic effect as the elimination of heparin is known to be dose dependent (Gilman et al., 1985).

Administration of such a dose of heparin, however, would most likely be limited by its hemorrhagic tendencies. At doses which were antithrombotically equivalent, pentasaccharide was observed to have the longest duration of antithrombotic activity of any of the agents tested, producing a significant antithrombotic effect 6 hours post-administration. It has been shown previously that ATIII bound pentasaccharide has a similar half-life to native antithrombin III (van Amsterdam, 1994) while unbound pentasaccharide is rapidly cleared from the circulation. ATIII affinity alone, however, does not ensure a long plasma half-life. The antithrombotic activity of heparin was rapidly diminished. By 60 minutes, clot scores were nearly at baseline levels. Aprosulate and GL-522-Y-1 exhibited effective antithrombotic activity for time periods between the duration of action of heparin and pentasaccharide.

To compare the duration of the antithrombotic effect of each agent following intravenous administration, the time to reach a half maximal clot score (+ 1.45) was determined. Heparin exhibited the shortest duration of action. Following a 30 minute circulation time, clot scores of 1.5 were achieved. The duration of activity for aprosulate and GL-522-Y-1 were 2 to 3 fold longer than heparin. Pentasaccharide exhibited the longest duration of activity. A circulation time of nearly 3 hours was required before clot scores returned to half maximal levels. Following a 20 minute stasis period, the loss of

antithrombotic activity occurred more rapidly. For pentasaccharide, a half maximal clot score was attained after a 40 minute circulation time. The 22 minute time calculated for heparin was nearly the same as following 10 minutes stasis. Neither aprosulate nor GL-522-Y-1 produced clot scores which were below +1.8 after any circulation times.

The time course of antithrombotic activity was also determined following subcutaneous administration. At the doses chosen, GL-522-Y-1, heparin, and pentasaccharide produced identical antithrombotic activity after a 2 hour circulation time. Aprosulate produced a stronger antithrombotic action at 2 hours. This difference was not statistically significant. Heparin exhibited the shortest duration of antithrombotic efficacy, with clot scores returning to half maximal values following a circulation time of 160 minutes. GL-522-Y-1 and pentasaccharide required somewhat longer times to return to the half maximal clot score. Aprosulate exhibited the longest duration of activity. This is likely related to the increased activity observed after 2 hours circulation time. Following 20 minutes stasis time, only aprosulate decreased clot scores below the half maximal value of +1.8 after a 2 hour circulation time. With aprosulate, the time to return to a half maximal clot score was more than 4 hours.

Anticoagulant activity was measured in blood samples obtained during the course of the stasis thrombosis experiments in order to determine the relative importance of these effects on the antithrombotic activity. Anticoagulant activity was determined in whole blood using the ACT and TEG and in platelet poor plasma samples using global clotting assays. Following intravenous administration, the ACT was dose dependently increased by aprosulate and GL-522-Y-1. This increase in anticoagulant activity was

correlated with the antithrombotic activity observed at these doses (r = 0.96 and 0.99 for aprosulate and GL-522-Y-1, respectively). Neither heparin nor pentasaccharide produced significant increases in the clotting time following intravenous administration. The ACT values for animals treated with these agents did not correlate well with the antithrombotic activity observed. Clinically, the ACT is used to monitor high dose heparin therapy. In these situations, a clotting time of 300 seconds is considered to reflect a complete anticoagulant state. This clotting time corresponds to an approximate heparin concentration of 2 to 3 U/mL. The doses used in this study are, however, to low to cause an increase in the clotting time. The highest dose of heparin used was 50 μg/kg. By estimating the blood volume of a rabbit to be approximately 50 mLs/kg (Schalm et al., 1975), the maximal heparin concentration to be expected based on this assumption was 1 μg/kg ($\approx$ 0.1 U/mL).

Following subcutaneous administration, the antithrombotic activity of GL-522-Y-1 also demonstrated a strong correlation with the ACT determined *ex vivo* (r = 0.99). As in the intravenous studies, heparin treatment did not result in a strong correlation. The increase in ACT produced by pentasaccharide correlated with the antithrombotic activity (r = 0.90). The doses of pentasaccharide administered subcutaneously were five times higher than those in the intravenous study. Because of pentasaccharide's high bioavailability and slow clearance, plasma pentasaccharide levels were likely higher following subcutaneous administration than after intravenous administration. ACT measurements in native whole blood provide a more physiologic method to assess the anticoagulant actions of various agents.

The anticoagulant activity in whole blood was also determined using the TEG. The time to initial clot formation (R-time) was correlated with antithrombotic activity. For aprosulate and GL-522-Y-1, this correlation was high following intravenous administration ($r = 0.91$ and 0.99 for aprosulate and GL-522-Y-1, respectively). As with the ACT, heparin displayed a poorer correlation. Pentasaccharide did not produce a dose-dependent increase in R-time following intravenous administration. Following subcutaneous administration, GL-522-Y-1 demonstrated a weak dose-dependent increase in R-time which correlated well with antithrombotic activity ($r = 0.98$). Neither heparin nor pentasaccharide administration produced as good a correlation.

Ex vivo anticoagulant responses were also determined on plasma samples using the global clotting assays. None of the agents was able to dose-dependently prolong the prothrombin time following either intravenous or subcutaneous administration. It was observed *in vitro* that of these agents, only heparin demonstrated a weak prolongation of the clotting time in this assay. The doses used in the rabbits were, however, too low to cause an anticoagulant effect measurable by the PT. The prothrombin time in normal rabbit plasma was significantly increased by heparin only a concentrations above 6 μg/mL.

Using the APTT assay, only aprosulate produced a significant dose-dependent increase in clotting time. The anticoagulant activity of aprosulate correlated with the antithrombotic activity ($r = 0.85$). The APTT was not a sensitive measure of the activity of GL-522-Y-1 or pentasaccharide. While the APTT is used clinically to monitor heparin therapy, higher levels of heparin are needed to significantly prolong the clotting time.

Heparin exhibited a dose-dependent increase in APTT following subcutaneous administration ($r = 0.98$) which correlated to the antithrombotic activity.

In the Heptest®, coagulation is initiated by the addition of activated factor X to the test plasma. Agents capable of inhibiting this factor Xa would be expected to prolong the clotting time. Following both intravenous and subcutaneous administration of heparin and pentasaccharide, the Heptest® clotting time was dose-dependently prolonged. This dose-dependent anticoagulant state was correlated with antithrombotic activity for both agents ($r > 0.90$).

The inhibition of thrombin as measured by the 2.5 U thrombin time was correlated with antithrombotic activity of all agents with the exception of pentasaccharide. Following intravenous administration, aprosulate, GL-522-Y-1, and heparin all dose-dependently increased thrombin inhibition. The inhibition of thrombin correlated well with the antithrombotic activity ($r > 0.90$). Following subcutaneous administration, only heparin treatment led to an increase in thrombin inhibition which correlated with antithrombotic activity.

The release of TFPI was measured in rabbit plasma samples obtained during the course of the intravenous dose-response studies with the stasis thrombosis model. For this analysis, samples from rabbits treated with the highest dose of each agent were analyzed. At these doses, equivalent antithrombotic activity was observed. To measure the TFPI levels, a functional assay was modified which was based on the assay of Sandset (Sandset et al, 1987). In this assay, incubation times were lengthened to compensate for the decreased anticoagulant potency of rabbit TFPI (Warn-Cramer et al., 1992). Aprosulate,

GL-522-Y-1, and pentasaccharide produced mild, statistically insignificant increases in the TFPI levels post-administration despite being administered at higher gravimetric dosages than heparin. This is in contrast to the stronger release observed with these agents in other species (Jeske et al., 1995). Heparin was the only agent which significantly increased TFPI levels relative to control treatment.

The *ex vivo* pharmacologic studies performed on the blood samples collected from animals treated with heparin or its analogues, were suggestive of the relevance of plasmatic effects of each agent to their antithrombotic activity. The degree of thrombin or Xa inhibition was not proportional to the expected antithrombotic activity using heparin as a reference drug. This again suggests that heparin is a polyfunctional drug targeting multiple sites while the analogues produce their actions at certain sites related to their affinity and specificity for various cofactors. For each analogue, a distinct *ex vivo* assay effect-antithrombotic response is obtained. Thus a single assay to predict the antithrombotic actions of heparin and its analogues may not be practical.

The *ex vivo* analysis also indicates that the vascular interactions of heparin and its analogues are not measurable by *ex vivo* assays. The TFPI release is also agent specific. Additional effects on platelets and white cells may also be contributing to the antithrombotic actions of these agents.

The formation of a thrombus may not be solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a nonthrombogenic surface over which the blood flows. The nonthrombogenic properties of the endothelium are in part due to release of such agents as prostacyclin which

prevents platelet aggregation, the presence of TFPI and heparin-like glycosaminoglycans, and the synthesis of fibrinolytic activators (Roberts et al., 1992). Disruption of the endothelium not only limits the beneficial effects enumerated above, but also exposes subendothelial tissue factor and collagen which serve to activate the coagulation and platelet processes, respectively. This process was modelled using a rat model of jugular vein clamping. In this model, repeated clamping of the jugular vein with a hemostat causes endothelial disruption. This vessel damage has previously been shown histologically (Raake et al., 1989). These studies clearly demonstrated endothelial damage which eventually results in the exposure of collagen and tissue factor. The number of clampings required to cause vascular occlusion are an index of antithrombotic activity. For this study, an experimental procedure was stopped when the clamping number reached 15. Above 15 clampings, excessive mechanical damage of the vessel led to bleeding from the clamping site, preventing an accurate determination of the time for thrombus formation. Potency was assessed in this model by determining the dose required to double the number of clampings needed in saline treated rats to cause vascular occlusion.

Intravenous and subcutaneous treatment protocols were used to study the antithrombotic properties of the heparin analogues in response to this thrombogenic trigger. Following intravenous administration, each agent exhibited a dose-dependent increase in the number of vascular clampings required to induce thrombus formation. The same rank order potency was observed as in the stasis thrombosis model with heparin being the most potent of the agents studied. The effective doses in the jugular vein

clamping model were higher than the doses required prevent clot formation in the stasis thrombosis model. 25 nmol/kg heparin was required to double control clamping numbers. Pentasaccharide, aprosulate, and GL-522-Y-1 were 3, 12, and 150 fold less potent than heparin, respectively. For each agent this dose exceeded the dose in the stasis thrombosis model which completely inhibited clot formation following intravenous administration.

In the subcutaneous administration protocol, the pharmacologic activity of heparin was hindered by its low bioavailability. Pentasaccharide was the most potent agent with this treatment protocol. The dose of pentasaccharide which doubled the baseline clamping number following subcutaneous administration was only 50 % higher than the dose determined following intravenous administration. GL-522-Y-1 was also well absorbed following subcutaneous administration. The dose to double baseline was less than two fold higher than the dose producing the same effect following intravenous administration. Heparin was nearly as potent as pentasaccharide following subcutaneous administration. The dose to double the number of baseline clampings, however, was nearly 6 fold higher than the dose determined following intravenous administration.

The antithrombotic profile of heparin and its analogues is also highly suggestive that non-serpin mediated actions contribute to the overall mechanism of heparin and its analogues. TFPI and other release mediators are known to significantly modulate these effects. Additional factors such as the modulation of selectin may also contribute to the antithrombotic actions of these agents.

I. Hemorrhagic Effects

Hemorrhagic complications are associated with anticoagulant therapy and to a greater degree when an anticoagulant in overdosed. Most commonly, this bleeding tendency is observed as oozing from a surgical incision or from a site of catheter insertion. To mimic these clinical situations a rabbit ear bleeding model was utilized which has previously been used to measure the hemorrhagic tendency of a wide variety of anticoagulant agents (Cade et al., 1984). In this model, five incisions were made in the rabbit ear avoiding major vessels. Red blood cells were collected in a saline bath for a standard amount of time and quantitated by hemocytometer.

The dose-response relationship was determined following intravenous administration of each agent. A bleeding index was determined to compare the potency of these agents. The bleeding index was calculated as the dose which elicited the loss of threefold more blood cells than observed in saline treated rabbits. Heparin and GL-522-Y-1 were observed to dose-dependently increase blood loss. For heparin, a bleeding index of 45 nmol/kg was determined. This dose was nearly 10 fold higher than the dose of heparin which completely inhibited clot formation in the stasis thrombosis model. For GL-522-Y-1, a bleeding index of 1.8 μmol/kg was determined. In the rabbit stasis thrombosis model, this dose did not to completely inhibit clot formation following intravenous administration. By comparing the slopes of the blood cell *vs.* dose curves for GL-522-Y-1 and heparin, it is noted that heparin produces a more potent hemorrhagic effect. A slope of 2.5 x 10^9 RBCs/L/(μmol/kg) was determined. For GL-522-Y-1, a slope of 0.13 x 10^9 RBCs/L/(μmol/kg) was determined. Aprosulate doubled the amount

of blood loss relative to saline treatment. This increase was not dose-dependent over the dose range studied. Pentasaccharide did not significantly increase the amount of blood loss in this model at doses 50 fold higher than the dose which was completely effective in the stasis thrombosis model.

In the subcutaneous administration protocol, both heparin and GL-522-Y-1 produced a hemorrhagic effect. As following intravenous administration, the bleeding index for heparin was higher than the antithrombotically effective dose ($\approx$ 7 fold). For GL-522-Y-1, the dose determined for the bleeding index was antithrombotically ineffective in the stasis thrombosis model. Aprosulate administration promoted a statistically significant increase in bleeding at doses above 4 μmol/kg. Blood loss did not reach levels of 3 fold baseline at doses below 8.4 μmol/kg. Pentasaccharide did not produce a significant blood loss a dose 40 fold higher than that which was completely effective in the stasis thrombosis model.

The time dependence on blood loss was examined following intravenous and subcutaneous administration. At five minutes post-intravenous administration, heparin, GL-522-Y-1, and aprosulate produced statistically significant increases in blood loss. By 60 minutes post-administration, neither heparin nor aprosulate produced a significant blood loss. GL-522-Y-1 was observed to have a longer duration of hemorrhagic effects than the other agents. GL-522-Y-1 significantly increased bleeding at time points out to 120 minutes post-intravenous administration and following subcutaneous administration, GL-522-Y-1 induced blood loss which was progressively decreased such that by 6 hours baseline values were observed.

It has been shown that the topical administration od heparin results in enhanced bleeding from small blood vessels (Cruz et al., 1967). As the hemorrhagic tendencies persisted following irrigation of the wound, it was suggested that heparin may be binding to a specific site in the wound. Heparin was shown to inhibit the hydrolysis of ATP by myosin ATPase (Cruz et al., 1967) and subsequently it was demonstrated that heparin binds to myosin ATPase with high affinity (Tersariol et al., 1992). It was also shown in human patients that topical administration of ATP reduces blood loss produced by heparin following cardiopulmonary bypass operations (Garcia et al., 1994). ATP is believed to displace heparin from the surgical lesions which may be related to its hemorrhagic actions.

To determine the relative safety of each agent, the bleeding index was divided by the ED_{50} calculated from the dose response curves in the stasis thrombosis model. Following intravenous administration, a ratio of 26.5 was calculated for heparin. The ratio for GL-522-Y-1 was nearly 10 fold lower than calculated for heparin. Exact values could not be determined for aprosulate and pentasaccharide due to their minimal bleeding effects. Based on the data obtained, aprosulate was at least as safe as heparin with a ratio calculated to be greater than 22. Pentasaccharide was much safer than any of the other agents tested with a ratio calculated to be greater than 140 following intravenous administration. Following subcutaneous administration, the ratio calculated for heparin was lower than following intravenous administration. This ratio was determined to be 15. The ratio for GL-522-Y-1 could not be calculated as all doses tested produced hemorrhagic effects greater than the bleeding index level. Due to low levels of bleeding,

exact ratios for aprosulate and pentasaccharide could not be determined.

From these studies, it appears that hemorrhagic actions can be separated from anticoagulant and antithrombotic activities. While heparin was the most potent antithrombotic agent and exhibited anticoagulant activity in all global assays, it did not promote bleeding at antithrombotically effective doses. Pentasaccharide also displayed potent antithrombotic activity in both animal models and a similar anti-Xa activity as heparin, yet did not promote bleeding at doses more than 10 fold higher than those which were antithrombotically effective. Aprosulate also did not promote a dose-dependent bleeding effect despite requiring higher doses than heparin or pentasaccharide to prevent thrombus formation (Sugidachi et al., 1993). This is in contrast to GL-522-Y-1 which promoted a strong hemorrhagic effect at doses which did not completely block thrombus formation.

J. Structure Activity Relationship

From the obvervations on the bleeding actions of heparin and its analogues, it is obvious that non-plasmatic processes also contribute to the bleeding effects. The release of TFPI and other vascular modulation may be important and should be investigated further. Because of the relatively inert effects of pentasaccharide in the bleeding models, it can be stated that agents with direct anti-Xa effects may not produce bleeding effects.

Heparin's chemical structure is complex in nature, containing iduronic acid residues which can adopt a number of energetically favorable solution conformations as

well as varyingly placed sulfate groups which result in a high degree of polyanionic character (Torri et al., 1985). On the other hand, sulfated glucuronic acid and glucosamine residues behave differently. These physicochemical characteristics of heparin allow it to interact with a variety of endogenous proteins and cellular sites.

The interaction with ATIII is dependent upon a specific sequence. The ATIII binding sequence represents a specific consensus region which is mimicked by the synthetic pentasaccharide in this dissertation. The interaction with other proteins such as HCII and tissue factor pathway inhibitor is dependent on other characteristics such as charge density and molecular size. One report has shown that a hexasaccharide component of heparin exhibits a high specific activity when determined with purified HCII (Linhardt et al., 1986). Additional studies designed to discover a minimal sequence which activates HCII have not confirmed this finding (Maimone et al., 1988). By designing agents which mimic various structural features of heparin, it is possible to mimic some of the specific biologic functions of this agent. Pentasaccharide, aprosulate, and GL-522-Y-1 are different analogues which were designed using such rationales.

