New Antithrombin-Based Anticoagulants

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DOI 10.1002/med.10058

Abstract: Clinically used anticoagulants are inhibitors of enzymes involved in the coagulation pathway, primarily thrombin and factor Xa. These agents can be either direct or indirect inhibitors of clotting enzymes. Heparin-based anticoagulants are indirect inhibitors that enhance the proteinase inhibitory activity of a natural anticoagulant, antithrombin. Despite its phenomenal success, current anticoagulation therapy suffers from the risk of serious bleeding. The need for safer and more effective antithrombotic agents clearly exists. The past decade has seen enormous effort directed toward discovering and/or designing new molecules with anticoagulant activity. These new molecules can be classified into (a) antithrombin and its mutants, (b) natural polysaccharides, (c) synthetic modified heparins and heparin-mimics, (d) synthetic oligosaccharides, and (e) synthetic non-sugar antithrombin activators. This review focuses on these efforts in designing or discovering new molecules that act through the antithrombin pathway of anticoagulation.
 Med Res Rev, 24, No. 2, 151–181, 2004

Key words: antithrombin; heparin; anticogulants; heparin mimics

1. INTRODUCTION

Clotting is a defense mechanism preventing excessive loss of blood and ingestion of microbes. Yet, inadvertent formation and dislocation of clots may be harmful. Antithrombotic drugs prevent the formation and growth of clots. Clot formation is a co-ordinated interplay of two fundamental processes, aggregation of platelets and formation of fibrin. Platelet aggregation involves association of platelets through physical forces following their activation (Fig. 1A), whereas fibrin formation involves the chemical synthesis of fibrin polypeptide through the action of several enzymes and co-factors (Fig. 1B). Thus, there are two classes of antithrombotic agents—antiplatelets and

Contract grant sponsor: American Heart Association–Mid-Atlantic Affiliate; *Contract grant number:* 0256286U; *Contract grant sponsor:* National Institutes of Health; *Contract grant number:* HL69975; *Contract grant sponsor:* A.D. Williams Funds
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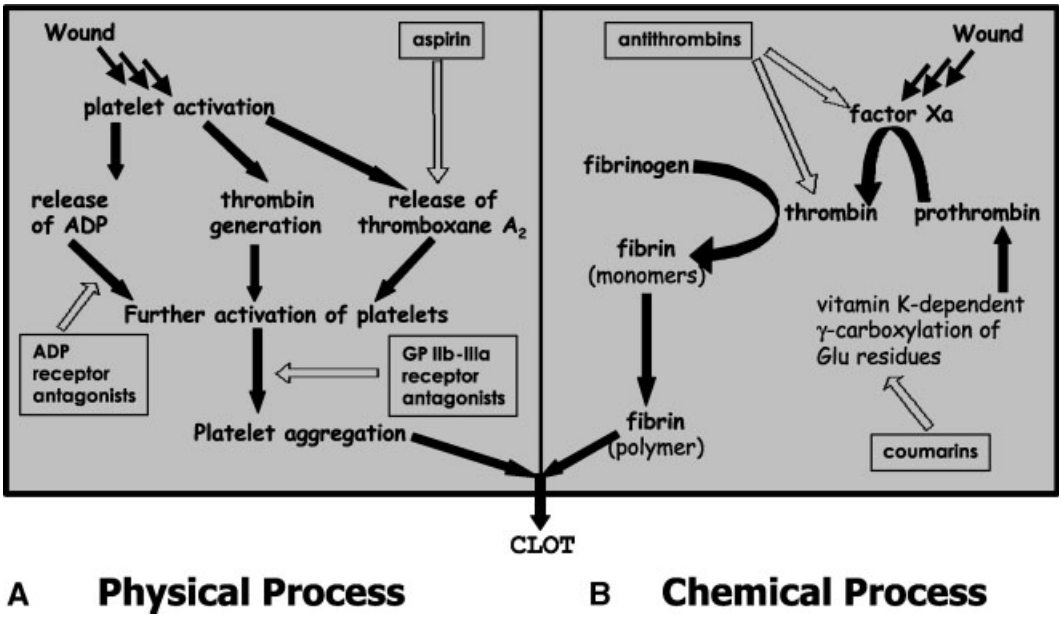

Figure 1. Flowchart showing the key elements of physical (**A**) and chemical (**B**) processes leading to clot formation. Both processes are more complex than depicted here. Further, the two processes are not completely independent as shown here. There is significant cross-talk between the two, exemplified by thrombin that is significantly involved in the activation of platelets. Inhibitors of clotting are shown in boxed text with arrows indicating their site of action.

anticoagulants. Antiplatelet molecules block the physical process (traditionally referred as the cellular process), whereas anticoagulants inhibit the chemical process (also referred as the humoral process).

Several antiplatelet drugs have been clinically tested. These include an inhibitor of synthesis of thromboxane A2—aspirin,[1] glycoprotein IIb/IIIa receptor antagonists—abciximab, tirofiban, eptifibatide, and inhibitors of ADP—ticlopidine and clopidogrel[2] (Fig. 1A). Although the effectiveness of aspirin alone is limited because it blocks only one pathway of platelet aggregation, it is hailed as a wonder drug in reducing the risk of fatal heart attack. Synergistic action of aspirin and glycoprotein IIb-IIIa antagonists is expected to be more effective.

Anticoagulants include direct and indirect inhibitors of enzymes involved in the coagulation pathways, primarily thrombin and factor Xa (Fig. 1B). Direct inhibitors interact with the procoagulant enzyme's active site or an exosite blocking its proteinase activity. Direct inhibitors of coagulation being clinically tested include thrombin inhibitors, hirudin, bivalirudin, argatroban, efegatran and inogratran, and factor Xa inhibitors, tick anticoagulant peptide (TAP), antistasin, and DX-9065a.[3–5] In contrast, indirect inhibitors enhance the proteinase inhibitory activity of natural anticoagulants, antithrombin, and heparin co-factor II. Indirect inhibitors currently available include unfractionated heparin (UFH), low-molecular weight heparins (LMWHs), and heparin pentasaccharide SR90107a.[6–9] Another major category of anticoagulant that work in an indirect manner include the coumarins and 1,3-indanediones, which prevent the biosynthesis of active forms of procoagulant proteinases (Fig. 1B).

Despite its phenomenal success, current anticoagulation therapy still suffers from the risk toward serious bleeding.[10,11] A direct correlation exists between the intensity of anticoagulation and severity of bleeding. In addition, each anticoagulant is associated with additional problems specific to its class. The need for safer and more effective antithrombotic agents clearly exists. This review focuses on

recent efforts in designing or discovering molecules that act through the antithrombin pathway of anticoagulation.

2. ANTITHROMBIN

Antithrombin is a major regulator of blood clotting. Antithrombin is a plasma protein (MW ~58,200) that inactivates a number of proteinases of the coagulation cascade, especially thrombin and factor Xa.[12] Although the rates of antithrombin inhibition of these enzymes are relatively slow under physiological conditions, the high plasma concentration (~2.3 μM) of the inhibitor coupled with its interaction with cell-surface polysaccharide species, such as heparan sulfate, result in rapid inactivation of the procoagulant proteinases.[13] This is a major mechanism for normal hemostasis. Homozygous antithrombin knockout appears to be incompatible with life, whereas heterozygous mutations can produce variants with functional defects.[14,15]

Antithrombin is a glycoprotein with 432 residues. The major form α-antithrombin circulating in plasma has four Asn residues glycosylated, whereas in the minor form (β-antithrombin) Asn135 is not glycosylated. Recombinant antithrombin can be expressed in a number of cell systems, including baculovirus infected insect cells and mammalian cells, with properties similar to the plasma protein. Antithrombin (AT) is also a member of the serine proteinase inhibitors (serpin) superfamily of proteins. Thus, it shows structural and functional similarity with homologous serpin members including α_1-proteinase inhibitor, heparin co-factor II, and plasminogen activator inhibitor-I.[16,17]

Several crystal structures of antithrombin are available. The structures of intact, uncleaved free antithrombin show 9 α-helices surrounding 3 β-sheets.[18–20] Of these secondary structures, two features are striking—a dominant five-stranded β-sheet A approximately in the center of the inhibitor and an exposed 15-residue sequence containing the reactive bond Arg393-Ser394, the so-called reactive center loop (RCL) at the ''top'' of the molecule (Fig. 2A). These features are common to all serpins.[16,17,21,22] Antithrombin shows, in addition, a unique feature known to be present in only one other serpin to-date, heparin co-factor II.[23] Two residues, P15-P14 (Gly379-Ser380) at the N-terminal end of the reactive center loop, are inserted as a short β-strand in-between strands 3 and 4 of β-sheet A in the inhibitor (Fig. 2A). This feature is called the partial insertion of RCL and undergoes some major changes during the process of proteinase inhibition (see below).

The structure of antithrombin cleaved at the reactive bond is very similar to intact antithrombin except for the complete insertion of RCL as strand 4a in β-sheet A (Fig. 2B). This structural change results in the movement of the P1 residue[a] from the ''top'' of the molecule to the ''bottom,'' a distance of approximately 70 Å. This dramatic conformational change following cleavage by the target enzyme leads to significant thermodynamic stabilization of the molecule.[25,26] Recent crystal structure of the α_1-proteinase inhibitor–trypsin complex[27] and biochemical results[28,29] indicate that the dramatic structural change following cleavage of the P1-P1′ bond is critical for the disruption of the catalytic triad of the proteinase, thereby resulting in the inactivation of the enzyme.