Additional strategies include hypersulfation of naturally occurring polymers resulting in such products as pentosan polysulfate and MPS. These agents still exhibit structural heterogeneity and produce their pharmacologic effects at several sites. Thus, these agents would not be suitable to determine the structure activity relationship in heparin. These agents have been investigated for their non-ATIII mediated effects.

The synthetic analogues used in this research offer specific probes to delineate the role of SERPINs as well as the degree of sulfation on the biologic effects of heparin.

The heparin analogues chosen for these studies exhibit more selective interactions with the endogenous inhibitors ATIII and HCII than heparin. In addition, while the anionic character of both pentasaccharide and aprosulate is derived from sulfate groups as in heparin, GL-522-Y-1 possesses sulfonate groups.

The synthetic pentasaccharide represents the minimal heparin sequence which binds ATIII with high affinity. It was shown by fractionation of native heparin that this sequence was required for the activation of ATIII (Rosenberg et al., 1979; Lindahl et al., 1979; Choay et al., 1980). This irregular sequence of heparin contains a single glucosamine unit which is sulfated at the 3-O position and gives heparin its ability to bind ATIII (Atha et al., 1985; Petitou, 1984). Removal of this sulfate group has been shown to abolish the interaction of pentasaccharide with ATIII. Due to its low molecular weight, pentasaccharide-ATIII complexes only inhibit Xa and Xa amplified processes. It was speculated that due to the critical role of Xa in the formation of thrombin, a specific Xa inhibitor would be an effective antithrombotic agent. This agent is currently in phase II clinical development for the prophylaxis of deep venous thrombosis. Additional indications for its use may include the prophylaxis or treatment of arterial thrombosis and the treatment of stroke. Initial studies have also demonstrated that pentasaccharide may be useful in preventing clotting in extracorporeal circuits such as in hemodialysis.

Because of its low molecular weight, pentasaccharide exhibits an almost 100 % bioavailability after subcutaneous administration. Additional molecular manipulations of pentasaccharide have resulted in the development of "super pentasaccharides" with higher anti-Xa potencies (up to 1200 U/mg) and much longer half-lives (Meuleman et al.,

1991). These pentasaccharide derivatives are made by adding additional 3-O sulfate groups to the other glucosamine residues of the molecule. Kinetics studies with these derivatives has indicated that the increased anti-Xa potencies observed are primarily due to an increase in ATIII affinity brought about by the addition of 3-O sulfate groups. It is therefore projected that several pentasaccharide analogues will be developed for indication specific applications in thrombotic and cardiovascular disorders (Carrie et al., 1994).

It remains speculative as to the potential that the super pentasaccharides may release TFPI upon administration. It has been shown in this work and in the work of others (Boneu et al., 1995) that plasma TFPI levels are not affected by pentasaccharide administration. It is also known, however, that the release of TFPI into the plasma is dependent upon the anionic charge density of the glycosaminoglysan. In addition, it has been shown in this dissertation that low molecular weight polyanions of high charge density release TFPI. One could, therefore, hypothesize that the superpentasaccharides may also be releasers of TFPI.

Aprosulate is produced from low molecular weight building blocks with the purpose of creating an agent which is antithrombotic yet exhibits reduced anticoagulant activity. The high sulfate content of this agent not only allows for its interaction with plasma proteins, but also eliminates structural heterogeneity. which can preclude dosing of the agent on a gravimetric basis.

Aprosulate provided a unique tool to differentially investigate the role of HCII in the mediation of the antithrombotic actions of heparin. Owing to its low molecular

weight and high degree of sulfation, this agent mimicked heparin and produced sizable antithrombotic actions in various models which warranted its clinical development. Aprosulate also produced a sizable release of TFPI from vascular sites. This observation confirmed the role of charge density on TFPI release as pentasaccharide did not produce the same effect. Despite a similar molecular weight to pentasaccharide, the function of aprosulate differed markedly from pentasaccharide and provided a tool to differentiate the activity of this agent in terms of HCII activity. The clinical development of this agent and its analogues is in progress and is contingent upon a favorable safety to efficacy ratio.

GL-522-Y-1 is an aromatic polysulfonate whose structure is markedly different from aprosulate and pentasaccharide. Studies with other sulfonate containing polymers, most notably polyvinyl sulfonate, have indicated that such agents provide an antithrombotic surface when they are surface immobilized. This molecule provided a tool where the functional properties of a different type of sulfoxide group can be studied. One of the objectives for studying this agent was to investigate the vascular uptake and endogenous binding of sulfur containing agents in the mediation of antithrombotic actions.

In the GPC studies, it is clear that this agent is capable of binding to solid matrices. It appears that sulfonate containing molecules can also produce antithrombotic effects. This agent also provided evidence that the activation of HCII is not specific to sulfate molecules, but also sulfonate moieties. Polysulfonates have been developed as antiviral agents but have also shown their antithrombotic and hemostatic compromising

actions. These agents, therefore, can also mimic some of heparin's actions. Currently, polysulfonates are developed for various indications including the production of non-thrombogenic surfaces.

Sulfate groups play an important role in mediating the biologic activities of glycosaminoglycans. Desulfated heparins exhibit weaker anticoagulant and antithrombotic activities than their normally sulfated precursors. Sulfate groups on heparin have been shown to interact with positively charged amino acids of ATIII. Loss of one sulfate group in particular has been shown to completely abrogate the anticoagulant and antithrombotic activity of pentasaccharide while oversulfation has been shown to increase the affinity of the molecule for ATIII and to increase the anti-Xa potency of this agent. Increased sulfate content also results in higher affinity to HCII and increases the release of TFPI from vascular stores. Sulfate content is also thought to determine an agent's potential to cause heparin induced thrombocytopenia. Higher sulfate content leads to a larger HIT response (Greinacher et al., 1992). The degree and type of sulfation was observed to determine the effects of glycosaminoglycans on platelet and leukocyte function (Rajtar et al., 1993). For many of these effects, the saccharidic backbone of the agent is not as important as its anionic character. Other negatively charged agents such as defibrotide which derive their polyanionic character from phosphate groups are also known to modulate the vascular system.

The synthetic analogues used in these studies were of similar size, but of distinctly different structure. GL-522-Y-1 contains an aromatic backbone whereas pentasaccharide and aprosulate and saccharidic in nature. Different sugar residues make

up pentasaccharide and aprosulate and the conformational characteristics of the agents are different. The higher molecular weight of aprosulate relative to GL-522-Y-1 and pentasaccharide is largely due to the additional sulfate residues it contains. The distinct chemical structures of these agents translate into differing biologic profiles.

Each of the synthetic analogues is single targeting with respect to the plasma SERPINs ATIII and HCII. In both *in vitro* anticoagulant assays and *in vivo* antithrombotic models, such single targeting agents were less potent than heparin. The importance of ATIII is observed in both models of thrombosis. In each case, the agents which were capable of inhibiting serine proteases via ATIII exhibited more potent antithrombotic activity than those that did not. In this case, the single targeting of factor Xa provided a potent inhibition of thrombogenesis. Additional increases in the chain length of pentasaccharide may provide further evidence on the differential role of thrombin and Xa inhibition in the control of thrombogenesis. Hypersulfation of pentasaccharide may also further support the role of charge density in the release of TFPI and the interaction with HCII. Additional manipulations of the structure of pentasaccharide may provide a useful approach in the development of heparin analogues with specific therapeutic applications.

Sole targeting of HCII produces a distinctly weaker *in vivo* biologic effect than that observed following the activation of ATIII. This can be the result of HCII's ability to only inhibit thrombin as opposed to the multiple sites of inhibition of ATIII. In addition, the widely differing biologic profiles of GL-522-Y-1 and aprosulate suggest the importance of other factors in mediating the actions of these agents. One such action may

be the inhibition of the factor IXa/VIIIa complex. Aprosulate was observed to inhibit this function 20 fold more potently than GL-522-Y-1. A second possible difference in the mechanisms of action of these agents is their interaction with TFPI. Both agents have been shown to increase plasma TFPI levels following administration. Reports in the literature have shown that heparin and other glycosaminoglycans can potentiate the protease inhibitory actions of TFPI, thus making TFPI another heparin cofactor. Aprosulate is made of saccharidic groups and is sulfated like heparin. It can be postulated that based on these structural similarities, that aprosulate may also be capable of potentiating the actions of TFPI. No such effect has yet been shown for aromatic polysulfonates.

Selectins are known to play a role in the inflammatory process (Rosen et al., 1994) and may also be involved in some of the cellular interactions involved in the hemostatic process (Turner, 1992). Heparin interaction with P- and L-selectins has been demonstrated (Skinner et al., 1989). The binding of heparin oligosaccharides to these selectins has been shown to be size dependent with a hexasulfated tetrasaccharide shown to be an effective inhibitor (Nelson et al., 1993). Additionally, it was shown in this study that tetrasaccharides with more sulfate moieties bound selectins better than those with less sulfate groups. It was concluded in this study that small, non-anticoagulant heparin oligosaccharides can effectively block neutrophil accumulation in models of acute inflammation. In another study, it has been shown that substitution of sulfate groups for sialic acid on Lewis x molecules results in enhanced selectin affinity (Yuen et al., 1994). These studies point to the potential use of sulfated heparin analogues for the modulation

of selectin function.

From the SAR standpoint, it appears that the overall pharmacologic actions of heparin and its analogues depends on their molecular and structural makeup. While serpin affinity may be important in the case of heparin and pentasaccharide, other analogues such as aprosulate and GL 522-Y-1 produce several direct effects on both the cellular and plasmatic components. There is no report on the contribution of various functional groups and their relevance to the biochemical and pharmacologic effects of heparin and its analogues. However, it is clear that charge density, molecular weight and specific oligosaccharide consensus sequence interaction with ligands play a crucial role in the mediation of the effects of these agents. From the integrated studies carried out in the dissertation, it can be generalized that the serpin interactions represent one of many different effects of these agents. The overall antithrombotic/anticoagulant actions of these agents not only depend on the structure, but also endogenous interactions and their relative pharmacodynamic interactions determine the pharmacologic actions. Thus, it is possible to develop selective modulators of the hemostatic process by considering the SAR relationship which is exhibited by these agents.

CHAPTER VI

SUMMARY

1. Heparin and pentasaccharide mediated antiprotease activities via activation of ATIII. Due to molecular weight considerations, pentasaccharide was limited to inhibiting factor Xa. Heparin, aprosulate, and GL-522-Y-1 promoted thrombin inhibition via HCII activation whereas pentasaccharide did not promote this process.

2. Heparin exhibited the broadest anticoagulant activity due to its ability to directly and indirectly modulate the coagulation process at multiple sites in the coagulation network as measured by using various global and specific clotting tests.

3. The anticoagulant activity of each heparin analogue was relatively weaker in comparison to heparin. Despite their differing specificity towards ATIII and HCII, both the pentasaccharide and aprosulate directed their anticoagulant activity against the intrinsic pathway of coagulation. GL-522-Y-1 exhibited almost no anticoagulant activity measurable by global clotting assays.

4. Heparin produced the strongest inhibition of protease generation in both ATIII and non-ATIII mediated systems. Pentasaccharide exhibited only limited actions against Xa in the intrinsically activated systems whereas aprosulate exhibited inhibition of both Xa

and thrombin generation in the intrinsically activated systems.

5. GL-522-Y-1 also demonstrated a potent inhibition of protease generation in most assay systems used. While the complete mechanism of action for this agent remains unclear, this effect may be related to inhibition of the factor IXa/VIIIa complex.

6. Heparin, aprosulate, and GL-522-Y-1 promoted an increase in heparan sulfate by cultured endothelial cells. On the other hand, pentasaccharide did not produce any such effect. None of the agents exhibited any effects on glycosaminoglycan synthesis in smooth muscle cell cultures.

7. The high degree of sulfation of aprosulate resulted in a HIT response similar to that produced by heparin. Despite having the same backbone as heparin, pentasaccharide did not produce a HIT response. In the experimental system used, GL-522-Y-1 did not exhibit a HIT response.

8. Protease inhibition mediated by ATIII led to a strong antithrombotic effect by heparin and pentasaccharide. Activation of HCII by aprosulate and GL-522-Y-1 produced a relatively weaker antithrombotic effect. A rank order potency of heparin > pentasaccharide > aprosulate > GL-522-Y-1 was observed following intravenous and subcutaneous administration in the rabbit stasis thrombosis model.

9. In the time dependent studies, the duration of antithrombotic activity was shortest for heparin. Following intravenous administration, pentasaccharide was observed to have the longest duration of action. However, following subcutaneous administration, aprosulate exhibited the longest duration of action.

10. When tissue factor initiated thrombogenesis as seen in the rat model of thrombosis, comparatively higher doses of each agent were required to prevent clot formation as those which were effective in the stasis thrombosis model. In the intravenous studies, the same rank order potent as in the stasis thrombosis model was observed. In the subcutaneous studies, pentasaccharide was observed to be more potent than heparin in this model. Aprosulate and GL-522-Y-1 were both less potent than heparin following subcutaneous administration.

11. The hemorrhagic effects of these agents appears not to be unrelated to their effects on SERPINs. Heparin and GL-522-Y-1 promoted blood loss in a rabbit ear bleeding model. Neither aprosulate nor pentasaccharide were observed to produce an increased blood loss relative to control.

12. TFPI release in both human studies and animal models was observed to be dependent on both degree of anionic character and on molecular size as both aprosulate and GL-522-Y-1 caused an increase in plasmatic TFPI levels following parenteral administration. Pentasaccharide administration did not elevate TFPI antigen levels.

CHAPTER VII

CONCLUSIONS

Unfractionated heparin represents a polypharmacologic agent, targeting several endogenous sites including plasmatic SERPINs, vascular modulation through the release of such active substances as TFPI and tPA, the modulation of leukocytic and platelet selectins and by altering the charge density characteristics of the vasculature. The results obtained with the heparin analogues studied in this investigation support this hypothesis. The role of ATIII clearly appears to be important for the mediation of anticoagulant and antithrombotic effects whereas interactions with HCII play a relatively minor role in the mediation of the pharmacologic actions of heparin. The use of specific analogues of heparin with differential SERPIN interaction profiles provided unique molecular probes to accomplish the intended objective of this dissertation. It is concluded that pentasaccharide behaves as a specific analogue of heparin and it can be molecularly manipulated to exhibit additional properties of heparin through alteration of charge density and saccharidic chain extension. Additional molecular manipulation of pentasaccharide and related analogues in terms of increased degree of sulfation, positioning of the functional groups or branching of the chain may lead to agents with polyfunctional characteristics which may be useful in the control of the thrombotic process at multiple sites.

APPENDIX A.

HPLC PROFILES OF HEPARIN AND ITS ANALOGUES

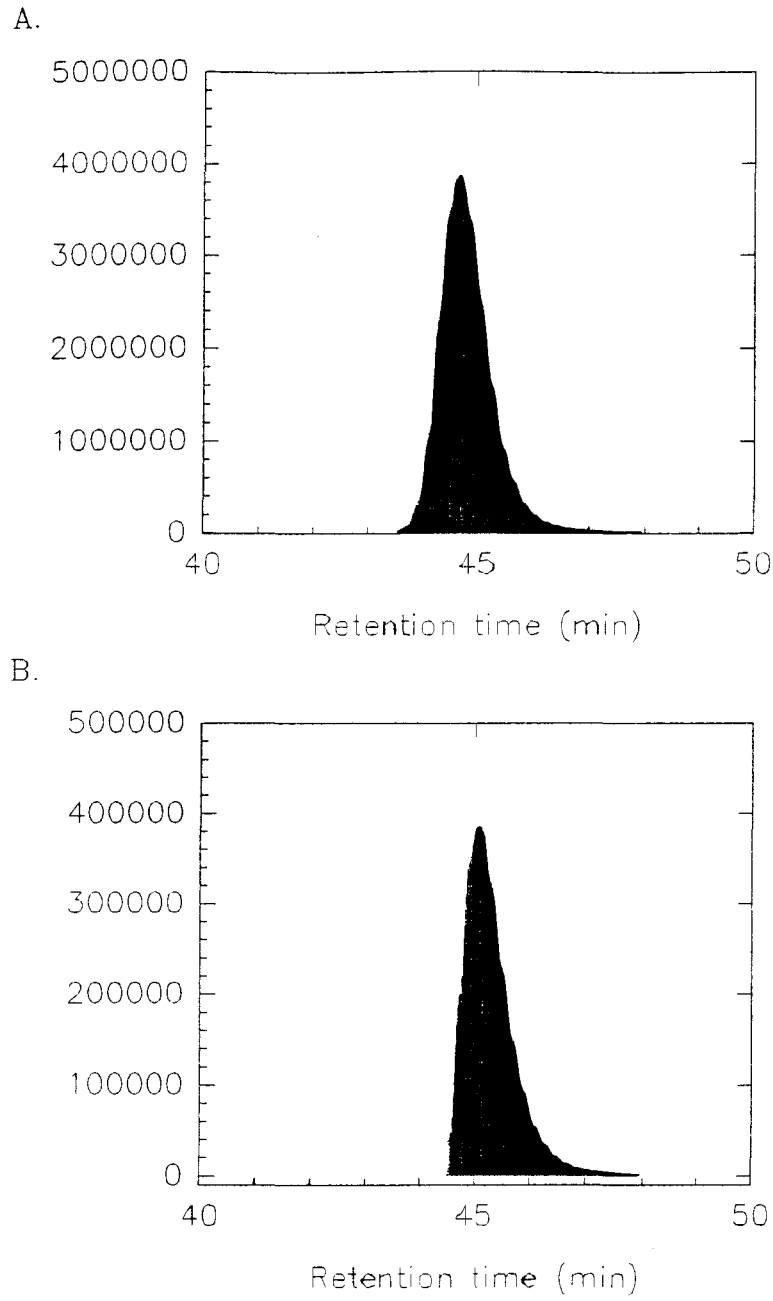


Figure 76. The HPLC elution profile of aprosulate as detected by refractive index (A.) and ultraviolet (B.) detectors.

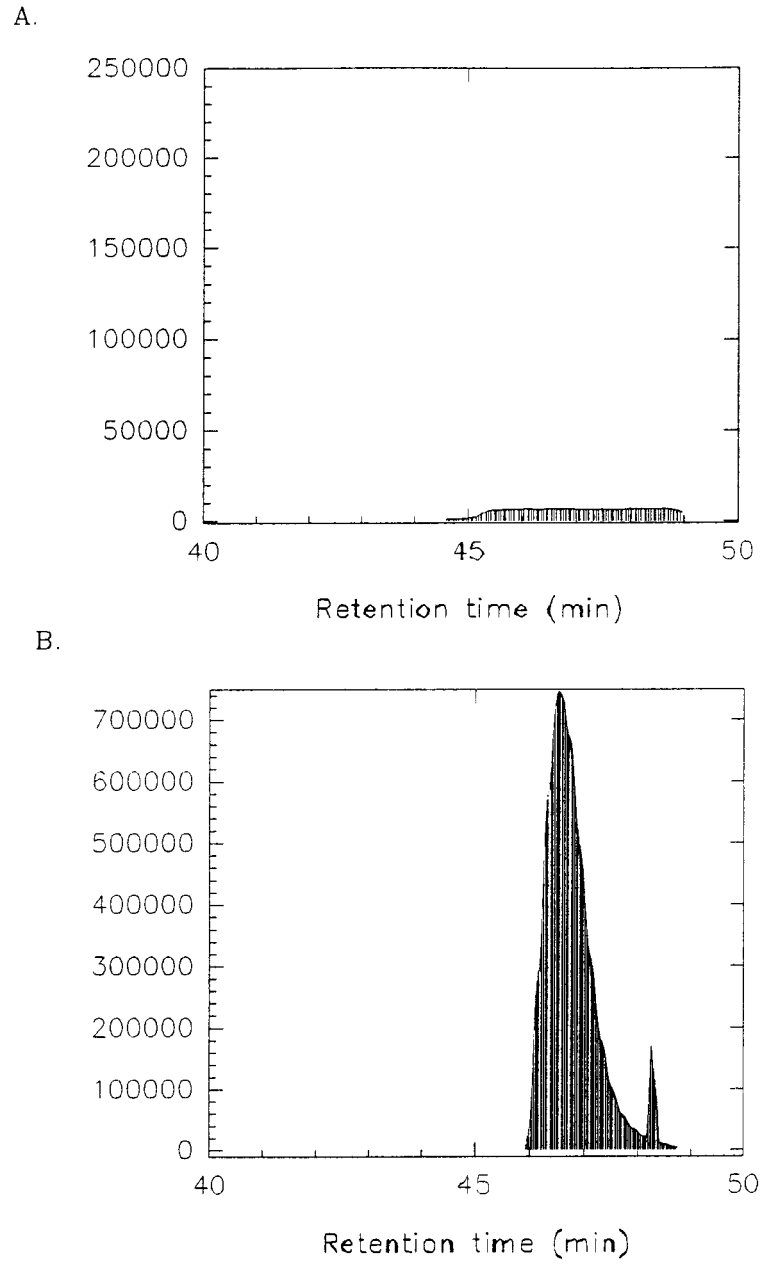


Figure 77. The HPLC elution profile of GL-522-Y-1 as detected by refractive index (A.) and ultraviolet (B.) detectors.

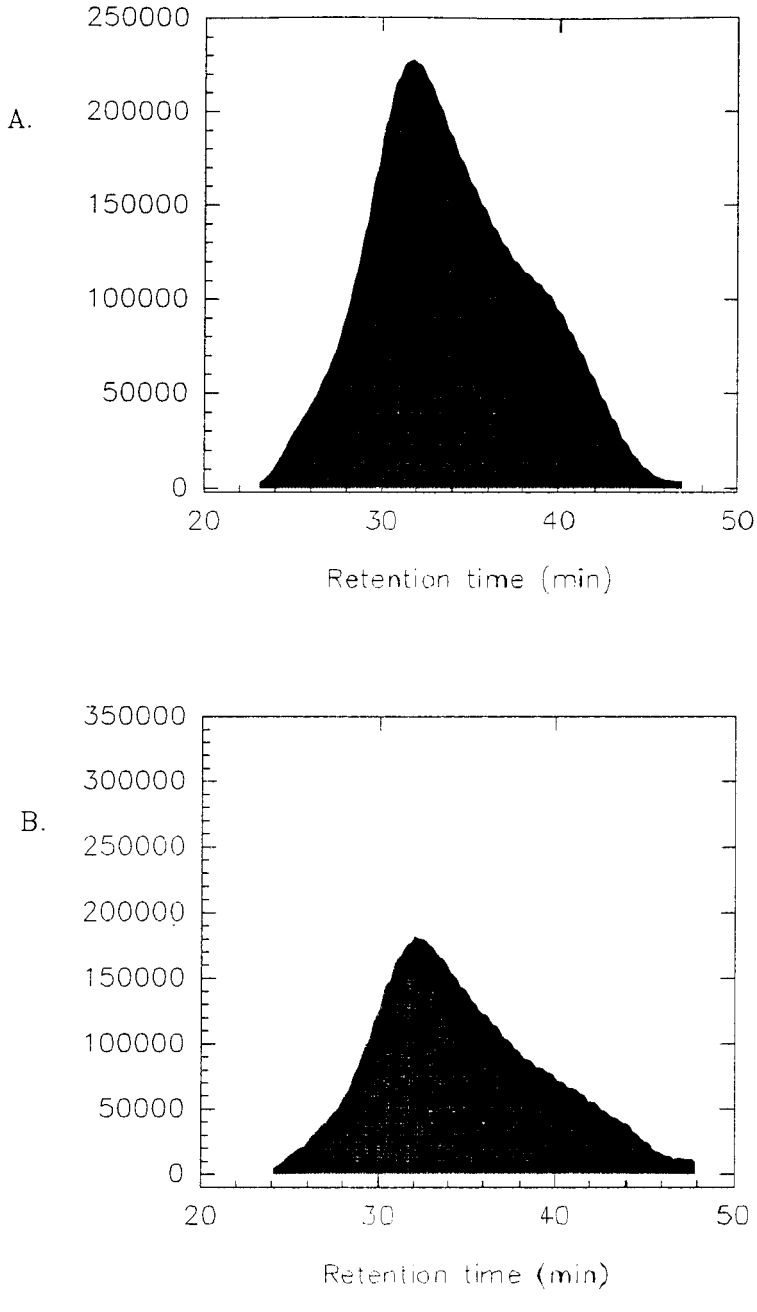


Figure 78. The HPLC elution profile of heparin as detected by refractive index (A.) and ultraviolet (B.) detectors.

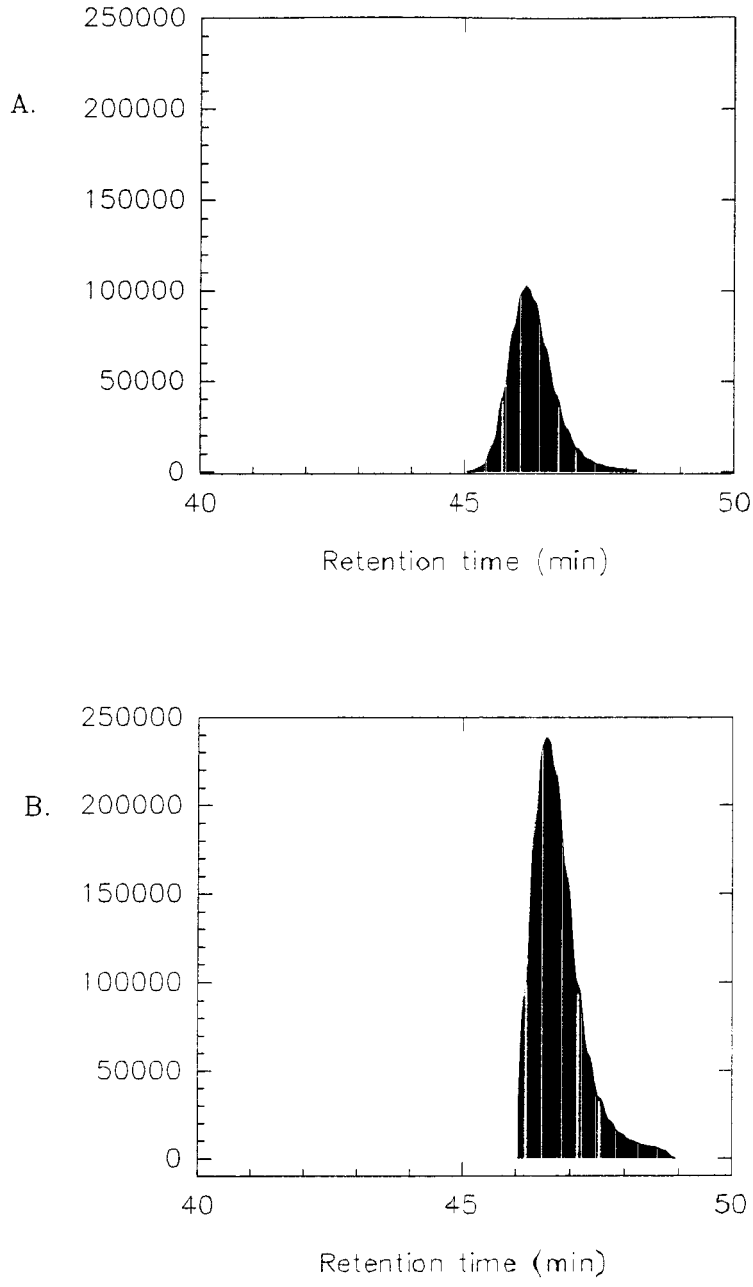


Figure 79. The HPLC elution profile of pentasaccharide as detected by refractive index (A.) and ultraviolet detectors (B.)

APPENDIX B.

PRODUCT SPECIFICATION SHEETS

26, Avenue Théophile Gautier 75752 Paris - Cedex 16
Tél. 524.36.77

INSTITUT CHOAY

BULLETIN D'ANALYSE

P 75.906

PRODUIT : HEPARINE

REFERENCE : Lot H 410

CARACTERES

Aspect	Conforme
Pouvoir rotatoire spécifique	- 33°

ESSAI

Limpidité de la solution à 5 p. 100	Conforme
pH de la solution à 5 p. 100	6,3
Métaux lourds	Conforme
Protéines	Conforme
Perte à la dessiccation	3,4 p. 100
Cendres sulfuriques	38,3 p. 100
Pyrogènes	Apyrogène
Recherche des substances histaminiques	Inf. à 10 mcg/g

DOSAGE

Azote	2,25 p. 100
Soufre	11,2 p. 100
Activité anticoagulante :	
- du produit tel quel	164 UI/mg
- du produit anhydre	170 UI/mg

Le 25 octobre 1984

J. ESTEVENIE
Pharmacien

recherche et
développement

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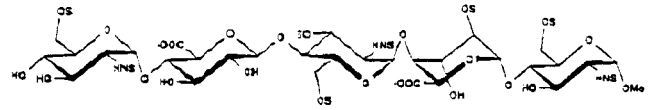
Toulouse, December 29, 1995



S : SO_3^- sodium salt

SR 90107A
batch N° : 2LS002

$C_{31}H_{43}N_3Na_{10}O_{49}S_8$

MW : 1723.09

TESTS :

Purity(HPLC) : 98.9%

$[\alpha]$ +52.8° (c 1 : H_2O)

anti Xa activity : 736UaXa/mg (pH 8.4)

LUITPOLD PHARMA
SANKYO GROUP

LUITPOLD PHARMA GMBH · 81366 MUNCHEN

81366 München
Zeilstartstraße 9
81379 München

Ihre Nachricht	Unser Zeichen	Durchwahl	Datum
		7608	11.03.1991

CERTIFICATE OF ANALYSIS

Product: Aprosulate Sodium Raw Material

Batch No.: 90416/7/72

TESTS	REQUIREMENTS	RESULTS
Description		
appearance	white powder	conforms
Identification		
IR-Spectrum	essentially identical to standard substance	conforms
metachromatic reaction with toluidineblue	conforms	conforms
Purity		
loss of drying [%]	8 - 13	9,38
free sulfate [µg/g]	not more than 200	< 25
pH-value (20 % solution (w/v))	6,5 - 8,0	7,95

2. Blatt zum Brief **C E R T I F I C A T E O F A N A L Y S I S**

Product: Aprosulate Sodium Raw Material

Batch No.: 90416/7/72

TESTS	REQUIREMENTS	RESULTS
Assay		
total sulfate [%]	62 - 66	62,3
total nitrogen [%]	1,0 - 1,3	1,18
sodium [%]	14,0 - 16,0	15,1
Metals		
heavy metals [µg/g]	not more than 20	< 20
Microbiological purity	conforms to DAB 10, Kat.3	conforms

Luitpold Pharma GmbH
i.V. i.V.

R. Klauser C. Meinetsberger

Dr. R. Klauser Dr. E. Meinetsberger

Genelabs Incorporated
505 Penobscot Drive
Redwood City, California 94063
UNITED STATES of AMERICA
(415) 369-9500 FAX (415) 368-0709

Certificate of Analysis

Compound: GL-522-Y-1 (Octamer)

Lot Number: Sabina #4

Molecular weight: 1488.42

Molecular formula: $C_{56}H_{48}O_{32}S_8$

Physical Description: neat, white solid

Chemical Purity: > 98% by HPLC

APPENDIX C.

LETTERS OF PERMISSION

SANOFI RECHERCHE
HAEMOBIOLOGY RESEARCH DEPARTMENT
195, ROUTE D'ESPAGNE
B.P. 1169 - F-31036 TOULOUSE CEDEX (FRANCE)
Tel.: (33) 62 14 23 62 FAX.: (33) 62 14 22 86

sanofi RECHERCHE

➠ *FACSIMILE MESSAGE*

Date	Toulouse, 28/12/95
to	Mr W. Jeske (C/O Dr Fareed)
Fax n°	(708) 216 6660
From	**Jean-Marc HERBERT**
	Haemobiology Research Dpt.
Number of Pages (including this one) :	Ref. JM-MA95

Dear Mr Jeske,

Thank you for your Fax dated 28.12.95.

Concerning your request of a technical specification sheet for SR 90107, I am sorry to tell you that these documents are confidential and cannot be included in your dissertation but I am sure that this will not affect the overall quality of your work.

Concerning the data generated by you at Loyola on SR 90107, of course, I will be pleased you include them as part of your dissertation but, as stated in the contract between Sanofi and Loyola, it is mandatory for you to submit any draft of publication that might arise from these works.

I wish you good luck for your thesis and a happy New Year.

Best regards,

JM Herbert
Haemobiology Res. Dept., Head.

LUITPOLD PHARMA
SANKYO GROUP

LUITPOLD PHARMA GMBH · 81366 MÜNCHEN

81366 München
Zeppelinstraße 9
81379 München

Walter Jeske
Loyola University Chicago
Medical Center
2160 South First Avenue
Maywood, Illinois 60153

January 11th, 1996

Dr. Ra./Sch 7808-412

Dear Mr. Jeske,

We were pleased to learn that some of the studies which are performed on aprosulate at the Loyola University have become part of your dissertation. We would like to inform you that we have no objections to the use of the results on aprosulate in your dissertation.
The requested technical specifications on aprosulate of batch 90416/7/72 are enclosed in this letter

We would like to wish you good luck for the completion of your dissertation.

Yours sincerely,

Dr. M. Hashimoto

Dr. W. Raake

LUITPOLD PHARMA GmbH
SANKYO GROUP
81366 MUNICH, ZELSTATTSTRASSE 9, 81379 MUNICH, GERMANY
PHONE: 089/7808-0, FAX: 089/7808-267, TELEX: 5212177

TELECOPY MESSAGE

AN/TO (COMPANY)	: Loyola University Chicago, Illinois	DATUM/DATE:	02.02.94
ZU HÄNDEN/TO THE ATTENTION OF	: Prof. Jawed Fareed		
FAX No.	: 001 708 216 6660		
VON/FROM	: LUITPOLD PHARMA GMBH		
ABTEILUNG/DEPARTMENT	: Clinical Research 2		
BEARBEITER/ORIGINATOR	: Dr. Eckenberger c/o Luitpold Pharma		
SEITENZAHL/TOTAL NUMBER OF PAGES	1		

Draft Report PALLAS-study

Dear Prof. Fareed,

By parcel service I am sending you the draft report of this study. If you have any comments or proposals for additional evaluations we can include this into the final version.

You may use the results of the coagulation measurements for the paper presented at the GTH-meeting and a final publication about the whole study as discussed earlier. I have already informed Walter Jeske, that he may use these data for his thesis.

As mentioned earlier the investigators involved in this study have been Andreas Kämmereit, Peter Wyld and Sylvia Haas as a scientific consultant and myself as the Luitpold Study Director. These persons should be considered as co-authors in further publications.

As you know, the project aprosulate has been stopped and I am working on another different project. Nevertheless I will try to take the opportunity to meet you and your co-workers at the Munich meeting.