Antithrombin inactivation of thrombin and factor Xa proceeds in similar manner.[30] The mechanism is referred to as the serpin 'mousetrap' mechanism in which antithrombin, the inhibitor (**AT**), acts as bait to trap the target enzyme (**E**) in an equimolar, covalent, inactive complex (**E*-AT***) (Fig. 3). The RCL first interacts with the active site of the proteinase as in a normal substrate reaction to form a Michaelis complex (**E:AT**). This is rapidly followed by cleavage of the scissile bond P1-P1′ in the RCL to form an acyl-enzyme intermediate (**E-AT**), which undergoes a major rearrangement to disrupt the enzyme's catalytic triad[27–30] resulting in inhibition (**E*-AT***) (Inhibition Pathway, Fig. 3). A competing process, called the substrate pathway (Fig. 3), may operate in parallel and diminish the

[a]Numbering is that of Schechter and Berger[24] in which the P1-P1′ bond is cleaved and residues are numbered P2, P3 ... toward the amino terminus and P2′, P3′ ... toward to carboxyl terminus.

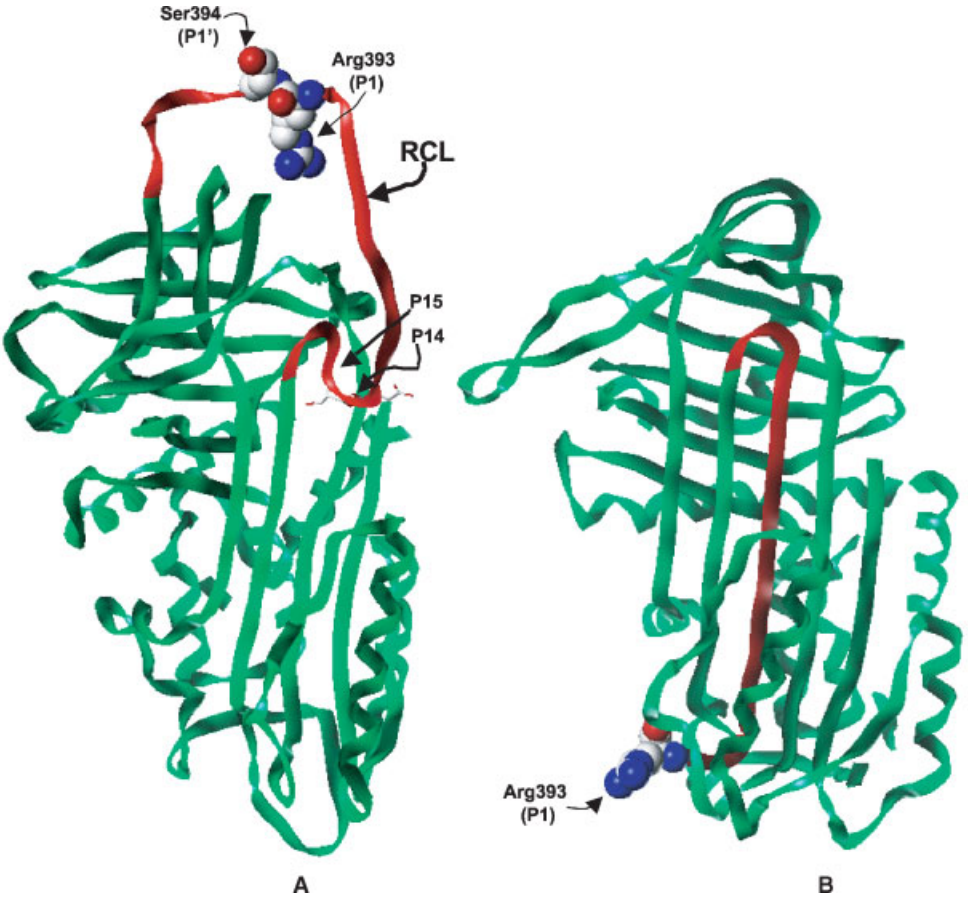

Figure 2. Ribbon diagram of native (**A**) and cleaved (**B**) plasma antithrombin. The structure of plasma antithrombin was obtained from PBD (file name '1ath'). The RCL is shown in red. Reactive bond residues Arg393 (P1)–Ser394 (P1′) is shown are space-fill representation, whereas P14-P15 residues (Glu381-Gly380) are shown in capped sticks form. The P1 residue, in an exposed orientation in the native structure, moves from the 'top' of the molecule to the 'bottom' following cleavage with a proteinase. The proteinase, e.g., factor Xa, moves with the P1 residue to the bottom of the inhibitor and gets covalently trapped. RCL is inserted as strand 4 in 6-stranded β-sheet A.

efficacy of inhibition. In the substrate pathway, structural perturbations in antithrombin, e.g., mutational changes, may facilitate rapid hydrolysis of the acyl enzyme intermediate **E-AT** to yield an active enzyme (**E**) and a cleaved inhibitor ($\mathbf{AT_C}$). Under normal experimental conditions, inhibition of factor Xa and thrombin by plasma antithrombin has no contribution from the substrate pathway. Thus, one molecule of antithrombin inactivates one molecule of the enzyme to give an inhibition stoichiometry of 1. (Under physiological conditions, approximately one-third of antithrombin reacts through the substrate pathway because of the bound heparin slowing the rate of the conformational change.) However, alterations in the structure of the inhibitor, e.g., introduction of mutations, may enhance the contribution of the substrate pathway, thereby increase the inhibition stoichiometry significantly. Such antithrombins may not function well as anticoagulants.

3. RATES OF ANTITHROMBIN INHIBITION OF FACTOR Xa/THROMBIN

Although most serpins inhibit their target enzymes with a second-order rate constant limited only by diffusion ($k_{INH} = 10^6$–$10^7\ M^{-1}\ sec^{-1}$),[16,17] the antithrombin inhibition of factor Xa and thrombin is

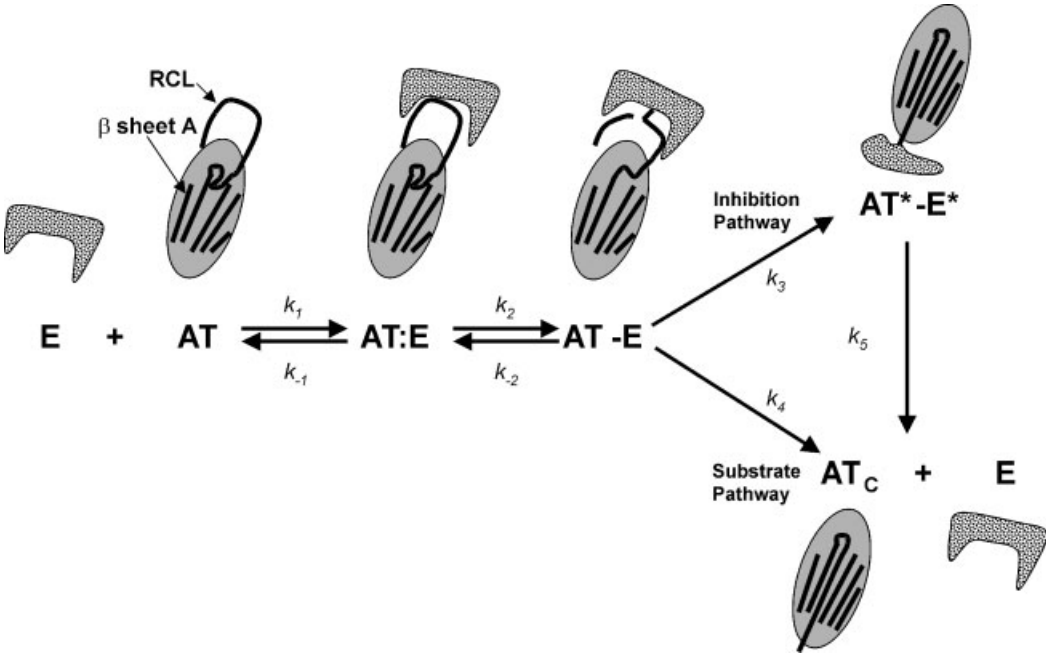

Figure 3. A model of the serpin 'mousetrap' mechanism of inhibition. Formation of a Michaelis complex (**AT:E**) is rapidly followed by cleavage of the scissile bond P1-P1′ (Arg393-Ser394) in the RCL of antithrombin to give an acyl-enzyme intermediate (**E-AT**). This serves as a bifurcating point for two competing processes, either inhibition or substrate reaction. Inhibition pathway traps the enzyme in a covalent form **E*-AT***, whereas substrate pathway releases an active enzyme and a cleaved inhibitor. For plasma antithrombin, the inhibition pathway dominates. However, natural or engineered mutations in inhibitor may invoke greater proportion of the substrate pathway. When substrate pathway contribution increases, the stoichiometry of inhibition (SI), the number of molecule of inhibitor needed to inactivate one molecule of the enzyme, increases rapidly from 1; **E**, target enzyme; **AT**, antithrombin; **E:AT**, Michaelis-Menten complex; **E-AT**, acyl-enzyme intermediate; **E*-AT***, antithrombin-enzyme complex; $\mathbf{AT_C}$ cleaved antithrombin; **RCL**, reactive center loop. Microscopic rate constants for individual steps are depicted using the letter 'k.'