Looking forward to your answer

best regards

Dr. P. Eckenberger

Genelabs Incorporated
505 Penobscot Drive
Redwood City, California 94063
UNITED STATES of AMERICA
(415) 369-9500 FAX (415) 368-0709

FACSIMILE COVER SHEET

TO: Prof. Jawed Fareed/ Walter Jeske

FROM: Bill Choy, Ph.D.
Manager - Asian Operations

COMPANY: Hemostasis Research Lab/Loyola U. Med Center

DATE: February 20, 1996 3:20pm PDST

FAX #: 708-216-6660

RE: Walter Jeske' dissertation

OF PAGES, INCLUDING THIS ONE: 1

I've read Chapter 3 of the thesis. Although the specific enumerations to the figures were left blank in the text, I can guess almost all of the figures which the text alludes to. On this basis, I find the information in this chapter support, directly or indirectly, GL522/Y-1 as an antithrombogenic agent as filed in Genelabs' patent applications. Thus, on behalf of Genelabs, I will allow you to publically release of the information I have read. As you promised last month, please send a full copy of this thesis to me after the defense.

cc: Jen Chen- VP/Asian Operations
Melinda Griffith- General Counsel

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
LOYOLA UNIVERSITY MEDICAL CENTER
Interoffice Communication
RESEARCH ADMINISTRATION OFFICE
Room 3945 -- Building 105 -- Extension 64288

TO: Jawed Fareed, Ph.D.

FROM: Mary Ann Jurgus

DATE: March 11, 1993

SUBJECT: LU# 5737

TITLE: Assessment of the Antithrombotic and Bleeding Effects of Three Synthetic Analogues of Heparin in Rabbits.

INVESTIGATOR(s): Jaweed Fareed, Ph.D. - Pathology
Walter Jeske, M.D. - Pharmacology

FUNDING SOURCE: Dept. Pathology, Hemostasis Research Labs

The above captioned IACUC was reviewed and APPROVED at the March meeting.

P/F# 454-3 has been issued approving the experimental design submitted. Upon funding of this project, please contact me at ex. 64288 with the appropriate account number responsible for the animal charges and an IACUC number will be issued approving the purchase of the animals requested.

Please verify the information below; if corrections are required, please notify me immediately. Animal purchases are processed via Barbara Isdale in the Animal Research Facility; she can be reached at ex. 69178.

Thank you for your cooperation.

sl

cc: Ms. R. O'Connor - ARF Mgr.
Mr. D. Boydston - G & C
Ms. D. Arendziak - Off. Mgr

LU#5737.apr

LU#: 5737
CATEGORY: B
SPECIES: Rabbits
ANIMALS APPR: 448
P/F#: 454-3
ACCT#:
IACUC#:
START/END DATES: 3/1/93-12/31/93
ORIG REVIEW DATE: 2/9/93

APPENDIX D.

DATA TABLES

TABLE 10

MOLECULAR WEIGHT DETERMINATION OF HEPARIN AND ITS SYNTHETIC ANALOGUES USING GEL PERMEATION CHROMATOGRAPHY

Calibrant: Nineteen Narrow Range Fractions
Detector: UV

	Wt. Avg. Mol. Wt. (Da)	HPLC/Formula Weight	Dispersity
Aprosulate	2,357	0.987	1.017
GL-522-Y-1	1,291	0.868	1.150
Heparin	10,523	—	1.228
Pentasaccharide	1,584	0.917	1.018

The molecular weight profile of aprosulate, GL-522-Y-1, heparin, and pentasaccharide was determined by gel permeation chromatography. Wt. Avg. Mol. Wt. is the weight average molecular weight as determined by HPLC. HPLC/Formula Weight is the ratio of the weight average molecular weight and the known formula weight of the agent. This is not determinable for heparin as the formula weight is not known.

TABLE 11

MOLECULAR WEIGHT DETERMINATION OF HEPARIN AND ITS SYNTHETIC ANALOGUES USING GEL PERMEATION CHROMATOGRAPHY

Calibrant: Nineteen Narrow Range Fractions
Detector: RI

	Wt. Avg. Mol. Wt. (Da)	HPLC/Formula Weight	Dispersity
Aprosulate	2,173	0.910	1.019
GL-522-Y-1	999	0.671	1.023
Heparin	10,062	---	1.329
Pentasaccharide	1,407	0.814	1.026

The molecular weight profile of aprosulate, GL-522-Y-1, heparin, and pentasaccharide was determined by gel permeation chromatography. Wt. Avg. Mol. Wt. is the weight average molecular weight as determined by HPLC. HPLC/Formula Weight is the ratio of the weight average molecular weight and the known formula weight of the agent. This is not determinable for heparin as the formula weight is not known.

TABLE 12

MOLECULAR WEIGHT DETERMINATION OF HEPARIN AND ITS SYNTHETIC ANALOGUES USING GEL PERMEATION CHROMATOGRAPHY

Calibrant: HMC
Detector: RI

	Wt. Avg. Mol. Wt. (Da)	HPLC/Formula Weight	Dispersity
Aprosulate	1,773	0.742	1.022
GL-522-Y-1	--	--	--
Heparin	9,877	--	1.413
Pentasaccharide	1,103	0.638	1.031

The molecular weight profile of aprosulate, GL-522-Y-1, heparin, and pentasaccharide was determined by gel permeation chromatography. Wt. Avg. Mol. Wt. is the weight average molecular weight as determined by HPLC. HPLC/Formula Weight is the ratio of the weight average molecular weight and the known formula weight of the agent. This is not determinable for heparin as the formula weight is not known.

TABLE 13

MOLECULAR WEIGHT DETERMINATION OF HEPARIN AND ITS SYNTHETIC ANALOGUES USING GEL PERMEATION CHROMATOGRAPHY

Calibrant: F913B
Detector: RI

	Wt. Avg. Mol. Wt. (Da)	HPLC/Formula Weight	Dispersity
Aprosulate	1,598	0.669	1.024
GL-522-Y-1	--	--	--
Heparin	12,782	--	1.639
Pentasaccharide	1,013	0.586	1.043

The molecular weight profile of aprosulate, GL-522-Y-1, heparin, and pentasaccharide was determined by gel permeation chromatography. Wt. Avg. Mol. Wt. is the weight average molecular weight as determined by HPLC. HPLC/Formula Weight is the ratio of the weight average molecular weight and the known formula weight of the agent. This is not determinable for heparin as the formula weight is not known.

TABLE 14

^{13}C NMR SIGNAL ASSIGNMENTS FOR UNFRACTIONATED HEPARIN

Chemical Shift (ppm)	Assignment
24	$-CH_3$
56	A-C-2-NAc
58.5	A-C-3-OSO_3
60	A-C-2-NSO_3
62	A-C-6-OH
69	A-C-6-OSO_3
99	A-C-1-NSO_3
101	IdA-C-1-$2SO_3$

^{13}C NMR spectrum of unfractionated heparin was detected using a Bruker AC300 NMR spectrometer at 75 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard. A = glucosamine, IdA = iduronic acid, NAc = N-acetlyated, OSO_3 = O-sulfated, NSO_3 = N-sulfated.

TABLE 15

^{1}H NMR SIGNAL ASSIGNMENTS FOR THE 3-O SULFATED AMINOSUGAR OF PENTASACCHARIDE

Chemical Shift (ppm)	Assignment
5.5	H-1
3.4	H-2
4.3	H-3
4.0	H-4
4.1	H-5
4.5	H-6

Proton NMR spectrum of the 3-0 sulfated amino sugar of pentasaccharide was detected using a Bruker AMX500 NMR spectrometer at 500 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard.

TABLE 16

^{13}C-NMR SIGNAL ASSIGNMENTS FOR APROSULATE

Chemical Shift (ppm)	Assignment
68.0	C6
68.5	C6'
73.5	C5
77.0	C4'
77.1	C4
77.2	C2'
77.5	C2
78.0	C3
78.3	C3'
79.5	C5'
103	C1

^{13}C NMR spectrum of aprosulate was detected using a Bruker AC300 NMR spectrometer at 75 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard. C2 is carbon nucleus 2 of the closed sugar ring of aprosulate. C2' is carbon nucleus 2 of the open chain sugar of aprosulate.

TABLE 17

^{1}H-NMR SIGNAL ASSIGNMENTS FOR APROSULATE

Chemical Shift (ppm)	Assignment
4.238	H5
4.395 / 4.416	H6
4.523 / 4.549	H2
4.658 / 4/679 / 4.689	H6'
4.713 / 4.722 / 4/731	H3
4.747 / 4.763	H4'
4.800	HOD (solvent)
5.005 / 5/021 / 5/037	H3"
5.073 / 5.082 / 5.099	H5'
5.146 / 5.163	H2'
5.223 / 5/233	H4

Proton NMR spectrum of aprosulate was detected using a Bruker AC300 NMR spectrometer at 300 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard. H2 is the proton on carbon 2 of the closed sugar ring of aprosulate. H2' is the proton on the second carbon of the open chain sugar of aprosulate.

TABLE 18

^{13}C NMR SIGNAL ASSIGNMENT FOR GL-522-Y-1

Chemical Shift (ppm)	Assignment
33.0	CH_2
128.8	CH
130.8	C-CH_2
138.0	C-OH
155.5	C-HSO_3

^{13}C NMR spectrum of GL-522-Y-1 was detected using a Bruker AC300 NMR spectrometer at 75 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard.

TABLE 19

^{1}H NMR SIGNAL ASSIGNMENTS FOR GL-522-Y-1

Chemical Shift (ppm)	Assignment
4.2	CH_2
4.7	HOD (solvent)
7.7	CH

^{1}H NMR spectrum of GL-522-Y-1 was detected using a Bruker AC300 NMR spectrometer at 300 MHz. All chemical shifts are relative to an internal sodium-3-(trimethylsilyl)-propionate standard.

TABLE 20

SERPIN MEDIATED INHIBITION OF THROMBIN AND FACTOR Xa BY APROSULATE

Concentration		% Inhibition		
μg/mL	μM	ATIII/IIa	ATIII/Xa	HCII/IIa
40	16.75	2.0 ± 2.2	12.8 ± 12.5	79.2 ± 2.2*
20	8.38	2.5 ± 2.5	7.6 ± 9.5	77.4 ± 1.9*
10	4.19	4.5 ± 3.5	4.6 ± 6.3	70.9 ± 2.6*
5	2.09	1.6 ± 2.8	3.2 ± 5.5	58.8 ± 4.6*
2.5	1.05	3.2 ± 2.8	5.0 ± 5.8	39.1 ± 8.0*
1.25	0.52	1.8 ± 1.6	2.6 ± 4.4	23.9 ± 5.1*
0.625	0.26	2.0 ± 2.5	4.5 ± 7.8	15.5 ± 4.5*
0.312	0.13	1.8 ± 2.2	3.3 ± 5.8	11.6 ± 4.3*

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 2388 Da. ATIII, antithrombin III; HCII, heparin cofactor II; IIa, thrombin; Xa factor Xa. All percent inhibitions were calculated based on an unsupplemented saline control. Statistically significant differences between treatment and control were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant. ATIII/IIa : $p = 0.636$, dF = 26, F = 0.767. ATIII/Xa : $p = 0.640$, dF = 26, F = 0.762. HCII/IIa : $p < 0.0001$, dF = 26, F = 152.9.

TABLE 21

SERPIN MEDIATED INHIBITION OF THROMBIN AND FACTOR Xa BY GL-522-Y-1

Concentration		% Inhibition		
μg/mL	μM	ATIII/IIa	ATIII/Xa	HCII/IIa
40	26.88	0 ± 0	25.0 ± 9.3	77.8 ± 2.3*
20	13.44	0.7 ± 1.2	21.7 ± 18.2	76.6 ± 1.6*
10	6.72	2.7 ± 4.6	18.3 ± 16.0	73.9 ± 0.8*
5	3.36	2.8 ± 2.8	15.6 ± 13.8	66.9 ± 0.5*
2.5	1.68	3.2 ± 4.6	10.2 ± 10.6	58.0 ± 3.2*
1.25	0.84	4.1 ± 5.7	10.6 ± 11.3	47.3 ± 2.8*
0.625	0.42	3.9 ± 5.8	10.0 ± 11.4	31.7 ± 3.2*
0.312	0.21	1.3 ± 2.3	5.6 ± 9.6	23.5 ± 5.4*

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 1488 Da. ATIII, antithrombin III; HCII, heparin cofactor II; IIa, thrombin; Xa factor Xa. All percent inhibitions were calculated based on an unsupplemented saline control. Statistically significant differences between treatment and control were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p < 0.05$ was considered statistically significant. ATIII/IIa : $p = 0.963$, dF = 26, F = 0.283. ATIII/Xa : $p = 0.316$, dF = 26, F = 1.27. HCII/ IIa : $p < 0.0001$, dF = 26, F = 302.8.

TABLE 22

SERPIN MEDIATED INHIBITION OF THROMBIN AND FACTOR Xa BY HEPARIN

Concentration		% Inhibition		
μg/mL	μM	ATIII/IIa	ATIII/Xa	HCII/IIa
40	3.81	--	--	80.9 ± 1.3*
20	1.90	--	--	80.8 ± 0.5*
10	0.95	--	--	80.0 ± 0.3*
5	0.48	--	--	78.3 ± 0.4*
2.5	0.24	--	--	76.3 ± 2.5*
1.25	0.12	94.4 ± 1.1*	98.0 ± 0.3*	68.4 ± 0.7*
0.625	0.06	93.9 ± 1.1*	95.0 ± 2.6*	55.0 ± 2.0*
0.312	0.03	93.2 ± 1.1*	93.1 ± 3.4*	40.0 ± 6.8*
0.156	0.015	92.3 ± 1.2*	76.2 ± 11.0*	--
0.078	0.0075	90.4 ± 0.9*	59.5 ± 16.6*	--
0.039	0.0038	84.0 ± 0.9*	35.2 ± 16.2*	--
0.020	0.0019	69.2 ± 0.9*	24.5 ± 18.6*	--
0.010	0.0009	48.5 ± 1.6*	16.3 ± 12.8	--

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a molecular weight of 10490. ATIII, antithrombin III; HCII, heparin cofactor II; IIa, thrombin; Xa factor Xa. All percent inhibitions were calculated based on an unsupplemented saline control. Statistically significant differences between treatment and control were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. * $p < 0.05$ was considered statistically significant. ATIII/IIa : $p < 0.0001$, dF = 26, F = 268.3. ATIII/Xa : $p < 0.0001$, dF = 26, F = 31.9. HCII/IIa : $p < 0.0001$, dF = 16, F = 337.8.

TABLE 23

SERPIN MEDIATED INHIBITION OF THROMBIN AND FACTOR Xa BY PENTASACCHARIDE

Concentration		% Inhibition		
μg/mL	μM	ATIII/IIa	ATIII/Xa	HCII/IIa
40	23.20	---	---	20.7 ± 3.0*
20	11.60	---	---	13.5 ± 7.7
10	5.80	---	---	12.0 ± 7.6
5	2.90	---	---	8.8 ± 7.0
2.5	1.45	---	---	6.6 ± 5.8
1.25	0.73	14.9 ± 2.1*	97.2 ± 0.3*	4.4 ± 5.5
0.625	0.36	11.4 ± 2.3*	97.1 ± 0.8*	6.5 ± 5.6
0.312	0.18	8.1 ± 0.4*	96.3 ± 0.5*	6.1 ± 5.5
0.156	0.09	5.2 ± 3.6	91.1 ± 4.1*	---
0.078	0.045	4.3 ± 1.9	80.5 ± 5.7*	---
0.039	0.023	3.2 ± 2.8	59.6 ± 8.1*	---
0.020	0.011	2.1 ± 2.8	36.0 ± 6.3*	---
0.010	0.0057	6.5 ± 3.1	21.1 ± 5.1*	---

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 1728 Da. ATIII, antithrombin III; HCII, heparin cofactor II; IIa, thrombin; Xa factor Xa. All percent inhibitions were calculated based on an unsupplemented saline control. Statistically significant differences between treatment and control were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test. * $p < 0.05$ was considered statistically significant. ATIII/IIa : $p < 0.0001$, dF = 26, F = 11.4. ATIII/Xa : $p < 0.0001$, dF = 16, F = 204.2. HCII/IIa : $p = 0.0182$, dF = 26, F = 3.24.

TABLE 24

IN VITRO ANTICOAGULANT ACTIVITY OF APROSULATE IN BLOOD BANK PLASMA

Concentration (μg/mL)	Concentration (μM)	PT (sec)	APTT (sec)	Heptest (sec)	5U TT (sec)	Anti-IIa (% Inhib)	Anti-Xa (% Inhib)
100.0	41.88	14.5 ± 0.3*	>300*	>300*	>300*	41.6 ± 4.0*	0.7 ± 0.6
50.0	20.94	13.3 ± 0.3*	>300*	173.1 ± 22.1*	>300*	38.3 ± 1.7*	0.7 ± 0.7
25.0	10.47	13.3 ± 0.2*	198.8 ± 49.5*	133.6 ± 43.5*	154.9 ± 36.7*	37.5 ± 2.3*	0.6 ± 0.5
12.5	5.23	13.0 ± 0.4*	138.2 ± 17.3*	48.8 ± 4.8	41.5 ± 5.8	33.4 ± 3.2*	0.5 ± 0.6
6.25	2.62	12.8 ± 0.3*	79.6 ± 2.9*	27.9 ± 2.3	28.7 ± 2.4	31.8 ± 3.7*	1.0 ± 1.1
3.12	1.31	12.9 ± 0.3*	60.5 ± 2.4	22.6 ± 3.8	24.4 ± 1.8	15.7 ± 3.2*	1.0 ± 0.9
1.56	0.65	12.4 ± 0.1	49.0 ± 3.1	19.9 ± 1.8	23.1 ± 2.1	12.9 ± 2.5*	0.7 ± 0.7
0.78	0.33	12.2 ± 0.2	44.6 ± 4.1	17.5 ± 1.5	23.1 ± 0.2	6.8 ± 2.6*	0.5 ± 0.7
0	0	12.0 ± 0.2	37.3 ± 4.0	16.5 ± 0.9	20.2 ± 0.7	0	0

All results represent the mean ± 1 standard deviation of four determinations. Molar values were calculated based on a formula weight of 2388 Da for aprosulate and represent the plasma aprosulate concentration. Statistical analysis of the data was made using one way ANOVA followed by the Newman-Keuls multiple comparison test for each assay. * indicates statistical significance compared to unsupplemented baseline plasma ($p < 0.05$). PT : dF = 35, F = 30.7, $P < 0.0001$. APTT : dF = 35, F = 147.9, $P < 0.0001$. Heptest : dF = 35, F = 145.0, $P < 0.0001$. 5U TT : dF = 35, F = 372.4, $P < 0.0001$. Anti-IIa : dF = 35, F = 152.4, $P < 0.0001$. Anti-Xa : dF = 35, F = 0.726, $P = 0.667$.