much slower. Typical uncatalyzed *in vitro* thrombin inhibition rates at pH 7.4 and 25°C lie in the range of $7-11 \times 10^3\ M^{-1}\ sec^{-1}$,[31–33] whereas those for factor Xa are $2-3 \times 10^3\ M^{-1}\ sec^{-1}$.[34] Thus, the uncatalyzed reaction of antithrombin does not contribute much under physiological conditions. The reason for the slow inhibition appears to originate from the structure of RCL in native antithrombin. Partial insertion of the RCL forces P1 and other important residues to adopt a conformation that is not conducive for rapid reaction with either factor Xa or thrombin.[35] Recently, mutation studies on several RCL residues suggested that proteinase specificity of antithrombin is encoded in an exosite that lies outside the RCL,[36] a result that was supported by steady-state and time-resolved fluorescence measurements on the conformational equilibrium of RCL.[37]

The slow rates of factor Xa and thrombin inhibition are dramatically increased in the presence of heparin, a natural linear sulfated polysaccharide (Fig. 4). The second-order rate constant for thrombin inhibition by antithrombin–heparin complex lies in the range of $1-4 \times 10^7\ M^{-1}\ sec^{-1}$ representing an acceleration of more than 2,000-fold, whereas that for factor Xa inhibition reaches $1.5 \times 10^6\ M^{-1}\ sec^{-1}$ representing an increase of some 600-fold.[38–41] Under physiological conditions, the acceleration in factor Xa inhibition may be even higher reaching some 2,400-fold because of the presence of calcium ions.[42] This enhancement in inhibition of thrombin and factor Xa forms the basis for heparin's clinical use as an anticoagulant. Only a small proportion (~33%) of chains in commercial heparin preparations bind antithrombin with high affinity. These heparin chains, called

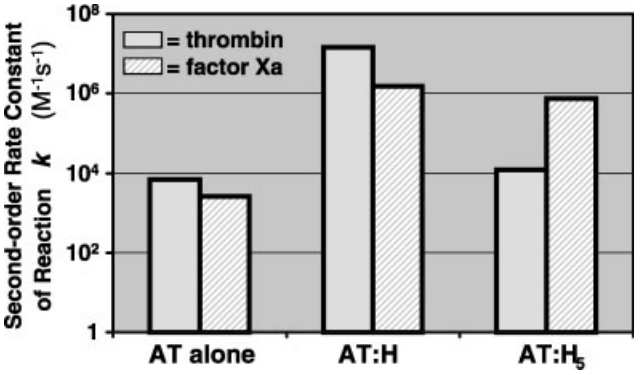

Figure 4. Comparison of second-order rate constants for antithrombin (AT) inhibition of thrombin (▭) and factor Xa (▨) in the presence of heparin (H) and heparin pentasaccharide (H_5). Heparin enhances ~500–2,000-fold antithrombin inhibition of both thrombin and factor Xa (~10^3–10^4 M^{-1} sec^{-1}). Likewise, pentasaccharide accelerates ~300-fold the inhibition of factor Xa, but only ~twofold the inhibition of thrombin. Note the logarithmic scale of y-axis.

high-affinity heparin (HAH), contain a specific sequence of five residues that contribute nearly 95% of antithrombin binding energy.[41] The five-residue sequence is called heparin pentasaccharide **H_5**. Pentasaccharide **H_5** accelerates antithrombin inhibition of factor Xa nearly 300-fold, only twofold less than full-length heparin, whereas it accelerates thrombin inhibition only 1.7-fold (Fig. 4). Thus, **H_5** selectively inactivates factor Xa in contrast to heparin.

4. MECHANISM OF HEPARIN ACTIVATION OF ANTITHROMBIN

Nature has engineered two distinct mechanisms for heparin-activated antithrombin inhibition of factor Xa and thrombin. The interaction of high-affinity heparin, or pentasaccharide **H_5**, expels the partially inserted RCL residues thereby significantly changing the conformation of the P1-P1′ reactive center and exposing an exosite in antithrombin (Fig. 5).[30,34,36,40,41] This phenomenon is called the conformational activation of antithrombin. The altered RCL in heparin-antithrombin co-complex is better recognized by factor Xa resulting in accelerated cleavage of the P1-P1′ bond and rapid formation of the covalent inhibited complex (**E*-AT*** in Fig. 2). Thus, conformational activation of antithrombin is necessary and sufficient for accelerated factor Xa inhibition.

Thrombin inhibition, in contrast, is accelerated only twofold through the conformational activation mechanism (see Fig. 4).[41] The predominant effect of heparin in accelerating thrombin inhibition arises from a bridging mechanism. Tight binding of antithrombin to the **H_5** sequence in full-length heparin is followed by the binding of thrombin to the same chain at non-specific sites to form an antithrombin–heparin–thrombin ternary complex (Fig. 5). Thrombin then diffuses along the polyanionic chain to encounter the inhibitor resulting in a ~2,000-fold acceleration in inhibition under physiological conditions. A saccharide length of ~18 residues is needed to simultaneously hold thrombin and antithrombin for the accelerated inhibition.[43,44] Thus, while sequence-specific **H_5** is necessary for tight binding of heparin chains, **H_5** alone cannot potentiate antithrombin inhibition of thrombin.

Recent investigations suggest that such a template mechanism may also play an important role *in vivo* for antithrombin inhibition of factor Xa. In the presence of physiological concentrations of Ca^{2+}, full-length heparin was found to accelerate the inhibition of factor Xa some 40-fold[42] suggesting that longer chains bind to an exosite on the enzyme. Likewise, a bell-shaped dependence of acceleration on the concentration of heparin chains, characteristic of a bridging mechanism, was observed.

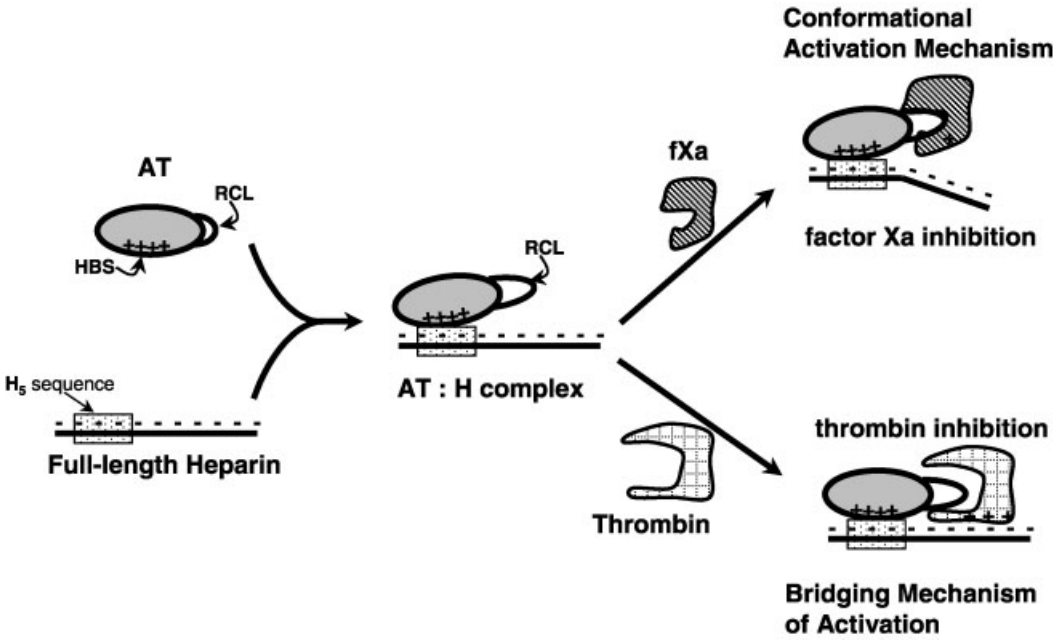

Figure 5. Mechanism of antithrombin inhibition of factor Xa and thrombin in the presence of full-length heparin. An antithrombin–heparin complex (AT:H) is formed following an interaction of the high-affinity pentasaccharide sequence (H_5) in heparin with the pentasaccharide-binding site (PBS). This causes expulsion of the reactive center loop (RCL) that better recognizes factor Xa (fXa). This mechanism is called the conformational activation mechanism. Thrombin inhibition, in addition to conformational activation, requires the bridging of thrombin and antithrombin on a full-length heparin chain. An exosite (shown as '+ + +') in thrombin binds non-specifically to some negative charges available at the extended heparin chain. This mechanism is called the bridging mechanism of activation and is the dominant contributor to inactivation of thrombin.