TABLE 25

IN VITRO ANTICOAGULANT ACTIVITY OF GL-522-Y-1 IN BLOOD BANK PLASMA

Concentration (μg/mL)	Concentration (μM)	PT (sec)	APTT (sec)	Heptest (sec)	5U TT (sec)	Anti-IIa (% Inhib)	Anti-Xa (% Inhib)
100.0	67.2	22.8 ± 1.9*	112.0 ± 4.4*	34.0 ± 8.3*	21.5 ± 8.2	7.0 ± 2.3*	5.9 ± 2.4*
50.0	33.6	19.5 ± 1.4*	79.2 ± 9.2*	22.0 ± 3.8	19.3 ± 5.5	5.0 ± 1.7*	0 ± 0
25.0	16.8	15.8 ± 1.4*	59.3 ± 6.7*	19.3 ± 1.1	19.5 ± 4.9	1.0 ± 0.8	0 ± 0
12.5	8.4	14.1 ± 1.0	48.8 ± 3.5*	17.2 ± 1.0	20.1 ± 5.9	0 ± 0	0 ± 0
6.25	4.2	12.2 ± 1.4	42.4 ± 2.7	15.6 ± 1.3	20.9 ± 5.9	0 ± 0	0 ± 0
3.12	2.1	12.7 ± 0.6	38.9 ± 1.3	17.5 ± 1.1	20.3 ± 5.3	0 ± 0	0 ± 0
1.56	1.05	12.3 ± 0.2	35.8 ± 3.1	18.0 ± 1.5	19.3 ± 8.2	0 ± 0	0 ± 0
0.78	0.53	12.2 ± 0.9	34.5 ± 2.2	18.2 ± 1.6	20.8 ± 5.6	0 ± 0	0 ± 0
0	0	12.9 ± 0.7	35.4 ± 1.7	17.0 ± 1.4	20.1 ± 3.9	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of four determinations. Molar values were calculated based on a formula weight of 1488 Da for GL-522-Y-1 and represent the plasma GL-522-Y-1 concentration. Statistical analysis of the data was made using one way ANOVA followed by the Newman-Keuls multiple comparison test for each assay. * indicates statistical significance compared to unsupplemented baseline plasma ($p < 0.05$). PT : dF = 35, F = 128.6, $P < 0.0001$. APTT : dF = 35, F = 393.1, $P < 0.0001$. Heptest : dF = 35, F = 30.7, $P < 0.0001$. 5U TT : dF = 35, F = 0.181, $P < 0.996$. Anti-IIa : dF = 35, F = 28.7, $P < 0.0001$. Anti-Xa : dF = 35, F = 24.2, $P < 0.0001$

TABLE 26
IN VITRO ANTICOAGULANT ACTIVITY OF HEPARIN IN BLOOD BANK PLASMA

Concentration		PT	APTT	Heptest	5U TT	Anti-IIa	Anti-Xa
(μg/mL)	(μM)	(sec)	(sec)	(sec)	(sec)	(% Inhib)	(% Inhib)
100.0	9.53	214.5 ± 99.6*	>300*	>300*	>300*	94.1 ± 0.9*	97.4 ± 0.2*
50.0	4.77	82.2 ± 44.7	>300*	>300*	>300*	93.6 ± 0.5*	97.3 ± 0.3*
25.0	2.38	28.4 ± 6.8	>300*	>300*	>300*	93.3 ± 0.3*	96.5 ± 0.5*
12.5	1.19	18.48 ± 2.2	>300*	>300*	>300*	92.6 ± 0.6*	80.5 ± 0.6*
6.25	0.60	15.1 ± 0.8	272.9 ± 31.3*	155.7 ± 10.8*	>300*	92.4 ± 0.7*	64.8 ± 4.7*
3.12	0.30	13.8 ± 1.1	95.6 ± 21.5*	96.4 ± 5.5*	>300*	87.6 ± 1.5*	43.7 ± 3.7*
1.56	0.15	13.8 ± 0.3	52.6 ± 5.1	67.3 ± 4.8*	246.2 ± 107.6*	69.1 ± 6.1*	27.9 ± 5.3*
0.78	0.07	13.8 ± 2.3	40.4 ± 2.4	37.3 ± 7.5*	90.5 ± 72.3*	24.8 ± 3.7*	11.2 ± 3.4*
0	0	14.6 ± 2.1	31.1 ± 4.0	17.7 ± 1.0	23.7 ± 1.5	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of four determinations. Molar values were calculated based on a molecular weight of 10492 Da for heparin and represent the plasma heparin concentration. Statistical analysis of the data was made using one way ANOVA followed by the Newman-Keuls multiple comparison test for each assay. * indicates statistical significance compared to unsupplemented baseline plasma ($p < 0.05$). PT : dF = 35, F = 155.4, $P < 0.0001$. APTT : dF = 35, F = 395.3, $P < 0.0001$. Heptest : dF = 35, F = 2474.3, $P < 0.0001$. 5U TT : dF = 35, F = 238.0, $P < 0.996$. Anti-IIa : dF = 35, F = 808.7, $P < 0.0001$. Anti-Xa : dF = 35, F = 696.6, $P < 0.0001$

TABLE 27

IN VITRO ANTICOAGULANT ACTIVITY OF PENTASACCHARIDE IN BLOOD BANK PLASMA

Concentration		PT	APTT	Heptest	5U TT	Anti-IIa	Anti-Xa
(μg/mL)	(μM)	(sec)	(sec)	(sec)	(sec)	(% Inhib)	(% Inhib)
100.0	58.0	13.7 ± 0.2	79.3 ± 9.3*	299.6 ± 0.9*	36.4 ± 11.2*	0.7 ± 0.6	97.8 ± 0.1*
50.0	29.0	12.9 ± 0.6	60.8 ± 5.6*	256.8 ± 50.2*	28.8 ± 5.8	0.5 ± 0.5	97.8 ± 0.1*
25.0	14.5	12.9 ± 0.7	46.2 ± 9.7	197.8 ± 66.9*	27.2 ± 2.3	1.4 ± 0.3	97.2 ± 0.2*
12.5	7.25	12.9 ± 0.8	43.8 ± 6.6	163.9 ± 37.7*	25.9 ± 1.5	0.7 ± 1.0	96.8 ± 0.7*
6.25	3.63	12.4 ± 0.9	40.9 ± 5.0	162.4 ± 49.3*	25.5 ± 0.5	1.1 ± 1.3	94.3 ± 2.1*
3.12	1.81	12.6 ± 1.1	38.5 ± 5.7	135.1 ± 37.9*	24.2 ± 0.5	1.0 ± 0.7	85.5 ± 1.5*
1.56	0.91	12.0 ± 1.0	35.4 ± 5.6	112.2 ± 34.3*	23.7 ± 1.3	0.4 ± 0.4	64.3 ± 3.9*
0.78	0.45	12.0 ± 0.9	35.2 ± 5.7	93.3 ± 21.1*	23.9 ± 3.1	0.4 ± 0.4	41.9 ± 3.4*
0	0	12.2 ± 1.3	31.8 ± 3.1	18.0 ± 3.4	22.9 ± 1.5	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of four determinations. Molar values were calculated based on a formula weight of 1728 Da for pentasaccharide. Statistical analysis of the data was made using one way ANOVA followed by the Newman-Keuls test for each assay. * indicates statistical significance compared to unsupplemented baseline plasma ($p < 0.05$). PT : dF = 35, F = 1.53, $P = 0.194$. APTT : dF = 35, F = 21.6, $P < 0.0001$. Heptest : dF = 35, F = 18.7, $P < 0.0001$. 5U TT : dF = 35, F = 3.46, $P = 0.0072$. Anti-IIa : dF = 35, F = 1.55, $P = 0.186$. Anti-Xa : dF = 35, F = 1235, $P < 0.0001$

TABLE 28

IN VITRO ANTICOAGULANT ACTIVITY OF APROSULATE IN NORMAL RABBIT PLASMA

Concentration (μg/mL)	Concentration (μM)	PT (sec)	APTT (sec)	Heptest (sec)	5U TT (sec)	Anti-IIa (% Inhib)	Anti-Xa (% Inhib)
100.0	41.88	7.4 ± 0.1*	>300*	133.7 ± 7.9*	>300*	20.5 ± 2.6*	0
50.0	20.94	7.3 ± 0.1*	260.6 ± 16.5*	93.1 ± 21.7*	>300*	12.1 ± 5.6*	0
25.0	10.47	6.9 ± 0.2*	153.5 ± 18.4*	45.4 ± 5.0	>300*	4.4 ± 2.4	0
12.5	5.23	6.8 ± 0.1	122.1 ± 20.6*	38.1 ± 4.7	117.8 ± 3.1*	0.7 ± 1.2	0
6.25	2.62	6.8 ± 0.3	88.4 ± 2.7*	33.3 ± 6.3	45.8 ± 2.7*	0	0
3.12	1.31	6.2 ± 0.3	69.2 ± 4.4	29.5 ± 5.9	36.6 ± 5.2*	5.1 ± 8.8	0
1.56	0.65	6.5 ± 0.2	56.7 ± 8.1	27.5 ± 4.8	33.1 ± 2.5*	0	0
0.78	0.33	6.4 ± 0.1	50.5 ± 0.8	27.0 ± 3.3	29.1 ± 2.0	0	0
0	0	6.3 ± 0.4	45.0 ± 2.3	25.7 ± 2.5	27.8 ± 0.5	0	0

All results represent the mean ± 1 standard deviation of three determinations. Molar values were calculated based on a formula weight of 2388 Da for aprosulate and represent the plasma aprosulate concentration. Statistical differences versus control were determined for each assay using one way ANOVA followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ *vs.* control. PT : dF = 26, F = 10.6, $p < 0.0001$. APTT : dF = 26, F = 211 $p < 0.0001$. Heptest : dF = 26, F = 55.1, $p < 0.0001$. 5U TT : dF = 26, F = 8222.5, $p < 0.0001$. Anti-IIa : dF = 26, F = 11.2, $p < 0.0001$. Anti-Xa : dF = 26, F = 1.0, P = 1.0.

TABLE 29

IN VITRO ANTICOAGULANT ACTIVITY OF GL-522-Y-1 IN NORMAL RABBIT PLASMA

Concentration (μg/mL)	Concentration (μM)	PT (sec)	APTT (sec)	Heptest (sec)	5U TT (sec)	Anti-IIa (% Inhib)	Anti-Xa (% Inhib)
100.0	67.2	7.0 ± 0.1	49.4 ± 1.1	26.7 ± 5.5	29.6 ± 0.9	9.2 ± 1.4*	0 ± 0
50.0	33.6	6.6 ± 0.2	49.4 ± 0.3	26.8 ± 3.9	28.4 ± 0.9	0 ± 0	0 ± 0
25.0	16.8	6.4 ± 0.1	48.8 ± 1.1	26.6 ± 3.7	28.3 ± 0.4	0.7 ± 1.2	0 ± 0
12.5	8.4	6.8 ± 0.5	48.6 ± 0.8	26.3 ± 3.5	27.7 ± 0.9	0.5 ± 0.9	0 ± 0
6.25	4.2	6.3 ± 0.2	46.5 ± 1.4	26.0 ± 3.2	27.2 ± 1.2	0 ± 0	0 ± 0
3.12	2.1	5.9 ± 0.6	44.1 ± 3.4	25.9 ± 2.7	25.7 ± 2.3	0 ± 0	0 ± 0
1.56	1.05	6.0 ± 0.2	43.7 ± 2.0	25.6 ± 2.8	26.1 ± 2.0	0 ± 0	0 ± 0
0.78	0.53	6.0 ± 0.3	46.2 ± 3.4	26.1 ± 2.0	26.1 ± 1.2	0 ± 0	0 ± 0
0	0	6.3 ± 0.4	45.0 ± 2.3	25.7 ± 2.5	27.8 ± 0.5	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. Molar values were calculated based on a formula weight of 1488 Da for GL-522-Y-1 and represent the plasma GL-522-Y-1 concentration. Statistical differences versus control were determined for each assay using one way ANOVA followed by the Newman-Keuls multiple comparison test. $^*p < 0.05$ *vs.* control. PT : dF = 26, F = , p = . APTT : dF = 26, F = 4057, p < 0.0001. Heptest : dF = 26, F = 0.0482, p = 0.9999. 5U TT : dF = 26, F = 2.97, p = 0.0263. Anti-IIa : dF = 26, F = 58.8, p < 0.0001. Anti-Xa : dF = 26, F = 1.0, P = 1.0000.

TABLE 30

IN VITRO ANTICOAGULANT ACTIVITY OF HEPARIN IN NORMAL RABBIT PLASMA

Concentration		PT	APTT	Heptest	5U TT	Anti-IIa	Anti-Xa
(μg/mL)	(μM)	(sec)	(sec)	(sec)	(sec)	(% Inhib)	(% Inhib)
100.0	9.53	16.9 ± 1.2*	>300*	>300*	>300*	90.2 ± 2.2*	95.6 ± 0.8*
50.0	4.77	14.7 ± 0.4*	>300*	>300*	>300*	90.6 ± 2.5*	95.5 ± 0.4*
25.0	2.38	11.9 ± 0.5*	>300*	>300*	>300*	91.3 ± 0.4*	93.4 ± 0.5*
12.5	1.19	9.9 ± 0.5*	>300*	>300*	>300*	91.7 ± 1.1*	90.3 ± 1.1*
6.25	0.60	7.9 ± 0.5*	>300*	>300*	>300*	87.1 ± 1.9*	74.0 ± 2.7*
3.12	0.30	7.0 ± 0.2	>300*	204.4 ± 7.7*	>300*	76.8 ± 2.4*	44.2 ± 2.6*
1.56	0.15	6.6 ± 0.3	104.5 ± 7.1*	73.8 ± 10.0*	>300*	50.4 ± 1.3*	33.9 ± 3.5*
0.78	0.07	6.3 ± 0.1	74.5 ± 5.5*	39.2 ± 1.4*	>300*	36.3 ± 2.0*	8.2 ± 2.1*
0	0	6.3 ± 0.4	45.0 ± 2.3	25.7 ± 2.5	27.8 ± 0.5	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. Molar values were calculated based on a molecular weight of 10492 Da for heparin and represent the plasma heparin concentration. Statistical differences versus control were determined for each assay using one way ANOVA followed by the Newman-Keuls multiple comparison test. $^*p < 0.05$ *vs.* control. PT : dF = 26, F = 159.2, $p < 0.0001$. APTT : dF = 26, F = , $p < 0.0001$. Heptest : dF = 26, F = 2457.7, p = 0.0001. 5U TT : dF = 26, F = 889114.1, $p < 0.0001$. Anti-IIa : dF = 26, F = 1041, $p < 0.0001$. Anti-Xa : dF = 26, F = 1225.2, $P < 0.0001$.

TABLE 31

IN VITRO ANTICOAGULANT ACTIVITY OF PENTASACCHARIDE IN NORMAL RABBIT PLASMA

Concentration (μg/mL)	Concentration (μM)	PT (sec)	APTT (sec)	Heptest (sec)	5U TT (sec)	Anti-IIa (% Inhib)	Anti-Xa (% Inhib)
100.0	58.0	6.4 ± 0.1	110.9 ± 5.8*	185.0 ± 10.7*	43.9 ± 1.8*	10.5 ± 0.7*	97.8 ± 0.1*
50.0	29.0	6.2 ± 0.3	99.8 ± 2.2*	164.4 ± 9.7*	39.2 ± 1.4*	10.0 ± 2.0*	97.8 ± 0.1*
25.0	14.5	6.3 ± 0.1	87.3 ± 3.4*	139.6 ± 0.6*	38.2 ± 0.4*	0 ± 0	97.2 ± 0.2*
12.5	7.25	6.4 ± 0.2	73.7 ± 8.8*	123.6 ± 12.5*	31.4 ± 1.2*	0 ± 0	96.8 ± 0.7*
6.25	3.63	6.4 ± 0.1	65.3 ± 8.2*	99.2 ± 1.1*	31.1 ± 0.2*	0 ± 0	94.3 ± 2.1*
3.12	1.81	6.4 ± 0.1	64.5 ± 0.4*	84.4 ± 3.1*	28.2 ± 0.5	0 ± 0	85.5 ± 1.5*
1.56	0.91	6.2 ± 0.3	60.9 ± 4.4*	65.2 ± 4.2*	27.5 ± 0.4	0 ± 0	64.3 ± 3.9*
0.78	0.45	6.3 ± 0.5	53.6 ± 6.4	46.8 ± 2.7*	26.5 ± 1.0	0 ± 0	41.9 ± 3.4*
0	0	6.3 ± 0.4	45.0 ± 2.3	25.7 ± 2.5	27.8 ± 0.5	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. Molar values were calculated based on a formula weight of 1728 Da for pentasaccharide and represent the plasma pentasaccharide concentration. Statistical differences versus control were determined for each assay using one way ANOVA followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ *vs.* control. PT : dF = 26, F = 0.283, p = 0.9643. APTT : dF = 26, F = 49.2, p < 0.0001. Heptest : dF = 26, F = 192.1, p = 0.0001. 5U TT : dF = 26, F = 123.8, p < 0.0001. Anti-IIa : dF = 26, F = 122.9, p < 0.0001. Anti-Xa : dF = 26, F = 926.2, P < 0.0001.