5. HEPARIN BINDING SITE AND REACTIVE CENTER LOOP IN ANTITHROMBIN

The heparin binding site in antithrombin, located some 20 Å away from the reactive center loop, is an engineering marvel. This binding site specifically recognizes $\mathbf{H_5}$ with high-affinity, yet is capable of binding to numerous structurally different polysaccharide chains of heparin that contain the pentasaccharide. The heparin-binding domain in antithrombin is formed by positively charged residues of helices A and D, and the polypeptide N-terminus. The crystal structure of antithrombin-pentasaccharide co-complex shows that residues Arg47, Lys114, Lys125, and Arg129 in this region interact with $\mathbf{H_5}$.[45] This conclusion is also supported by biochemical studies with antithrombin mutants.[46–50] The domain formed by Arg47, Lys114, Lys125, and Arg129 is called the pentasaccharide-binding site (PBS, Fig. 6). In addition to interacting with PBS, full-length heparin binds to an extended region formed by residues Arg132, Lys133, and Arg136 at the C-terminal end of helix D. This extended region is designated as the extended heparin-binding site (EHBS).[51] The interaction of $\mathbf{H_5}$ (or full-length heparin) with PBS results in transmission of binding energy for conformational change in the RCL,[45,52] suggesting that heparin activation of antithrombin is an allosteric phenomenon.

It is important to recognize that the allosteric activation phenomenon involves the conformational changes at both ends—at the RCL end as well as at the heparin-binding site end. At a molecular level, following heparin binding at least three changes occur in the HBS. One, a short 3_{10} P helix is formed at

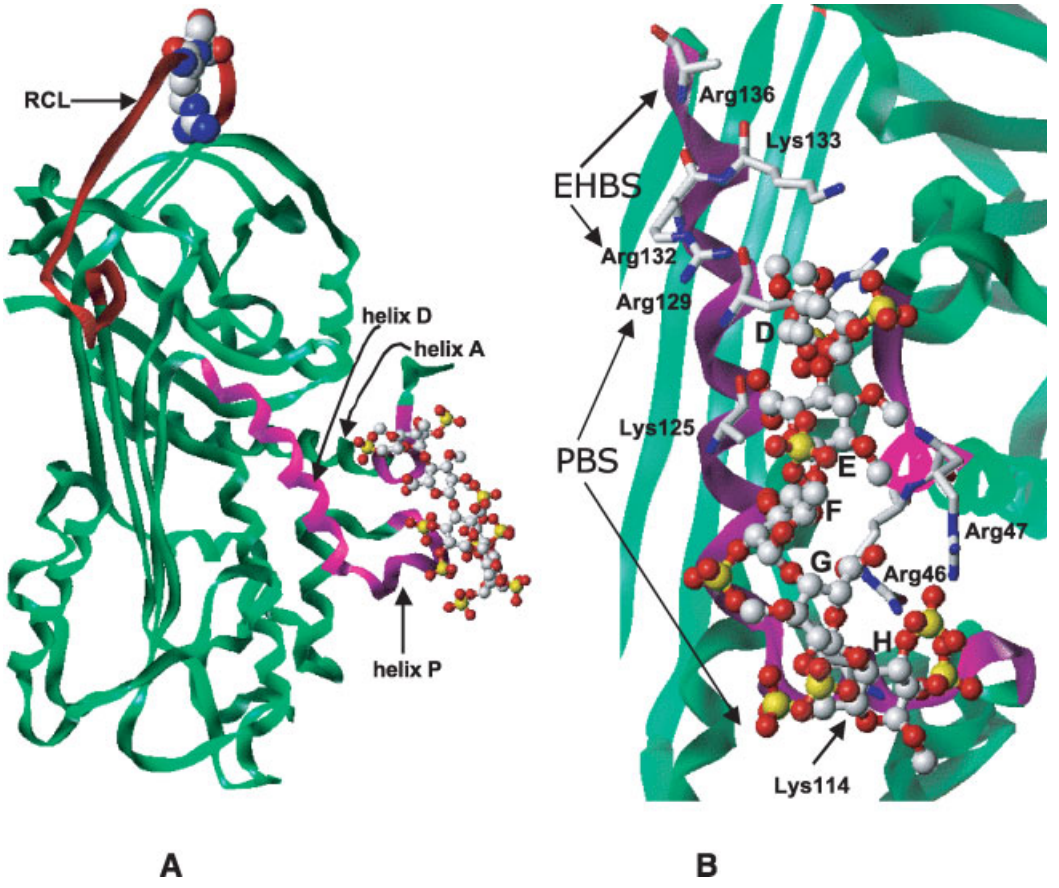

Figure 6. Ribbon diagram of plasma antithrombin complexed to natural pentasaccharide DEFGH (**A**) and a close-up view of the heparin-binding site (**B**). The structure of co-complex was obtained from PDB (filename '1e03'). In both figures, green ribbon shows antithrombin, red represents RCL, and magenta represents the heparin-binding site. Pentasaccharide DEFGH is shown in ball-and-stick representation and individual residues are marked in B). Helices D, P, and A (C-terminal end) form the heparin-binding site. Helix D extends by 1.5 turns in the complex as compared to free antithrombin (Fig. 2). In addition, helix P is not present in uncomplexed antithrombin. Arg46, Arg47, Lys114 (hidden in B)), Lys125, and Arg129 form the pentasaccharide binding site (PBS), whereas Lys132, Arg133, and Lys136 form the extended heparin-binding site (EHBS).

the N-terminal end of helix D. Two, a small kink present in helix D before heparin binding is straightened out and three, helix D is extended by 1.5 turns.[45,53] These changes in the heparin-binding site in turn affect the conformation of RCL and possibly other regions. Details regarding the molecular mechanism of transmission of heparin binding energy to the RCL remain unclear at the present time.

The P1-P1′ bond in the RCL of antithrombin is Arg393-Ser394. The P1 residue is a key residue for target proteinase recognition and is the reason for antithrombin's inhibition of several coagulation proteinases.[12,54] Because antithrombin inhibits several other procoagulant enzymes, the effect of heparin is also felt at the reactions catalyzed by them. Whereas such activities are good for rapid termination of a coagulation signal, it may also lead to bleeding complications. The Ser394 residue does not seem to be as important. Although the primary specificity of antithrombin arises from the P1 residue, other residues are likely to be important. Although wild-type P2 residue is glycine, its replacement with a proline residue apparently enhances recognition of thrombin.[55] Extensive work is progressing toward identifying the critical determinants of specificity in antithrombin.

6. CLASSES OF ANTICOAGULANTS

Although heparin has been the most successful antithrombin-based anticoagulant to-date, it is by no means the only molecule researched. The past decade has seen enormous effort directed toward discovering and/or designing new molecules with anticoagulant activity. These new molecules can be classified into (a) antithrombin and its mutants, (b) natural polysaccharides, (c) synthetic modified heparins and heparin-mimics, (d) synthetic oligosaccharides, and (e) synthetic non-sugar antithrombin activators. This classification is primarily based on the structure of molecules and does not represent an iron-clad segregation between the classes. Some overlap remains unavoidable.

7. ANTITHROMBIN MUTANTS

A simple approach to achieve higher rates for inhibition of factor Xa and thrombin would be to engineer an antithrombin mutant in which the RCL and other important domains have assumed a structure necessary for enhanced recognition of factor Xa and thrombin. β-Antithrombin, a minor form present in plasma, shows a 5-fold higher heparin affinity and a 2-fold better factor Xa inhibition (k_{UNCAT}) than the α-form.[56] Thrombin inhibition was indistinguishable for the two forms. This variant of plasma antithrombin lacks a carbohydrate chain at the Asn135 position, which is within the heparin-binding site (see below). Similar profile was also noted for recombinant wild-type antithrombin prepared in BHK or CHO cell lines in which the carbohydrate chains were enzymatically removed[57,58] as well as for antithrombin mutants in which Asn135 was mutated to either Ala or Gln.[46,54]

The slightly higher factor Xa inhibition rate for these antithrombin variants most probably originates from a change in the structure of RCL induced by small changes in the heparin-binding domain, although it is possible that presence of the carbohydrate chain at 135-position hinders the conformational change.[59] In other words, the 'native' ↔ 'activated' conformational equilibrium may be affected by removal of *N*-glycosylation at 135-position. This observation that a structural change introduced in HBS could beneficially affect the RCL has not exploited further.

A second region open to engineering for inducing enhanced rates of factor Xa and thrombin inhibition is the RCL sequence. Considerable evidence indicates that the conformation of RCL in antithrombin is likely to be constrained. This is borne out by a comparative analysis of the loops of antithrombin and a closely related inhibitor serpin, α_1-proteinase inhibitor (α_1-PI).[19] Whereas α_1-PI shows a much more exposed P1 side chain, antithrombin's P1 Arg side chain (Arg393) is turned inward forming a salt bridge with the underlying Glu255. Further, heparin-dependent modification of the P1 Arg residue by the enzyme, peptidylarginine deiminase, indicated a sub-optimal P1 conformation.[60] Thus, it was expected that appropriate modifications in the RCL residues should induce better inhibition rates. However, this expectation has not been realized.

Relative to its wild-type parent, Glu255 → Ala antithrombin mutant had a fivefold higher rate of thrombin inhibition but no increase in the rate of factor Xa inhibition.[61] Likewise, mutation of P1 Arg residue to tryptophan, histidine, leucine, and methionine converted the specificity of antithrombin from a trypsin inhibitor to a chymotrypsin inhibitor, as expected. However, heparin pentasaccharide did not further enhance the inhibition of the enzyme by these antithrombin mutants, as one would expect on the basis of the constrained RCL hypothesis.[54] Further, mutating the P6-P3′ reactive center loop sequence, excluding the P1-P1′ residues, to a more optimal thrombin recognition sequence has an at most ninefold enhanced thrombin specificity and no effect on factor Xa rates. Simultaneous deletion of two residues, P7′ and P8′ (Val400 and Thr401), from antithrombin's longer RCL increased the factor Xa inhibition rates nearly tenfold.[62] Yet, these deletions compromised the inhibitor's ability to inactivate thrombin. In combination, the mutational studies suggest that whereas the specificity of factor Xa and thrombin is dependent on the P1-P1′ recognition site, it is weakly

dependent on the flanking RCL residues and possibly more dependent on an unrecognized exosite on the inhibitor.[36]

Aside from the exposed RCL sequence, two key structural elements are involved in the inhibition mechanism—the heparin-binding site and the partially inserted P14-P15 residues. The heparin-binding domain is a wide area and to-date all amino acid replacements in this region, whether natural or man-made (other than Asn135 mutants), result in either no effect or weaker inactivation of thrombin and factor Xa in comparison to the wild-type.[16,30,50,63–65] The triggering of the antithrombin conformational change arises from this region and inability to engineer a better inhibitor suggests an optimal evolutionary construction of the region.

The hinge region residues P14 and P15 possibly represent the most direct and easiest locales to effect a change in the antithrombin conformational equilibrium. If expulsion of P14-P15 residues initiates the sequence of events that result in an enhanced rate of factor Xa inhibition,[66] then engineering antithrombin in which the residues remain expelled will likely make a better inhibitor. Several attempts have been made to realize this possibility. A P14 Ser → Trp antithrombin and a P14 Ser → Cys mutant were made to test the inherently activated antithrombin hypothesis.[67–69] Neither variant was satisfactory. Thus, the P14 Ser → Trp mutant showed only a small increase in reactivity toward factor Xa.[67] In contrast, the P14 Ser → Cys mutant, derivatized with a bulky fluorophore, was much more reactive with factor Xa. However, the bulky fluorophore interfered with the insertion of RCL (inhibition pathway, Fig. 3) in β-sheet A and greatly enhanced the hydrolysis of the E*-I* complex (substrate pathway, Fig. 3).[68] Likewise, the P14 Ser → Glu variant has ∼200-fold increased basal rate of factor Xa inhibition, after a major correction for the increased stoichiometry of inhibition, whereas the basal rate of thrombin inhibition is similar to wild-type antithrombin.[69]

8. NATURAL POLYSACCHARIDES

A. Heparin

Heparin has been the anticoagulant of choice since 1940s until the mid-90s when LMWHs started gaining popularity.[70,71] Heparin is a 1 → 4-linked linear copolymer of glucosamine (2-amino-2-deoxyglucopyranose) and uronic acid (pyranosyluronic acid) residues that are variously sulfated[b] (Fig. 7). This structure, called glycosaminoglycan (GAG), is obtained by chemical processing of proteoglycan heparin present in porcine or bovine intestinal mucosa and lung.[72] GAG heparin is a complex mixture of molecules. The structural complexity arises primarily from the biosynthesis of proteoglycan heparin, in which multiple enzymes systems are involved,[73] and secondarily from the preparative processing, in which harsh treatment with alkali, enzymes and bleaching agents are used.

GAG heparin, referred to as simply heparin, consists of polysaccharide chains having molecular weights in the range of 5,000–40,000 with an average MW of ∼14,000. Several substitutions are possible in the glucosamine and uronic acid residues. The β-D-glucosamines may be *N*-sulfated or acetylated, and may also contain sulfates at 2-, 3-, and 6-positions, whereas the uronic acid residues may be either β-D-glucuronic acid (GlcAp) or α-L-iduronic acid (IdoAp), and may be sulfated at 2-position (Fig. 7). Typically there are more *N*-sulfated glucosamines than *N*-acetylated, and more IdoAp residues than GlcAp. Heparin is the most negatively charged molecule in our body carrying an average of ∼80 negative charges at physiological pH.[c] The molecular weight dispersity, structural variability, and polyanionic character introduce some novel and challenging properties in heparin.

[b]In this review, the $NHSO_3^-$ group is called N-sulfate and the OSO_3^- group is called sulfate.

[c]The average molecular mass of heparin disaccharide is ∼600 and using the average molecular weight of 14,000 per heparin chain, the degree of polymerization is 23.3. The average disaccharide contains ∼2.5 sulfate groups and 1 carboxylate. Thus, the total negative charge per disaccharide is 3.5. One can thus infer that the number of negatively charged groups in an average heparin chain is 81.6.

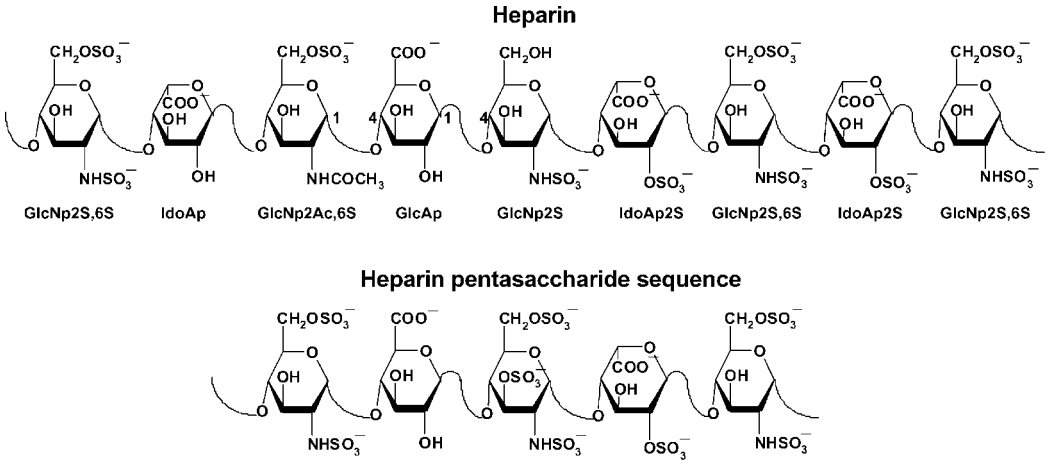

Figure 7. Structure of a heparin chain, labeled heparin, and the sequence-specific heparin pentasaccharide DEFGH. Note the variations in the structure of glucosamine (GlcNp) and uronic acid residues (IdoAp or GlcAp). Numbers 1 and 4 refer to saccharide positions.

Heparin is an anticoagulant because it recognizes plasma antithrombin with high-affinity and activates the inhibitor several hundred-fold, as discussed earlier. The high-affinity interaction arises from a specific sequence $\mathbf{H_5}$ in the polymeric heparin chain, called high-affinity heparin (HAH). $\mathbf{H_5}$ is composed of three 2-*N*- and 6-sulfated glucosamines (β-D-GlcNp2S,6S) interspersed with a 2-sulfated iduronic acid (α-L-IdoAp2S) and a glucuronic acid (β-D-GlcAp), in which the central glucosamine residue has a unique 3-sulfate group (Fig. 7). This sequence is abbreviated as GlcNp2S,6S (1 → 4) IdoAp2S (1 → 4) GlcNp2S,3S,6S (1 → 4) GlcAp (1 → 4) GlcNp2S,6S and labelled as **DEFGH**, in line with the history of its identification.[74–77] This five-residue sequence containing high-affinity heparin chains bind to plasma antithrombin with ∼10 nM K_D at physiological pH in which ionic interactions between the sulfate and carboxylate groups of DEFGH and positively charged residues in the PBS contribute ∼40% of the binding energy.[41]

Heparin chains devoid of the DEFGH sequence, called low-affinity heparin (LAH), bind antithrombin with nearly 1,000-fold lower affinity.[78] Further, the maximal acceleration induced in antithrombin inhibition of factor Xa and thrombin by LAH was reduced significantly indicating a critical role played by the DEFGH sequence in conformational activation and bridging phenomenon (Fig. 5). Both LAH and HAH are capable of binding to many proteins in our body because of their polyanionic character. Studies indicate that heparin interacts with many cationic sequences with sub-micromolar affinity.[79–81] A conservative estimate would put the number of heparin-binding proteins in our body at more than 100, although the significance of all these interactions under physiological conditions could be questioned.[80,82] Yet, studies demonstrate that heparin chains interact well with platelet factor 4 (PF4), a plasma protein, to form a tetrameric complex that initiates an immune response.[83,84] This interaction is the basis for heparin-induced thrombocytopenia (HIT), an adverse condition that affects nearly 10% of patients on heparin therapy.