TABLE 32

EFFECT OF APROSULATE ON THE FVIII:C MEDIATED GENERATION OF FACTOR Xa GENERATION

Concentration		
μM	μg/mL	% Inhibition
41.88	100.0	98.4 ± 1.1*
20.94	50.0	92.6 ± 5.0*
10.47	25.0	84.8 ± 7.2*
5.23	12.5	74.7 ± 5.6*
2.62	6.2	64.5 ± 9.2*
1.31	3.1	52.9 ± 9.8*
0.65	1.6	44.8 ± 9.6*
0.33	0.8	28.6 ± 7.7*

All results represent the mean ± 1 standard deviation of three determinations. μM concentrations were calculated based on a formula molecular weight of 2388 Da. All percent inhibitions were based on an unsupplemented control. Statistically significant differences between treatment and control were determined by one way ANOVA (dF = 23, F = 65, $p < 0.0001$) followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ was considered statistically significant.

TABLE 33

EFFECT OF GL-522-Y-1 ON THE FVIII:C MEDIATED INHIBITION OF FACTOR Xa GENERATION

Concentration		
μM	μg/mL	% Inhibition
67.20	100.0	99.7 ± 0.5*
33.60	50.0	58.8 ± 4.2*
16.80	25.0	32.8 ± 1.7*
8.40	12.5	21.2 ± 3.3*
4.20	6.2	18.6 ± 2.1*
2.10	3.1	12.0 ± 3.7*
1.05	1.6	13.7 ± 0.7*
0.53	0.8	3.6 ± 0.0

All results represent the mean ± 1 standard deviation of three determinations. μM concentrations were calculated based on a formula molecular weight of 1488 Da. All percent inhibitions were based on an unsupplemented control. Statistically significant differences between treatment and control were determined by one way ANOVA (dF = 23, F = 975, $p < 0.0001$) followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ was considered statistically significant.

TABLE 34

EFFECT OF HEPARIN ON THE FVIII:C MEDIATED INHIBITION OF FACTOR Xa GENERATION

Concentration		
μM	μg/mL	% Inhibition
0.038	0.4	99.7 ± 0.3*
0.019	0.2	99.7 ± 0.3*
0.010	0.1	99.7 ± 0.3*
0.005	0.05	96.4 ± 2.0*
0.0025	0.025	61.2 ± 8.0*
0.0012	0.012	39.2 ± 8.4*
0.0006	0.006	36.8 ± 1.0*
0.0003	0.003	30.3 ± 0.4*

All results represent the mean ± 1 standard deviation of three determinations. μM concentrations were calculated based on a weight average molecular weight of 10492 Da. All percent inhibitions were based on an unsupplemented control. Statistically significant differences between treatment and control were determined by one way ANOVA (dF = 23, F = 975, $p < 0.0001$) followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ was considered statistically significant.

TABLE 35

EFFECT OF PENTASACCHARIDE ON THE FVIII:C MEDIATED INHIBITION OF FACTOR Xa GENERATION

Concentration		
μM	μg/mL	% Inhibition
58.00	100.0	82.4 ± 10.9*
29.00	50.0	77.3 ± 8.3*
14.50	25.0	74.9 ± 7.7*
7.25	12.5	70.8 ± 8.7*
3.63	6.2	67.9 ± 7.0*
1.81	3.1	67.9 ± 6.5*
0.91	1.6	62.3 ± 6.8*
0.45	0.8	52.6 ± 7.1*

All results represent the mean ± 1 standard deviation of three determinations. μM concentrations were calculated based on a formula molecular weight of 1728 Da. All percent inhibitions were based on an unsupplemented control. Statistically significant differences between treatment and control were determined by one way ANOVA (dF = 23, F = 346, $p < 0.0001$) followed by the Newman-Keuls multiple comparison test. *$p < 0.05$ was considered statistically significant.

TABLE 36

EFFECT OF APROSULATE ON PROTEASE GENERATION FOLLOWING ACTIVATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS

Concentration		% Inhibition			
μM	μg/mL	Extrinsic IIa	Extrinsic Xa	Intrinsic IIa	Intrinsic Xa
33.3	79.5	11.1 ± 2.1*	5.2 ± 1.5*	75.6 ± 4.8*	91.3 ± 3.7*
16.7	39.8	8.1 ± 2.9*	0.7 ± 0.7	70.6 ± 4.6*	92.2 ± 3.1*
8.4	19.9	2.0 ± 1.6	0.1 ± 0.1	65.4 ± 5.2 *	92.0 ± 4.2*
4.2	9.9	0.6 ± 1.4	0 ± 0	55.1 ± 10.6*	91.5 ± 4.6*
2.1	5.0	0 ± 0	0 ± 0	17.2 ± 25.5	67.7 ± 24.0*
1.05	2.5	0 ± 0	0 ± 0	0 ± 0	42.7 ± 27.2*
0.52	1.2	0 ± 0	0 ± 0	0 ± 0	20.5 ± 18.7
0.26	0.6	0 ± 0	0 ± 0	0 ± 0	8.4 ± 10.0
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 2388 Da. All percent inhibitions were calculated based on an unsupplemented control. Statistically significant differences between supplemented and control samples were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test (Ext IIa; dF = 26, F = 27.3, $p < 0.0001$. Ext Xa; dF = 26, F = 29.2, $p < 0.0001$. Int IIa; dF = 26, F = 37.9, $p < 0.0001$. Int Xa; dF = 26, F = 22.4, $p < 0.0001$). * $p < 0.05$ was considered statistically significant.

TABLE 37

EFFECT OF GL-522-Y-1 ON PROTEASE GENERATION FOLLOWING ACTIVATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS

Concentration		% Inhibition			
μM	μg/mL	Extrinsic IIa	Extrinsic Xa	Intrinsic IIa	Intrinsic Xa
33.3	49.6	97.7 ± 3.9*	98.3 ± 3.0*	100 ± 0*	99.9 ± 0.2*
16.7	24.8	77.8 ± 7.0*	80.4 ± 9.5*	100 ± 0*	100 ± 0*
8.4	12.4	54.7 ± 5.7*	57.2 ± 5.2*	99.4 ± 1.0*	96.3 ± 3.2*
4.2	6.2	26.6 ± 14.2*	36.8 ± 13.1*	84.0 ± 5.1*	93.8 ± 6.9*
2.1	3.1	10.8 ± 0.1	14.6 ± 7.5	28.4 ± 33.5*	81.9 ± 7.0*
1.05	1.5	4.7 ± 3.1	1.6 ± 1.0	0.4 ± 0.7	27.2 ± 19.8*
0.52	0.8	0.2 ± 0.2	0.5 ± 0.6	0 ± 0	7.4 ± 6.6
0.26	0.4	0 ± 0	0.2 ± 0.3	0 ± 0	2.1 ± 3.0
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 1488 Da. All percent inhibitions were calculated based on an unsupplemented control. Statistically significant differences between supplemented and control samples were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test (Ext IIa; dF = 26, F = 123.2, $p < 0.0001$. Ext Xa; dF = 26, F = 111.3, $p < 0.0001$. Int IIa; dF = 26, F = 55.5, $p < 0.0001$. Int Xa; dF = 26, F = 102.9, $p < 0.0001$). * $p < 0.05$ was considered statistically significant.

TABLE 38

EFFECT OF HEPARIN ON PROTEASE GENERATION FOLLOWING ACTIVATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS

Concentration		% Inhibition			
μM	μg/mL	Extrinsic IIa	Extrinsic Xa	Intrinsic IIa	Intrinsic Xa
33.3	349.4	100 ± 0*	97.3 ± 0.9*	29.7 ± 1.7*	79.6 ± 1.0*
16.7	174.7	98.7 ± 1.1*	96.2 ± 1.0*	29.2 ± 2.5*	80.1 ± 2.5*
8.4	87.4	93.4 ± 2.1*	94.9 ± 0.4*	29.7 ± 2.0*	81.9 ± 1.6*
4.2	43.7	54.2 ± 5.4*	92.6 ± 0.3*	28.9 ± 3.3*	80.7 ± 2.5*
2.1	21.8	1.7 ± 1.9	85.6 ± 0.7*	24.1 ± 4.9*	81.3 ± 2.8*
1.05	10.9	0 ± 0	68.5 ± 2.3*	23.5 ± 5.9*	84.7 ± 1.3*
0.52	5.6	0 ± 0	47.8 ± 2.3*	7.8 ± 13.5	86.7 ± 2.1*
0.26	2.7	0 ± 0	27.7 ± 2.2*	0 ± 0	68.3 ± 6.5*
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 10492 Da. All percent inhibitions were calculated based on an unsupplemented control. Statistically significant differences between supplemented and control samples were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test (Ext IIa; dF = 26, F = 1577.5, $p < 0.0001$. Ext Xa; dF = 26, F = 1880.7, $p < 0.0001$. Int IIa; dF = 26, F = 16.9, $p < 0.0001$. Int Xa; dF = 26, F = 294.4, $p < 0.0001$). * $p < 0.05$ was considered statistically significant.

TABLE 39

EFFECT OF PENTASACCHARIDE ON PROTEASE GENERATION FOLLOWING ACTIVATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS

Concentration		% Inhibition			
μM	μg/mL	Extrinsic IIa	Extrinsic Xa	Intrinsic IIa	Intrinsic Xa
33.3	57.5	0 ± 0	0 ± 0	0 ± 0	53.3 ± 12.7*
16.7	28.8	0 ± 0	0 ± 0	0 ± 0	29.2 ± 13.7*
8.4	14.4	0 ± 0	0 ± 0	0 ± 0	16.3 ± 9.5
4.2	7.2	0 ± 0	0 ± 0	0 ± 0	8.9 ± 6.1
2.1	3.6	0 ± 0	0 ± 0	0 ± 0	4.1 ± 3.2
1.05	1.8	0 ± 0	0 ± 0	0 ± 0	1.2 ± 0.6
0.52	0.9	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.4
0.26	0.4	0 ± 0	0 ± 0	0 ± 0	1.3 ± 1.3
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

All results represent the mean ± 1 standard deviation of three determinations. μM amounts are final assay concentrations based on a formula molecular weight of 1728 Da. All percent inhibitions were calculated based on an unsupplemented control. Statistically significant differences between supplemented and control samples were determined by one way ANOVA followed by the Newman-Keuls multiple comparison test (Ext IIa; dF = 26, F = 1.0, p = 1.0 Ext Xa; dF = 26, F = 1.0, p =1.0. Int IIa; dF = 26, F = 1.0, p = 1.0. Int Xa; dF = 26, F = 17.9, $p < 0.0001$). * $p < 0.05$ was considered statistically significant.

TABLE 40

EFFECT OF APROSULATE ON GLYCOSAMINOGLYCAN SYNTHESIS IN ENDOTHELIAL CELL CULTURE

Dose (μM)	CS Med[1]	HS Med[2]	CS Cell[3]	HS Cell[4]
0	2.71 ± 0.43	1.06 ± 0.26	0.84 ± 0.13	1.30 ± 0.21
0.04	1.87 ± 0.31	0.77 ± 0.12	0.76 ± 0.01	1.20 ± 0.17
0.41	2.22 ± 0.06	0.92 ± 0.07	0.85 ± 0.09	1.17 ± 0.10
4.18	2.72 ± 0.13	1.32 ± 0.23	0.73 ± 0.02	1.12 ± 0.04
20.9	2.87 ± 0.10	1.61 ± 0.28	1.02 ± 0.16	0.97 ± 0.21
41.8	2.99 ± 0.06	2.14 ± 0.01*	1.46 ± 0.04*	0.91 ± 0.15

All results represent the mean ± SEM of three individual culture dishes. Confluent rabbit endothelial cells were incubated with aprosulate and ^{35}S containing buffer for 18 hours. Glycosaminoglycan production was determined by scintillation counting. [1] Chondroitin sulfate in culture media (C.P.M.'s x 10^5). [2] Heparan sulfate in culture media (C.P.M.'s x 10^6). [3] Chondroitin sulfate on endothelial cells (C.P.M.'s x 10^5). [4] Heparan sulfate on endothelial cells (C.P.M.'s x 10^6). Statistical comparison of the C.P.M.'s at each concentration with that of unsupplemented controls was made by one way ANOVA followed by the Newman-Keuls test. (CS Med; dF = 17, F = 10.5, $p = 0.0005$. HS Med; dF = 17, F = 21.2, $p < 0.0001$. CS Cell; dF = 17, F = 25.4, $p < 0.0001$. HS Cell; dF = 17, F = 2.56, $p = 0.0847$). *$p < 0.05$ *vs.* control is considered statistically significant.

TABLE 41

EFFECT OF GL-522-Y-1 ON GLYCOSAMINOGLYCAN SYNTHESIS IN ENDOTHELIAL CELL CULTURE

Dose (μM)	CS Med[1]	HS Med[2]	CS Cell[3]	HS Cell[4]
0	1.45 ± 0.12	0.33 ± 0.10	0.95 ± 0.08	0.76 ± 0.02
0.07	1.29 ± 0.22	0.31 ± 0.06	1.31 ± 0.01	1.14 ± 0.02
0.67	1.43 ± 0.02	0.42 ± 0.03	1.51 ± 0.11*	1.36 ± 0.04*
6.70	1.30 ± 0.16	0.78 ± 0.08	1.30 ± 0.25	1.24 ± 0.29
33.5	1.70 ± 0.36	1.10 ± 0.19*	1.70 ± 0.08*	1.81 ± 0.13*
67.0	1.58 ± 0.19	1.11 ± 0.15*	1.70 ± 0.06*	1.91 ± 0.12*

All results represent the mean ± SEM of three individual culture dishes. Confluent rabbit endothelial cells were incubated with GL-522-Y-1 and ^{35}S containing buffer for 18 hours. Glycosaminoglycan production was determined by scintillation counting. [1] Chondroitin sulfate in culture media (C.P.M.'s x 10^5). [2] Heparan sulfate in culture media (C.P.M.'s x 10^6). [3] Chondroitin sulfate on endothelial cells (C.P.M.'s x 10^5). [4] Heparan sulfate on endothelial cells (C.P.M.'s x 10^6). Statistical comparison of the C.P.M.'s at each concentration with that of unsupplemented controls was made by one way ANOVA followed by the Newman-Keuls test (CS Med; dF = 17, F = 1.80, $p = 0.1867$. HS Med; dF = 17, F = 31.6, $p < 0.0001$. CS Cell; dF = 17, F = 16.5, $p < 0.0001$. HS Cell; dF = 17, F = 28.3, $p < 0.0001$). *$p < 0.05$ *vs.* control is considered statistically significant.

TABLE 42

EFFECT OF HEPARIN ON GLYCOSAMINOGLYCAN SYNTHESIS IN ENDOTHELIAL CELL CULTURE

Dose (μM)	CS Med[1]	HS Med[2]	CS Cell[3]	HS Cell[4]
0	0.76 ± 0.05	2.60 ± 0.24	0.55 ± 0.07	8.40 ± 1.18
0.0095	0.93 ± 0.20	2.86 ± 0.13	0.56 ± 0.07	8.44 ± 0.59
0.095	1.08 ± 0.32	3.73 ± 0.20	0.69 ± 0.5	9.48 ± 0.66
0.95	1.03 ± 0.22	4.21 ± 0.53*	0.88 ± 0.26	7.56 ± 2.02
4.77	0.93 ± 0.25	5.77 ± 0.62*	0.52 ± 0.06	9.45 ± 3.03
9.50	0.84 ± 0.07	6.76 ± 0.18*	1.05 ± 0.08	9.24 ± 0.69

All results represent the mean ± SEM of three individual culture dishes. Confluent rabbit endothelial cells were incubated with heparin and ^{35}S containing buffer for 18 hours. Glycosaminoglycan production was determined by scintillation counting. [1] Chondroitin sulfate in culture media (C.P.M.'s x 10^5). [2] Heparan sulfate in culture media (C.P.M.'s x 10^5). [3] Chondroitin sulfate on endothelial cells (C.P.M.'s x 10^5). [4] Heparan sulfate on endothelial cells (C.P.M.'s x 10^5). Statistical comparison of the C.P.M.'s at each concentration with that of unsupplemented controls was made by one way ANOVA followed by the Newman-Keuls test (CS Med; dF = 17, F = 0.959, $p = 0.4793$. HS Med; dF = 17, F = 59.9, $p < 0.0001$. CS Cell; dF = 17, F = 2.44, $p = 0.0950$. HS Cell; dF = 17, F = 0.655, $p = 0.6636$). *$p < 0.05$ *vs.* control is considered statistically significant.

TABLE 43

EFFECT OF PENTASACCHARIDE ON GLYCOSAMINOGLYCAN SYNTHESIS IN ENDOTHELIAL CELL CULTURE

Dose (μM)	CS Med[1]	HS Med[2]	CS Cell[3]	HS Cell[4]
0	7.70 ± 1.17	8.52 ± 0.28	6.65 ± 0.53	3.28 ± 0.54
0.058	7.74 ± 0.30	8.61 ± 0.34	6.55 ± 1.03	3.36 ± 0.48
0.58	7.58 ± 0.44	9.33 ± 0.04	6.80 ± 0.01	4.09 ± 0.43
5.8	7.83 ± 0.39	9.09 ± 0.32	8.58 ± 1.38	3.85 ± 0.05
29.0	7.53 ± 1.32	7.51 ± 0.57	8.50 ± 0.02	3.76 ± 0.40
58.0	6.63 ± 0.28	8.84 ± 0.18	9.78 ± 0.07	4.71 ± 0.70

All results represent the mean ± SEM of three individual culture dishes. Confluent rabbit endothelial cells were incubated with pentasaccharide and ^{35}S containing buffer for 18 hours. Glycosaminoglycan production was determined by scintillation counting. [1] Chondroitin sulfate in culture media (C.P.M.'s x 10^4). [2] Heparan sulfate in culture media (C.P.M.'s x 10^5). [3] Chondroitin sulfate on endothelial cells (C.P.M.'s x 10^4). [4] Heparan sulfate on endothelial cells (C.P.M.'s x 10^5). Statistical comparison of the C.P.M.'s at each concentration with that of unsupplemented controls was made by one way ANOVA followed by the Newman-Keuls test (CS Med; dF = 17, F = 0.964, $p = 0.4770$. HS Med; dF = 17, F = 11.0, $p = 0.0004$. CS Cell; dF = 17, F = 9.86, $p = 0.0006$. HS Cell; dF = 17, F = 3.63, $p = 0.0313$). $^*p < 0.05$ *vs.* control is considered statistically significant.