Anticoagulation regimen with full-length heparin is associated with many complications in addition to HIT. The single major problem is the risk of hemorrhage arising from significant variation in, nearly unpredictable, patient-to-patient response. This complication originates from the polyanion's non-specific interactions, compounded by the myriad structural differences in heparin chains and the significant variation in individual preparations. Heparin's non-specific interactions result in its neutralization by plasma proteins, the concentration and composition of which may differ significantly from person to person.[85–90]

In addition to these problems with heparin therapy, it has been found that heparin cannot inactivate clot bound thrombin.[91,92] This can result in reactivation of thrombosis after treatment discontinuation. A plausible mechanism that explains this phenomenon involves the binding of thrombin to γ′-fibrin through its exosite II.[93] The non-availability of exosite II on thrombin for interaction with full-length heparin precludes the formation of antithrombin-heparin-thrombin ternary complex. Thus, clot-bound thrombin remains unaffected by antithrombin-heparin complex.

B. Low Molecular Weight Heparins

LMWHs represent the past decade's answer to some of the problems accompanying heparin therapy.[7,94,95] LMWHs are much smaller (M_R 4,000–6,000) and are produced from heparin through chemical or enzymatic depolymerization aided in some cases by size exclusion chromatography (Table I). Heparin is cleaved at the glucosamine residue containing an *N*-sulfate group by nitrous acid. When attempted in a controlled manner where the rate of cleavage is slow, some glucosamine *N*-sulfates are more susceptible than others resulting in partial depolymerization. This method apparently leaves the glucosamine residue containing 3-sulfate residue (residue F in DEFGH) intact, thus retaining most of the antithrombin-binding sequences present in parent heparin. The terminal residue produced through nitrous acid deamination is a ring-contracted anhydromannose, an unnatural sugar residue.

Heparin can also be enzymatically cleaved by heparinases. Three heparinases have been obtained from bacteria[96] and their substrate specificities elucidated.[97–99] Heparinase II shows broad specificity for GAG chains with considerable variation in sulfation pattern, and is possibly of least commercial value. In contrast, heparinase I is specific for heparin-like (higher sulfation) chains, whereas heparinase III (heparitinase) is more specific for heparan sulfate-like (lower sulfation) chains. These heparinases target the α-L-iduronic acid residue by abstracting the acidic proton α to the carboxylate group. The α-carbanion then rearranges to a α,β-double bond by releasing the *trans*-oriented sugar chain at the 4-position. Thus, one of the resulting low molecular weight heparin chains contains an unsaturated sugar residue at the non-reducing terminus.

Similar β-elimination can also be accomplished chemically through a benzyl ester derivative of the uronic acid residues followed by treatment with alkali. One may expect that such alkaline degradation of heparin is relatively indiscriminate resulting in greater polydispersity and heterogeneity.

LMWHs have chemical compositions similar, not identical, to heparin and their mechanism of interaction with antithrombin is similar too.[100,101] The binding affinity and anti-factor Xa activity of LMWH for antithrombin varies depending on the fraction of DEFGH sequences present in each preparation. Likewise, their activity for accelerated thrombin inhibition varies according to the proportion of high-affinity chains that have length greater than 18 saccharides. The overall goal is to

Table I. Comparative Properties of Low Molecular Weight Heparin Preparations*

	Manufacturer	*Key reagent in depolymerization*	M_R	*Ratio anti-fXa: anti-Iia*
Ardeparin	Wyeth-Ayerst, US	Peroxides	5,300	2.0
Dalteparin	Pharmacia, US	Nitrous acid	6,000	1.9–3.2
Enoxaparin	Aventis, US	Alkali	4,500	3.3–5.3
Nadroparin	Sanofi, France	Nitrous acid	4,300	2.5–4.0
Reviparin	Knoll AG, Germany	Nitrous acid	3,900	3.6–6.1
Tinzaparin	Leo Labs, Ireland	Heparinase	6,500	1.5–2.5

*Compiled from manufacturer's data and Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, Granger C, Ohman EM, and Dalen JE. (2001). Heparin and low-molecular weight heparins: Mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy and safety. Chest 119, 64S–94S.

reduce the average heparin chain-length, corresponding to a high anti-fXa: anti-factor IIa ratio. This can be achieved when processing minimally affects the DEFGH sequence whereas reducing the length of the polysaccharide chain. Demonstrated advantages of LMWHs over heparin are the greater bioavailability at low doses, better pharmacokinetics, and the more predictable dose response, which allows for fixed doses to be administered without laboratory monitoring.[7,88–90,94,95]

The reduction in M_W reduces their length dispersity and lowers their non-specific interactions. Indeed, thrombocytopenia associated with UFH is significantly reduced with LMW heparins.[90,102,103] However, the expectation that LMW heparins might eliminate the risk of bleeding was not confirmed. In fact, it appears that little difference exists between UFH and LMW heparins when comparing pre-operative hemorrhagic risk.[90,104–107] In addition, other concerns originating from the structure of LMW-heparins exist. For example, different methods of preparation may introduce considerable variation in *in vivo* efficacy among the LMW heparins (see also Table I).[108] Chemical methods may introduce non-native structures increasing their structural heterogeneity, a cause for non-specific interactions. On the other hand, enzymatic methods may destroy the anticoagulant's active site, as found with heparinase I.[109,110] In fact, the FDA has suggested that each clinical LMWH be treated as an unrelated independent drug, and not be exchanged with another member of its class during therapy.

Despite these apparent problems, newer LMWHs are being developed at a feverish pace. As our understanding and technology improves, the LMWHs are expected to possess better structural and biological profiles. Recently, rationally designed LMWHs were created through controlled partial depolymerization of heparin with a mixture of heparinases.[111] These rationally designed LMWHs showed better anti-fXa to anti-fIIa ratio in comparison to dalteparin and enoxaparin.

C. Covalent Glycosaminoglycan–Inhibitor Complexes

Heparin exhibits its anticoagulant effect through antithrombin. However, the inhibitor is typically not available at places other than the vasculature, whereas clot formation may occur at extravascular sites in a variety of organs contributing to morbidity. To induce antithrombotic activity in heparin at such locales, covalent antithrombin–heparin complexes were developed. These complexes relied on the formation of a Schiff base between the reducing end aldose present in a subpopulation of heparin chains and the amino terminus of certain lysine residues in antithrombin. The Schiff base was then reduced with sodium cyanoborohydride to give a covalent, stable antithrombin–heparin complex.[112] The complex had high anti-factor Xa and anti-factor IIa activities compared with non-covalent mixtures of antithrombin and heparin (861 and 753 U/mg vs. 209 and 198 U/mg, respectively). An added advantage with these complexes was their extended intravenous half-life (2.6 hr) as compared to heparin (0.32 hr). Interestingly, the stoichiometry of thrombin inhibition was 1, despite the requirement that the bound heparin should be released following reactive center loop insertion in antithrombin. Further, the overall mechanism of the covalent complex was similar to that of the non-covalent antithrombin–heparin species.[113] Although a novel concept, these GAG-serpin complexes are less likely to be useful because reversal of anticoagulation might be more difficult.

D. Other Carbohydrate-Based Polymers

Several carbohydrate-based polymers have been investigated for their anticoagulant activities. These include heparan sulfate, dermatan sulfate (DS), chondroitin sulfate, hyaluronan, fucan sulfate, chitosan sulfate, dextran sulfate, and sulfated mannan. Typically these polymers are either isolated from natural sources as sulfated molcules or a neutral carbohydrate backbone is sulfated. To-date not a single polymer has been identified that acts as an antithrombin-based anticoagulant without some form of sulfation. Each polymer is generally a complex mixture of carbohydrate chains with multiple micro-structural sequences. Thus in effect, each polymer is structurally complex and identification of active sequence(s) has been difficult. Further, the myriad structural sequences present in this group target antithrombin as well as heparin co-factor II.

DS, a member of the GAG family, is a 1 → 3 linked linear co-polymer of *N*-acetyl D-galactopyranose (D-GalpNAc), L-idopyranosyluronic acid (L-IdoAp), and D-glucopyranosyluronic acid (D-GlcAp) with sulfate groups most commonly found at the 4-position of D-GalpNAc residues (occasionally at the 6-position) and 2-position of L-IdoAp residues (Fig. 8). Typical uronic acid composition of DS is 9:1 IdoAp:GlcAp. DS is sometimes referred to as chondroitin sulfate B and differs from chondroitin sulfate A and C in the presence of L-IdoAp residues. DS contains approximately 1 sulfate group per disaccharide unit in comparison to nearly 2.5 present in heparin.[114] The therapeutic potential of dermatan suflate has been reviewed.[115]

DS does not function as an anticoagulant through the antithrombin pathway of inhibition of the coagulation enzymes. DS binds and activates heparin cofactor II (HCII) for accelerated inhibition of thrombin. HCII is a serpin, homologous to antithrombin in its three dimensional structure, that is specific for thrombin leaving factor Xa unaffected.[17,59] Hence, HCII-based molecules represent an interesting class of specific indirect thrombin inhibitors. A specific hexasaccharide sequence, (→ 3IdoAp2S (1 → 3) GalNpAc4S (1 →)$_3$, in polymeric DS is responsible for high-affinity binding and activation of heparin cofactor II.[116] Yet a minimum chain size of 12–14 U is necessary for inhibition of thrombin.[117,118]

Low molecular weight DSs have also been prepared with an aim of enhancing the bioavailability of these charged polymers.[115] Oversulfated low molecular weight DSs have been prepared to understand the relationship between charge, molecular weight and *in vivo* anti-thrombin activity.[119] Recently, covalent complexes of DS and heparin with heparin cofactor II have been prepared and shown to be good inhibitors of thrombin.[120] These covalent complexes were prepared in a manner similar to covalent heparin–antithrombin complexes.[112] The heparin cofactor II–DS complex has fast bimolecular rate constants approaching diffusion limits ($\sim 10^7$ M^{-1} sec^{-1}) for reactions with thrombin. A comparison of the conformational change induced in heparin cofactor II by heparin and DS using fluorescence spectroscopy suggested that DS stimulates heparin cofactor II better than heparin.[121] The specificity of DSs for accelerating the inhibition of thrombin only and its enhanced efficiency in comparison to heparin offers an additional anticoagulant pathway, which will likely be exploited in the future.

Recently, several antithrombotic DSs have been isolated. A DS from human placenta with MW ~40,000 was isolated from its proteoglycan, decorin, and proposed to function as a local regulator of

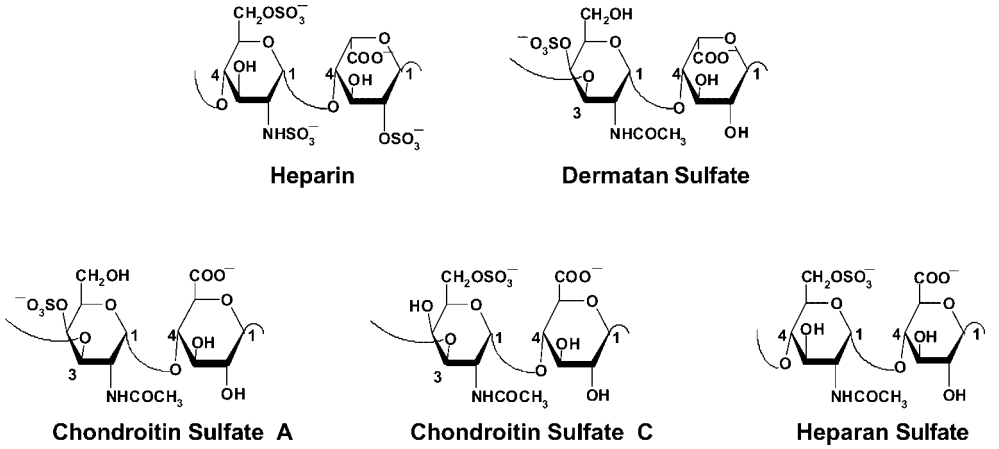

Figure 8. Structures of major disaccharide sequences of heparin, dermatan sulfate, chondroitin sulfate A, chondroitin sulfate C, and heparan sulfate. Each polysaccharide is an alternating co-polymer of a glycosamine and a uronic acid. Heparin and dermatan sulfate contain the flexible iduronic acid residue, whereas heparan sulfate and chondroitin sulfates A and C have the rigid glucuronic acid residue. Whereas the co-polymerization pattern in heparin and heparan sulfate is 1 → 4, it is 1 → 4 and 1 → 3 for the other three. In terms of sulfation, heparin is most sulfated with ~2.5 sulfates per disaccharide, whereas the sulfate density for the others is ~1.

high concentrations of thrombin released during pregnancy.[122] A DS has been isolated from avian crown that exhibits better antithrombotic activity than that from mammalian origin *in vivo*, possibly because of its longer half-life.[123,124] This DS had a mean MW of 16,000 and indicated presence of a disaccharide sequence that closely relates to the high-affinity hexasaccharide known to bind heparin cofactor II.[123] DSs from ascidians have been isolated containing high proportion of IdoAp2S residues.[125,126] The structures of these naturally oversulfated DS chains show the presence of 4- and 6-sulfated galactosamine units, however the anticoagulant activity is better with 4-sulfation. Despite the oversulfation, the ascidian DS chains do not exhibit any effect on thrombin or factor Xa inhibition through antithrombin.[126] Eight sulfated polysaccharides, which show heparin cofactor II-dependent thrombin inhibition, have also been isolated from chlorophyta.[127] Although these naturally occuring DS exhibit significant anticoagulant activity, their structural characterization remains incomplete.

Heparan sulfate is another GAG that is known to be antithrombotic. It is similar to heparin in terms of its carbohydrate backbone, except for three fundamental differences.[128] Whereas heparin contains greater proportion of iduronic acid residues in comparison to glucuronic acid residues, the reverse is true for heparan sulfate. The greater GlcAp content induces greater rigiditiy in the heparan sulfate polymer.[129] Secondly, heparan sulfate has more varied sulfation pattern generating significantly greater structural complexity (Fig. 8). The overall charge on a typical heparan sulfate polymer is much less than heparin, yet there might be sequences with local high charge density.[130] These local high charge sequences, exemplified by the DEFGH sequence, are implicated in heparan sulfate's antithrombotic activity. Finally, heparan sulfate chains are approximately twofold longer than heparin.[131] Heparan sulfate is ubiquitously distributed on cell surfaces and is an important component of the extracellular matrix.[132] These chains are responsible for binding a variety of proteins and contribute to several physiological processes including coagulation. The structure-activity relationship of heparan sulfate has been reviewed.[133]

Nature, particularly seaweeds, is a rich resources for sulfated polysaccharides possessing anticoagulant activity. Among them are fucoidin,[134–139] sulfated high rhamnoses,[140] and sulfated xylomannan.[141] Each of these sulfated polymers is a complex mixture of species with as yet unidentified sequence. The mechanism of anticoagulant action of these polysaccharides has not been deduced, yet it is likely that a majority of them function through the heparin cofactor II pathway rather than the antithrombin pathway. It is interesting that seaweeds have these sulfated polymers that prevent clot formation, although it is questionable whether anticoagulation is the purpose. It is more likely to be water retention and/or filtration of microbes through charge neutralization.

9. SYNTHETIC MODIFIED HEPARINS AND HEPARIN-MIMICS

Numerous attempts have been made to enhance anti-factor IIa and anti-factor Xa activity by chemically modifying the structure of the polyanion, heparin. Chemically modified heparins were also prepared to understand the structural basis of heparin's anticoagulant activity (Fig. 9). A heparin polymer containing carboxy-reduced uronic acid residues was devoid of anti-factor Xa activity.[142] Sulfation of this polymer to enhance the average number of sulfate groups per disaccharide by 1 did not induce antithrombin-mediated anticoagulant activity.[142] Heparins in which glucosamine residues were *N*-desulfated or *O*-desulfated at the 2- or 3-position indicated complete loss of anti-fXa activity.[143–145] These results were consistent with the idea that reducing the sulfate density lowered anticoagulant activity. Hence, GAGs, including DS and heparan sulfate that have lower charge densities, were over-sulfated. These polymers exhibited better anti-fXa activity than the starting molecules.[146] In each of these chemically modified species, except for carboxy-reduced heparin, only the sulfate content was modified and the basic natural GAG backbone remained unaltered.

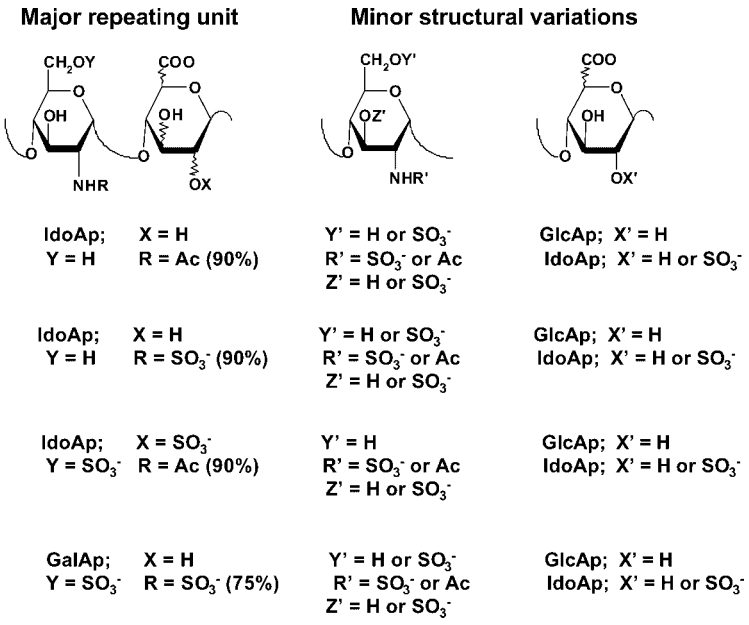

Figure 9. Structures of chemically modified heparins. Heparin was completely desulfated and then selectively modified to introduce either the $-OSO_3^-$, $-NHSO_3^-$, or $-NHCOCH_3$ groups. CDSNAc, completely desulfated *N*-acetylated heparin; CDSNS, completely desulfated *N*-sulfated heparin; CNDSNAc, completely *N*-desulfated, *N*-acetylated heparin; GalAHep, heparin containing α-L-galactopyranosyluronic acid GalAp that contains reversal of stereochemistry at both 2- and 3-positions as comparised to instead of α-L-idopyranosyl-uronic acid residue. The numbers in parenthesis represent the proportion of major disaccharide repeating unit in the polydisperse heterogeneous mixture.