TABLE 44

EFFECT OF APROSULATE, GL-522-Y-1, HEPARIN AND PENTASACCHARIDE ON AGONIST INDUCED PLATELET AGGREGATION IN PLATELET RICH PLASMA

	Aprosulate	GL-522-Y-1	Heparin	Pentasaccharide	Control
Epinephrine	68.1 ± 4.9	66.1 ± 4.1	73.9 ± 0.7	63.3 ± 7.4	66.0 ± 6.0
ADP 1:4	72.0 ± 3.9	69.1 ± 4.5	71.6 ± 1.6	66.9 ± 4.3	65.4 ± 3.8
ADP 1:8	61.0 ± 7.1	60.2 ± 6.4	68.6 ± 5.3	58.8 ± 7.5	53.9 ± 7.7
Arachidonic Acid	78.5 ± 0.8	68.8 ± 7.0	77.8 ± 0.8	69.3 ± 7.1	78.3 ± 1.1
Thrombin	46.6 ± 11.3*	91.8 ± 1.1	6.7 ± 0.8*	89.1 ± 2.7	93.7 ± 0.9
Collagen	76.4 ± 1.3	74.8 ± 1.3	75.5 ± 1.7	75.9 ± 1.6	74.7 ± 1.4

All results represent the mean (± SEM) percent aggregation of 10 volunteers. All test agents were supplemented at a final concentration of 10 μM. Epinephrine = 10 μg/mL; ADP = 1.15 and 0.58 μg/mL; Arachidonic acid = 300 μg/mL; Thrombin = 1 U/mL; Collagen = 0.8 μg/mL. All agonist concentrations represent final assay concentrations. Statistical comparisons vs. control for each agonist were made by one way ANOVA and the Newman Keuls test (Epinephrine; dF = 49, F = 5.98, $p = 0.0006$. ADP 1:4; dF = 49, F = 6.13 $p = 0.0005$. ADP 1:8; dF = 49, F = 5.98, $p = 0.0006$. Arachidonic Acid; dF = 49, F = 12.4, $p < 0.0001$. Thrombin; dF = 49, F = 532, $p < 0.0001$. Collagen; dF = 49, F = 2.15 $p = 0.0900$). *$p < 0.05$ was considered statistically significant.

TABLE 45

EFFECT OF APROSULATE, GL-522-Y-1, HEPARIN AND PENTASACCHARIDE ON AGONIST INDUCED PLATELET AGGREGATION IN PLATELET RICH PLASMA

	Aprosulate	GL-522-Y-1	Heparin	Pentasaccharide	Control
Epinephrine	22.5 ± 2.7	20.7 ± 2.6	25.5 ± 2.2	21.7 ± 3.1	21.8 ± 3.5
ADP 1:4	39.4 ± 1.8	39.5 ± 1.3	41.3 ± 1.0	38.6 ± 1.2	41.2 ± 1.9
ADP 1:8	31.2 ± 1.8	31.8 ± 1.9	36.5 ± 2.1	30.6 ± 2.3	31.5 ± 2.8
Arachidonic Acid	45.2 ± 2.3	42.4 ± 4.4	46.7 ± 2.2	41.8 ± 4.6	47.9 ± 2.8
Thrombin	21.9 ± 2.8*	74.6 ± 8.0	3.8 ± 0.2*	50.8 ± 4.7	61.5 ± 3.0
Collagen	40.9 ± 2.2	41.1 ± 1.9	40.4 ± 2.0	41.0 ± 1.9	43.1 ± 2.7

All results represent the mean (± SEM) slope of the aggregation response of 10 volunteers. All test agents were supplemented at a final concentration of 10 μM. Epinephrine = 10 μg/mL; ADP = 1.15 and 0.58 μg/mL; Arachidonic acid = 300 μg/mL; Thrombin = 1 U/mL; Collagen = 0.8 μg/mL. All agonist concentrations represent final assay concentrations. Statistical comparisons were made by one way ANOVA and the Newman Keuls test (Epinephrine; dF = 49, F = 4.10, $p = 0.0065$. ADP 1:4; dF = 49, F = 6.49 $p = 0.0003$. ADP 1:8; dF = 49, F = 11.6, $p < 0.0001$. Arachidonic Acid; dF = 49, F = 6.01, $p = 0.0006$. Thrombin; dF = 49, F = 410.6, $p < 0.0001$. Collagen; dF = 49, F = 2.32 $p = 0.0710$). *$p < 0.05$ was considered statistically significant.

TABLE 46

COMPARATIVE EFFECT OF SYNTHETIC HEPARIN ANALOGUES IN A HEPARIN INDUCED THROMBOCYTOPENIA SCREENING ASSAY

	μg/mL	μM	% Aggregation
Saline			8.3 ± 1.1
Aprosulate	5.5	2.30	9.0 ± 1.9
	11.0	4.61	18.9 ± 1.6*
	21.7	9.21	21.2 ± 4.2*
GL-522-Y-1	5.5	3.70	10.4 ± 3.3*
	11.0	7.39	7.8 ± 0.9
	21.7	14.78	5.0 ± 1.3
Heparin	5.5	0.52	11.6 ± 1.7*
	11.0	1.05	15.5 ± 1.4*
	21.7	2.10	22.2 ± 5.5*
Pentasaccharide	5.5	3.18	3.4 ± 0.2
	11.0	6.37	5.9 ± 0.3
	21.7	12.73	6.6 ± 1.1

Results represent the mean ± SEM of 10 volunteers. Aggregation was induced by the test agent and HIT positive serum. Statistical analysis was made by one way ANOVA followed by the Newman Keuls test (aprosulate; dF = 39, F = 70.8, $p < 0.0001$. GL-522-Y-1; dF = 39, F = 13.5, $p < 0.0001$. heparin; dF = 39.3, F = 39.3, $p < 0.0001$. pentasaccharide; dF = 39, F = 64.9, $p < 0.0001$). *$p < 0.05$ represents a statistically significant increase in aggregation *vs.* control.

TABLE 47

ANTITHROMBOTIC EFFECT OF SYNTHETIC HEPARIN ANALOGUES IN A RABBIT MODEL OF STASIS THROMBOSIS

	Dose		Clot Score 10 minutes	Clot Score 20 Minutes
	(nmol/kg)	(mg/kg)		
Saline	0.1 mL/kg		2.9 ± 0.1	3.6 ± 0.3
Aprosulate	42.0	0.10	2.8 ± 0.3	3.3 ± 0.3
	104.7	0.25	1.2 ± 0.2#	3.0 ± 0.0
	209.4	0.50	0.2 ± 0.2#	2.2 ± 0.4*
GL-522-Y-1	67.0	0.10	2.6 ± 0.2*	3.4 ± 0.1
	776.0	1.00	1.6 ± 0.3*	2.9 ± 0.1
	1940.0	2.50	1.0 ± 0.3#	3.2 ± 0.2
	3880.0	5.00	0.4 ± 0.2#	2.8 ± 0.2
Heparin	0.6	0.01	0.8 ± 0.2	3.6 ± 0.3
	1.2	0.01	1.8 ± 0.5	3.0 ± 0.0
	2.4	0.02	1.2 ± 0.6*	2.4 ± 0.2
	4.8	0.05	0.0 ± 0.0#	0.2 ± 0.2#
Pentasaccharide	7.2	0.01	2.4 ± 0.3	3.0 ± 0.0
	14.5	0.02	1.8 ± 0.4*	3.0 ± 0.0
	29.0	0.05	1.2 ± 0.2#	2.8 ± 0.2
	58.0	0.10	0.2 ± 0.2#	1.2 ± 0.2#

All doses, with the exception of saline, are expressed in nmol/kg administered 5 minutes prior to injection of the thrombogenic challenge. Clot Score 10 minutes and Clot Score 20 minutes are expressed as mean ± S.E.M. of the clot scores obtained after stasis times of 10 and 20 minutes, respectively. Each treatment group contained 5 rabbits. Statistical comparisons were made using the Kruskal-Wallis test for each agent followed by the Mann-Whitney U test for specific comparisons. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control.

TABLE 48

ANTITHROMBOTIC EFFECT OF SYNTHETIC HEPARIN ANALOGUES IN A RABBIT MODEL OF STASIS THROMBOSIS

Agent	Dose		Clot Score 10 minutes	Clot Score 20 Minutes
	(μmol/kg)	(mg/kg)		
Saline	0.1 mL/kg		2.9 ± 0.1	3.6 ± 0.3
Aprosulate	1.047	2.5	2.7 ± 0.1	2.9 ± 0.1
	2.094	5.0	1.4 ± 0.2#	2.0 ± 0.2*
	4.188	10.0	0.5 ± 0.1#	1.4 ± 0.1#
GL-522-Y-1	6.720	10.0	2.4 ± 0.2	3.6 ± 0.2
	13.440	20.0	1.2 ± 0.4#	3.2 ± 0.2
	20.160	30.0	0.8 ± 0.4#	3.8 ± 0.2
Heparin	0.024	0.25	2.6 ± 0.3	3.4 ± 0.3
	0.048	0.50	1.2 ± 0.2#	2.5 ± 0.2
	0.095	1.00	0.0 ± 0.0#	1.0 ± 0.4#
Pentasaccharide	0.073	0.12	1.8 ± 0.2#	3.0 ± 0.5
	0.145	0.25	1.2 ± 0.2#	2.4 ± 0.4
	0.290	0.50	0.4 ± 0.2#	1.8 ± 0.4*

All doses, with the exception of saline, are expressed in μmol/kg administered 2 hours prior to injection of the thrombogenic challenge. Clot Score 10 minutes and Clot Score 20 minutes are expressed as mean ± S.E.M. of the clot scores obtained after stasis times of 10 and 20 minutes, respectively. Each treatment group contained 5 rabbits. Statistical comparisons were made using the Kruskal-Wallis test for each agent followed by the Mann-Whitney U test for specific comparisons. *$p < 0.05$ *vs.* control. #$p < 0.01$ *vs.* control.

TABLE 49

TIME DEPENDENCE OF THE ANTITHROMBOTIC ACTIVITY OF SYNTHETIC HEPARIN ANALOGUES IN A RABBIT STASIS THROMBOSIS MODEL FOLLOWING INTRAVENOUS ADMINISTRATION

	Circulation Time	Clot Score 10 minutes	Clot Score 20 minutes
Saline	5 min.	2.9 ± 0.1	3.6 ± 0.3
Aprosulate (209 nmol/kg; 500 μg/kg)	5 min.	0.2 ± 0.2#	2.2 ± 0.4*
	60 min.	1.4 ± 0.3#	3.0 ± 0.0
	120 min.	2.4 ± 0.3	3.4 ± 0.3
GL-522-Y-1 (3880 nmol/kg; 5 mg/kg)	5 min.	0.4 ± 0.2#	2.8 ± 0.2
	60 min	1.4 ± 0.2*	2.8 ± 0.2
	120 min.	1.6 ± 0.2*	3.0 ± 0.0
Heparin (4.77 nmol/kg; 50 μg/kg)	5 min.	0.0 ± 0.0#	0.2 ± 0.2#
	30 min.	1.6 ± 0.3*	2.6 ± 0.3
	60 min.	2.4 ± 0.3	3.2 ± 0.2
Pentasaccharide (58.0 nmol/kg; 100 μg/kg)	5 min.	0.2 ± 0.2#	1.2 ± 0.2#
	120 min.	1.3 ± 0.3	3.3 ± 0.3
	240 min.	1.8 ± 0.2*	3.2 ± 0.2

Clot Score 10 minutes and Clot Score 20 minutes are expressed as mean ± S.E.M. of the clot scores obtained after stasis times of 10 and 20 minutes, respectively. Each treatment group contained 4-5 rabbits. Statistical comparisons were made using the Kruskal-Wallis test for each agent followed by the Mann-Whitney U test for specific comparisons. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control.

TABLE 50

TIME DEPENDENCE OF THE ANTITHROMBOTIC ACTIVITY OF SYNTHETIC HEPARIN ANALOGUES IN A RABBIT STASIS THROMBOSIS MODEL FOLLOWING SUBCUTANEOUS ADMINISTRATION

Agent (dose)	Circulation Time	Clot Score 10 minutes	Clot Score 20 minutes
Saline	5 min.	2.9 ± 0.1	3.6 ± 0.3
Aprosulate	2 hrs.	0.5 ± 0.1#	1.4 ± 0.1#
(4.19 μmol/kg; 10.0 mg/kg)	4 hrs.	0.8 ± 0.2#	1.8 ± 0.5*
	6 hrs.	2.2 ± 0.2	3.2 ± 0.2
GL-522-Y-1	2 hrs.	1.2 ± 0.4*	3.2 ± 0.2
(13.44 μmol/kg; 20.0 mg/kg)	3 hrs.	1.4 ± 0.3*	3.0 ± 0.0
	4 hrs.	2.4 ± 0.3	3.0 ± 0.0
Heparin	2 hrs.	1.2 ± 0.2#	2.5 ± 0.4
(0.048 μmol/kg; 500 μg/kg)	4 hrs.	2.0 ± 0.7	3.3 ± 0.4
	6 hrs.	2.4 ± 0.3	3.2 ± 0.2
Pentasaccharide	2 hrs.	1.2 ± 0.2*	2.4 ± 0.4
(0.145 μmol/kg; 250 μg/kg)	4 hrs.	1.5 ± 0.3*	2.8 ± 0.3
	6 hrs.	2.2 ± 0.2	3.4 ± 0.3

Clot Score 10 minutes and Clot Score 20 minutes are expressed as mean ± S.E.M. of the clot scores obtained after stasis times of 10 and 20 minutes, respectively. Each treatment group contained 4-5 rabbits. Statistical comparisons were made using the Kruskal-Wallis test for each agent followed by the Mann-Whitney U test for specific comparisons. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.01$ *vs.* control.

TABLE 51

ANTITHROMBOTIC EFFECT OF SYNTHETIC HEPARIN ANALOGUES FOLLOWING INTRAVENOUS ADMINISTRATION IN A RAT JUGULAR VEIN CLAMPING MODEL

	Dose		# of Clampings
	(μmol/kg)	(μg/kg)	
Saline	0.1 mL/kg		4.4 ± 0.3
Aprosulate	0.105	250	6.0 ± 0.7
	0.209	500	8.2 ± 0.4*
	0.419	1000	10.6 ± 0.8*
	1.047	2000	13.0 ± 1.9*
GL-522-Y-1	1.941	2500	6.4 ± 1.0
	3.882	5000	8.8 ± 0.4*
	7.764	10000	12.8 ± 0.8*
	11.646	15000	13.0 ± 1.1*
Heparin	0.012	125	5.6 ± 0.6
	0.024	250	8.0 ± 0.94*
	0.048	500	10.8 ± 1.1*
	0.095	1000	> 15*
Pentasaccharide	0.029	50	5.6 ± 0.5*
	0.058	100	8.4 ± 0.3*
	0.145	250	11.4 ± 0.5*
	0.290	500	13.6 ± 0.8*

All doses, with the exception of saline are expressed as μmol/kg. μmole dosages of each agent were calculated based on the formula molecular weights of 2388, 1488, and 1728 Da for aprosulate, GL-522-Y-1, and pentasaccharide, respectively, and a weight average molecular weight of 10492 for heparin. The number of clampings represent the mean ± S.E.M. of 5 rats. Statistical comparisons were made by one way analysis of variance followed by the Newman-Keuls test. *$p < 0.05$ *vs.* control.

TABLE 52

ANTITHROMBOTIC EFFECT OF SYNTHETIC HEPARIN ANALOGUES FOLLOWING SUBCUTANEOUS ADMINISTRATION IN A RAT JUGULAR VEIN CLAMPING MODEL

	Dose (μmol/kg)	Dose (mg/kg)	# of Clampings
Saline	0.1 mL/kg		4.4 ± 0.3
Aprosulate	1.047	2.5	6.8 ± 0.4*
	2.094	5.0	8.4 ± 0.7*
	4.188	10.0	11.8 ± 0.8*
	8.375	20.0	14.6 ± 0.5*
GL-522-Y-1	3.360	5.0	5.8 ± 0.4
	6.720	10.0	8.8 ± 0.4*
	13.441	20.0	11.2 ± 0.6*
	20.161	30.0	13.8 ± 0.9*
Heparin	0.060	0.6	6.4 ± 0.6
	0.119	1.25	7.4 ± 1.0*
	0.238	2.5	11.0 ± 0.7*
	0.477	5.0	12.8 ± 0.7*
Pentasaccharide	0.036	0.06	4.6 ± 0.3
	0.073	0.12	7.4 ± 0.5*
	0.145	0.25	10.6 ± 0.9*
	0.290	0.50	12.0 ± 0.6*

All doses, with the exception of saline are expressed as μmol/kg. μmole dosages of each agent were calculated based on the formula molecular weights of 2388, 1488, and 1728 Da for aprosulate, GL-522-Y-1, and pentasaccharide, respectively, and a weight average molecular weight of 10492 for heparin. The number of clampings represent the mean ± S.E.M. of 5 rats. Statistical comparisons were made by one way analysis of variance followed by the Newman-Keuls test. $^*p < 0.05$ *vs.* control.

TABLE 53

HEMORRHAGIC EFFECT OF HEPARIN ANALOGUES FOLLOWING INTRAVENOUS ADMINISTRATION

	Dose		RBC's 5 min	RBC's 15 min
	μmol/kg	mg/kg		
Saline	0.1 mL/kg		0.07 ± 0.04	0.07 ± 0.03
Aprosulate	0.42	1.0	0.16 ± 0.04*	0.09 ± 0.02
	1.05	2.5	0.15 ± 0.06*	0.08 ± 0.01
	2.09	5.0	0.17 ± 0.05*	0.10 ± 0.05
GL-522-Y-1	1.68	2.5	0.17 ± 0.06	0.15 ± 0.05
	3.36	5.0	0.54 ± 0.16*	0.54 ± 0.11*
	6.72	10.0	0.87 ± 0.13*	1.19 ± 0.23*
Heparin	0.02	0.25	0.11 ± 0.05	0.08 ± 0.04
	0.05	0.50	0.22 ± 0.10	0.07 ± 0.01
	0.10	1.00	0.31 ± 0.19*	0.13 ± 0.06
Pentasaccharide	0.58	1.00	0.05 ± 0.02	0.03 ± 0.01
	1.45	2.50	0.04 ± 0.01	0.05 ± 0.01
	2.90	5.00	0.04 ± 0.01	0.04 ± 0.02

All results represent the mean ± S.E.M. blood cell loss (x 10^9/liter) in five rabbits per treatment goup. Each agent was administered intravenously via the left marginal ear vein. After five minutes, five standardized incisions were made in the non-vascular portion of the right ear. Blood cells were collected for 10 minutes. After 10 minutes, five incisions were made on the left ear and the blood cells collected for 10 minutes. Blood cells were quantitated using a hemocytometer. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ *vs.* control was considered to be statistically significant.

TABLE 54

HEMORRHAGIC EFFECT OF HEPARIN ANALOGUES FOLLOWING SUBCUTANEOUS ADMINISTRATION

	Dose		RBC's (x 10^9/L)
	μmol/k	mg/kg	
Saline	0.1 mL/kg		0.11 ± 0.04
Aprosulate	1.05	2.5	0.09 ± 0.03
	2.09	5.0	0.16 ± 0.04
	4.19	10.0	0.18 ± 0.05*
	8.38	20.0	0.22 ± 0.05*
GL-522-Y-1	1.68	2.5	0.48 ± 0.13
	3.36	5.0	0.90 ± 0.38*
	6.72	10.0	0.97 ± 0.54*
	13.44	20.0	1.19 ± 0.55*
Heparin	0.24	2.5	0.22 ± 0.07
	0.48	5.0	0.21 ± 0.06
	0.95	10.0	0.55 ± 0.25*
	1.91	20.0	2.00 ± 0.01*
Pentasaccharide	5.80	10.0	0.18 ± 0.04
	11.60	20.0	0.15 ± 0.07

All results represent the mean ± S.E.M. blood cell loss (x 10^9/liter) in five rabbits per treatment group. Each agent was administered subcutaneously in the abdominal region. After 3 hours, five standardized incisions were made in the non-vascular portion of the right ear. Blood cells were collected for 10 minutes. Blood cells were quantitated using a hemocytometer. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ *vs.* control was considered to be statistically significant.

TABLE 55

TIME DEPENDENCE ON THE HEMORRHAGIC EFFECT OF HEPARIN ANALOGUES FOLLOWING INTRAVENOUS ADMINISTRATION

	Dose	Time	RBC's (x 10^9/L)
Saline	0.1 mL/kg	5 min.	0.10 ± 0.01
Aprosulate	0.42 μmol/kg	5 min.	0.15 ± 0.01*
	(1.0 mg/kg)	30 min.	0.15 ± 0.01*
		60 min.	0.11 ± 0.01
		120 min.	0.11 ± 0.01
GL-522-Y-1	6.72 μmol/kg	5 min.	0.87 ± 0.13*
	(10.o mg/kg)	60 min.	1.37 ± 0.29*
		120 min.	0.39 ± 0.12*
Heparin	0.95 μmol/kg	5 min.	3.06 ± 1.4*
	(10.0 mg/kg)	60 min.	0.82 ± 0.15
		120 min.	0.38 ± 0.08
Pentasaccharide	2.90 μmol/kg	5 min.	0.04 ± 0.02*
	(5.0 mg/kg)	30 min.	0.02 ± 0.01*
		60 min.	0.01 ± 0.01

All results represent the mean ± S.E.M. blood cell loss (x 10^9/liter) in five rabbits per treatment group. Each agent was administered intravenously via the left marginal ear vein. After varying circulation times, five standardized incisions were made in the non-vascular portion of the right ear. Blood cells were collected for 10 minutes. Blood cells were quantitated using a hemocytometer. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ *vs.* control was considered to be statistically significant.

TABLE 56

TIME DEPENDENCE OF THE HEMORRHAGIC EFFECT OF HEPARIN ANALOGUES FOLLOWING SUBCUTANEOUS ADMINISTRATION

	Dose	Time	RBC's (x 10^9/L)
Saline	0.1 mL/kg	3 hrs.	0.11 ± 0.04
Aprosulate	2.09 μmol/kg	1 hr.	0.16 ± 0.01*
	(5.0 mg/kg)	2 hrs.	0.20 ± 0.02*
		4 hrs.	0.19 ± 0.02*
		6 hrs.	0.13 ± 0.01
GL-522-Y-1	3.36 μmol/kg	3 hrs.	0.90 ± 0.38*
	(5.0 mg/kg)	4.5 hrs.	0.42 ± 0.16*
		6 hrs.	0.06 ± 0.02
Heparin	0.48 μmol/kg	3 hrs.	0.21 ± 0.02*
	(5.0 mg/kg)	6 hrs.	0.14 ± 0.01

All results represent the mean ± S.E.M. blood cell loss (x 10^9/liter) in five rabbits per treatment group. Each agent was administered subcutaneously in the abdominal region. After varying absorption times, five standardized incisions were made in the non-vascular portion of the right ear. Blood cells were collected for 10 minutes. Blood cells were quantitated using a hemocytometer. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ *vs.* control was considered to be statistically significant.

TABLE 57

EFFECT OF INTRAVENOUS ADMINISTRATION OF HEPARIN ANALOGUES ON THE CELITE ACT IN RABBITS

	Dose	n	Fold Increase
Aprosulate	100 μg/kg	5	1.01 ± 0.04
	250 μg/kg	5	1.05 ± 0.02
	500 μg/kg	4	1.12 ± 0.12
GL-522-Y-1	1.0 mg/kg	5	1.11 ± 0.03*
	2.5 mg/kg	5	1.15 ± 0.02*
	5.0 mg/kg	4	1.21 ± 0.02*
Heparin	6.25 μg/kg	5	1.00 ± 0.01
	12.5 μg/kg	4	1.03 ± 0.02
	25.0 μg/kg	3	1.03 ± 0.03
	50.0 μg/kg	5	0.98 ± 0.03
Pentasaccharide	12.5 μg/kg	4	1.00 ± 0.01
	25.0 μg/kg	5	1.02 ± 0.02
	50.0 μg/kg	5	1.08 ± 0.03
	100.0 μg/kg	5	1.05 ± 0.02

Celite activated clotting times were determined at baseline and at 5 minutes post-administration of the test agent to New Zealand white rabbits. Fold increases were calculated relative to individual baselines. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ *vs* saline treatment was considered statistically significant.

TABLE 58

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF HEPARIN ANALOGUES ON THE CELITE ACT IN RABBITS

	Dose	n	Fold Increase
GL-522-Y-1	10 mg/kg	5	1.06 ± 0.02
	20 mg/kg	4	1.15 ± 0.06
	30 mg/kg	5	1.21 ± 0.09
Heparin	250 μg/kg	3	1.15 ± 0.09
	500 μg/kg	5	1.16 ± 0.04*
	1000 μg/kg	5	1.14 ± 0.02
Pentasaccharide	125 μg/kg	5	1.06 ± 0.04
	250 μg/kg	5	1.06 ± 0.04
	500 μg/kg	4	1.12 ± 0.03

Celite activated clotting times were determined at baseline and at 2 hours post-administration of the test agent to New Zealand white rabbits. Fold increases were calculated relative to individual baselines. Statistical comparisons were made using one way ANOVA followed by the Newman-Keuls test. $^{*}p < 0.05$ *vs* saline treatment was considered statistically significant.

TABLE 59

EFFECT OF INTRAVENOUS ADMINISTRATION OF HEPARIN ANALOGUES ON THE R-TIME.

	Dose	n	R-time (fold increase)
Saline		5	1.05 ± 0.22
Aprosulate	100 μg/kg	5	0.78 ± 0.02
	250 μg/kg	4	1.54 ± 0.35
	500 μg/kg	5	3.97 ± 1.92
GL-522-Y-1	0.1 mg/kg	5	0.89 ± 0.49
	1.0 mg/kg	4	1.34 ± 0.23
	2.5 mg/kg	5	1.82 ± 0.29
	5.0 mg/kg	5	2.33 ± 0.71
Heparin	6.25 μg/kg	5	0.81 ± 0.18
	12.5 μg/kg	3	1.35 ± 0.39
	25.0 μg/kg	4	1.21 ± 0.21
	50.0 μg/kg	5	1.22 ± 0.30
Pentasaccharide	25.0 μg/kg	5	1.92 ± 0.54
	50.0 μg/kg	3	1.46 ± 0.33
	100.0 μg/kg	5	1.35 ± 0.22

TEG analysis of whole rabbit blood was made at baseline and after administration of the test agent. Fold increase was calculated relative to each rabbit's baseline. Statistical significance was assessed for each agent using one way ANOVA followed by the Newman-Keuls multiple comparison test. All p values are for treatment versus saline treated control animals.

Aprosulate : $p = 0.146$

GL-52-Y-1 : $p = 0.168$

Heparin : $p = 0.636$

Pentasaccharide : $p = 0.388$

TABLE 60

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF HEPARIN ANALOGUES ON THE R-TIME IN RABBITS

	Dose	n	R-time (Fold Increase)
Saline		5	1.05 ± 0.22
GL-522-Y-1	10 mg/kg	4	1.11 ± 0.26
	20 mg/kg	4	1.40 ± 0.68
	30 mg/kg	5	1.42 ± 0.26
Heparin	250 μg/kg	4	1.60 ± 0.39
	500 μg/kg	5	1.12 ± 0.21
	1000 μg/kg	5	2.52 ± 0.75
Pentasaccharide	125 μg/kg	3	1.31 ± 0.11
	250 μg/kg	5	1.18 ± 0.19
	500 μg/kg	4	1.58 ± 0.10

TEG analysis of whole rabbit blood was made at baseline and after administration of the test agent. Fold increase was calculated relative to each rabbit's baseline. Statistical significance was assessed for each agent using one way ANOVA followed by the Newman-Keuls multiple comparison test. All p values are for treatment versus saline treated control animals.

GL-52-Y-1 : $p = 0.842$

Heparin : $p = 0.113$

Pentasaccharide : $p = 251$

TABLE 61

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE PT ASSAY

	Dose (μg/kg)	Clotting time (fold increase)
Saline	---	1.04 ± 0.10
Aprosulate	100	0.93 ± 0.05
	250	0.86 ± 0.02
	500	1.06 ± 0.02
GL-522-Y-1	1000	1.13 ± 0.17
	2500	1.05 ± 0.03
	5000	1.15 ± 0.06
Heparin	12.5	0.97 ± 0.03
	25.0	0.96 ± 0.02
	50.0	1.03 ± 0.06
Pentasaccharide	25.0	1.04 ± 0.03
	50.0	1.03 ± 0.05
	100.0	1.02 ± 0.02

All treatments were administered intravenously 5 minutes prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA:

aprosulate; $p = 0.082$
GL-522-Y-1; $p = 0.836$
Heparin; $p = 0.723$
Pentasaccharide; $p = 0.994$

TABLE 62

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE APTT ASSAY

	Dose (μg/kg)	Clotting time (fold increase)
Saline	---	0.97 ± 0.04
Aprosulate	100	1.16 ± 0.06*
	250	1.20 ± 0.03*
	500	1.51 ± 0.06*
GL-522-Y-1	1000	1.36 ± 0.37
	2500	1.04 ± 0.17
	5000	1.00 ± 0.07
Heparin	12.5	1.28 ± 0.09
	25.0	1.03 ± 0.15
	50.0	1.19 ± 0.16
Pentasaccharide	25.0	1.15 ± 0.06
	50.0	1.22 ± 0.12
	100.0	1.10 ± 0.09

All treatments were administered intravenously 5 minutes prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. *$p < 0.05$ was considered statistically significant.

By ANOVA:

aprosulate; $p < 0.001$
GL-522-Y-1; $p = 0.534$
Heparin; $p = 0.277$
Pentasaccharide; $p = 0.227$

TABLE 63

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE HEPTEST ASSAY

	Dose (μg/kg)	Clotting time (fold increase)
Saline	---	0.89 ± 0.06
Aprosulate	100	1.60 ± 0.26
	250	1.30 ± 0.26
	500	1.35 ± 0.11
GL-522-Y-1	1000	1.13 ± 0.11
	2500	1.12 ± 0.02
	5000	1.38 ± 0.20
Heparin	12.5	1.13 ± 0.12
	25.0	1.05 ± 0.04
	50.0	1.42 ± 0.16*
Pentasaccharide	25.0	1.90 ± 0.31*
	50.0	2.38 ± 0.25*
	100.0	2.72 ± 0.09*

All treatments were administered intravenously 5 minutes prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. *$p < 0.05$ was considered statistically significant.

By ANOVA:

aprosulate; $p = 0.117$
GL-522-Y-1; $p = 0.070$
Heparin; $p = 0.020$
Pentasaccharide; $p < 0.001$

TABLE 64

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE 2.5 U TT ASSAY

	Dose (μg/kg)	Clotting time (fold increase)
Saline	---	1.14 ± 0.08
Aprosulate	100	1.17 ± 0.04
	250	1.38 ± 0.22
	500	2.57 ± 0.67
GL-522-Y-1	1000	1.50 ± 0.14
	2500	1.84 ± 0.34
	5000	1.86 ± 0.70
Heparin	12.5	1.46 ± 0.03
	25.0	2.80 ± 0.66*
	50.0	3.18 ± 0.15*
Pentasaccharide	25.0	1.19 ± 0.14
	50.0	1.14 ± 0.16
	100.0	1.21 ± 0.09

All treatments were administered intravenously 5 minutes prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. $^*p < 0.05$ was considered statistically significant.

By ANOVA:

aprosulate; $p = 0.036$
GL-522-Y-1; $p = 0.549$
Heparin; $p = 0.001$
Pentasaccharide; $p = 0.967$

TABLE 65

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE PT ASSAY

	Dose (mg/kg)	Clotting time (fold Increase)
Saline	---	1.04 ± 0.10
GL-522-Y-1	10.0	1.09 ± 0.05
	20.0	1.04 ± 0.03
	30.0	1.04 ± 0.03
Heparin	0.25	1.02 ± 0.04
	0.50	0.96 ± 0.08
	1.00	1.14 ± 0.06
Pentasaccharide	0.125	1.13 ± 0.11
	0.250	1.07 ± 0.09
	0.500	1.23 ± 0.13

All treatments were administered subcutaneously 2 hours prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA:

GL-522-Y-1; $p = 0.912$
Heparin; $p = 0.403$
Pentasaccharide; $p = 0.626$

TABLE 66

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE APTT ASSAY

	Dose (mg/kg)	Clotting time (fold Increase)
Saline	---	0.97 ± 0.04
GL-522-Y-1	10.0	1.00 ± 0.13
	20.0	0.79 ± 0.14
	30.0	1.27 ± 0.19
Heparin	0.25	1.14 ± 0.12
	0.50	1.21 ± 0.11
	1.00	1.34 ± 0.36
Pentasaccharide	0.125	1.30 ± 0.20
	0.250	1.24 ± 0.21
	0.500	1.20 ± 0.09

All treatments were administered subcutaneously 2 hours prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. $^{*}p < 0.05$ was considered statistically significant.

By ANOVA:

GL-522-Y-1; $p = 0.139$
Heparin; $p = 0.623$
Pentasaccharide; $p = 0.468$

TABLE 67

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE HEPTEST ASSAY

	Dose (mg/kg)	Clotting time (fold Increase)
Saline	---	0.89 ± 0.06
GL-522-Y-1	10.0	1.27 ± 0.13
	20.0	1.15 ± 0.10
	30.0	1.46 ± 0.18*
Heparin	0.25	1.07 ± 0.03
	0.50	1.42 ± 0.10
	1.00	2.01 ± 0.33*
Pentasaccharide	0.125	1.85 ± 0.32
	0.250	2.05 ± 0.34
	0.500	3.25 ± 0.45*

All treatments were administered subcutaneously 2 hours prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. *$p < 0.05$ was considered statistically significant.

By ANOVA:

GL-522-Y-1; $p = 0.036$
Heparin; $p = 0.002$
Pentasaccharide; $p = 0.001$

TABLE 68

EX VIVO ANTICOAGULANT ACTIVITY MEASURED IN RABBIT SAMPLES USING THE 2.5 U TT ASSAY

	Dose (mg/kg)	Clotting time (fold Increase)
Saline	---	1.14 ± 0.08
GL-522-Y-1	10.0	1.14 ± 0.07
	20.0	1.18 ± 0.28
	30.0	0.79 ± 0.10
Heparin	0.25	1.58 ± 0.15
	0.50	3.11 ± 0.85
	1.00	5.31 ± 1.00*
Pentasaccharide	0.125	1.16 ± 0.23
	0.250	1.37 ± 0.15
	0.500	1.25 ± 0.17

All treatments were administered subcutaneously 2 hours prior to administration of the thrombogenic challenge. Blood samples were drawn at baseline and immediately before administration of the thrombogenic challenge. All results represent the mean ± SEM of 5 rabbits. Statistical comparisons were made using one way ANOVA followed by the Newman Keuls test. $^*p < 0.05$ was considered statistically significant.

By ANOVA:

GL-522-Y-1; $p = 0.297$
Heparin; $p = 0.002$
Pentasaccharide; $p = 0.757$

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The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March 31/1996
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