Several unnatural heparins were synthesized through base-catalyzed displacement of the 2-sulfate group in IdoAp residue. The displacement proceeded to give a polymer containing IdoAp residues with 2,3-epoxide, which could be further hydrolyzed to an α-L-galactopyranosyluronic acid residue in which the geometry at both 2- and 3-positions was reversed. Each polymer was further sulfated to introduce heparin-like property.[147] Yet, the anticoagulant activity of these polymers was less than 20% of that achieved with natural heparin. It is interesting that these unnatural polymers had some anti-fXa activity, considering the fact that the epoxidation or epimerization at two centers are expected to be drastic structural and conformational modifications.

Recognizing that the probability of increasing high-affinity antithrombin-binding domain (DEFGH, Fig. 7) in a typical preparation increases with oversulfation, several GAGs were fully sulfated.[148,149] Whereas fully sulfated DS, chondroitin sulfates, hyaluronan, and heparan sulfate showed anti-factor Xa activity in the range of 10–80 U/mg comparable to some LMWHs, fully sulfated heparin completely lost its anticoagulant activity. Recently, controlled solvolytic desulfation of fully sulfated heparin was performed to afford modified heparins in which 40% of the IdoAp residues and GlcNp2S are sulfated at 2- and 3-position, respectively. These modified heparins were found to display *in vitro* anti-factor Xa activities equivalent or higher than conventional LMWHs.[150]

Several naturally available, neutral polysaccharides have been sulfated to introduce heparin-like features in the polymer. Hyaluronic acid is a high molecular weight neutral GAG. Chemical sulfation led to hyaluronic acid derivatives differing in sulfate content, chain length, and anti-factor IIa and anti-factor Xa activity.[151] Although anti-coagulant activity could be introduced in these neutral polymers, the activity was small and the possibilities of enhancing it further are less. Similarly, neutral carbohydrate polymers including chitosan,[152,153] dextran,[154–157] galactomannan,[158] and fucan,[159,160] have been sulfated, yet the anticoagulant activity level in each of these polymers is small.

10. SYNTHETIC OLIGOSACCHARIDES

Through controlled cleavage of full-length heparin followed by affinity fractionation, high-affinity oligosaccharides were isolated. Elucidation of structures of penta- to octasaccharides led to a core structure corresponding to DEFGH sequence. The identification of a small sequence in heparin, which is responsible for most of heparin's affinity for antithrombin, has led to a huge body of work in the past two decades devoted to the synthesis of oligosaccharides, especially DEFGH and its derivatives (reviewed in ref. 161).

A. Heparin Pentasaccharide DEFGH

The heparin pentasaccharide sequence DEFGH has some interesting features. The five-residue sequence can be thought of as composed of two domains, a trisaccharide DEF and a disaccharide GH domain. The GH sequence consisting of → 4) IdoAp2S (1 → 4) GlcNp2S6S represents the most abundant disaccharide sequence in heparin, whereas the trisaccharide DEF consisting of GlcNp2S,6S (1 → 4) GlcAp (1 → 4) GlcNp2S,3S,6S (1 → represents the least abundant sequence in heparin.[162,163] A structural feature that most characterizes the DEFGH sequence is the central glucosamine residue F consisting of three sulfates at 2-, 3-, and 6-positions. This residue is rarely present outside the heparin pentasaccharide sequence.

The two domains have different conformational flexibilities. Although the conformational preference across interglycosidic bonds in heparin remains relatively constant irrespective of the substitution pattern in the residues flanking the bond,[129] saccharide pucker shows some variation. The D, E, and F residues are predominantly present in the $^4C_1$ conformation in the ground state, whereas the IdoAp2S residue exhibits primarily two conformer populations in the ground state, the $^1C_4$ and $^2S_0$.[164–166] Of these two ground states, the $^2S_0$ conformer binds antithrombin with higher affinity.[167] This conformational flexibility of iduronic acid residue in DEFGH plays an important role in the activation of antithrombin.[168]

Finally, the two domains in pentasaccharide play a slightly different role in the conformational activation of antithrombin. The binding of DEFGH to antithrombin is a two-step process involving the formation of an initial low-affinity complex (**AT:H**, Fig. 10A) in rapid equilibrium. This is followed by a major conformational change to give a high-affinity **AT*:H** complex in which antithrombin has been conformationally activated (Fig. 10A).[41,169] This conformational change has been linked to the expulsion of the RCL for accelerated inhibition of factor Xa.[170] Studies with the truncated variants, trisaccharide DEF and tetrasaccharide EFGH″ (Fig. 10B) of pentasaccharide DEFGH indicate that the DEF sequence is critical for both the initial recognition of the heparin-binding site and conformational activation processes in antithrombin.[171,172] Further, full conformational activation of antithrombin (~300-fold acceleration) can be achieved with trisaccharide DEF under saturating conditions, except that the affinity of DEF for antithrombin is much weaker (K_D ~66 μM) under physiological conditions.[172] The disaccharide unit GH of the pentasaccharide appears to play an important role primarily in enhancing the affinity of the molecule for the conformationally activated inhibitor. Thus, the dissociation constant of DEFGH–plasma antithrombin complex decreases to ~50 nM.

The heparin pentasaccharide is a selective anti-factor Xa molecule, not affecting thrombin inhibition[41] (see Fig. 5). In fact, earlier it was debated whether factor Xa inhibition alone, or absence of thrombin inhibition, could lead to antithrombotic effects,[173] until antithrombotic effects were demonstrated through the use heparin pentasaccharide.[174] Recent clinical use suggests that the pentasaccharide is more effective than a LMWH in preventing venous thromboembolism and was equally safe.[175,176] The biochemical rationale and some clinical aspects of heparin pentasaccharide have been reviewed.[6,177]

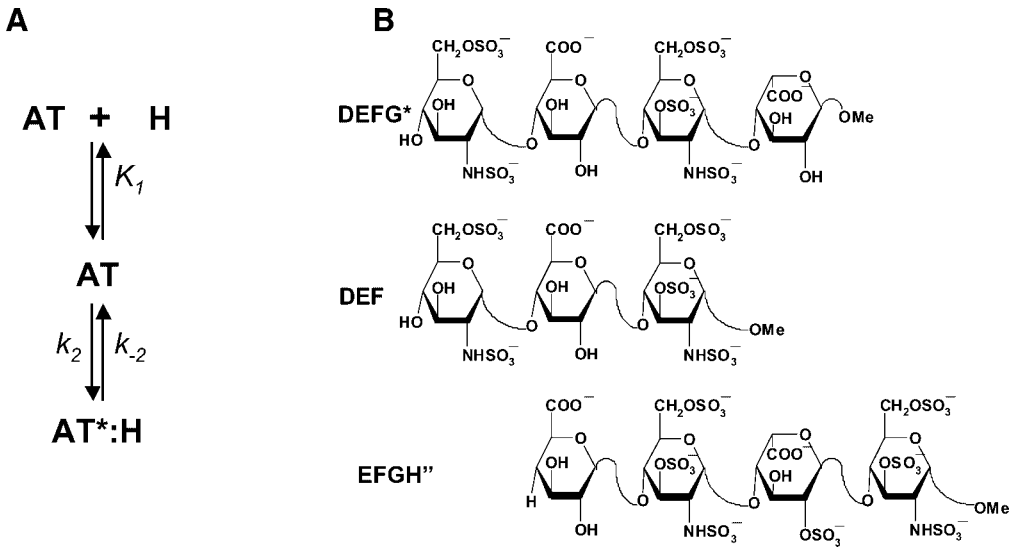

Figure 10. Two-step induced-fit mechanism of activation of antithrombin (**A**) and structures of truncated pentasaccharides DEFG*, DEF, and EFGH″ based on natural pentasaccharide DEFGH (**B**). H, heparin; AT, native antithrombin; AT*, conformationally activated antithrombin; K_1, rapid equilibrium dissociation constant of first step of the induced-fit pathway; k_2 and k_{-2} are forward and reverse rate constants for the conformational change step. Structures of truncated pentasaccharides. Truncated molecules with primes or asterisks have one or two individual saccharides whose structure differs slightly from natural pentasaccharide DEFGH.

Heparin is non-specific because it forms multi-dentate, indiscriminate, ionic interactions with numerous soluble proteins, and cell surface receptors. This non-specific binding, and the problems thereof, can be greatly reduced by decreasing the size and the highly anionic character of the activators. This is borne out by the observation that heparin pentasaccharide, a smaller and less anionic antithrombin activator shows reduced non-specific interactions in comparison to UFH.[6,178–180] Heparin pentasaccharide possesses several advantages, including the absence of adverse effects on platelet aggregation, absence of release of tissue factor pathway inhibitor, absence of release of lipoprotein lipase activity, and reduced non-specific interactions in comparison to LMW-heparins. Further, the bioavailability of pentasaccharide is better than LMW heparins.[6]

The first total synthesis of heparin pentasaccharide was accomplished in some 40 steps in very small overall yield.[181] This was a tremendous achievement and paved a way for quantitative structure-activity studies. Petitou and co-workers have invested a major effort in deducing these relationships and have made major inroads into pentasaccharide derivatives that are better than the native molecule.[161]

The natural pentasaccharide sequence has two variations of unequal activity, both of which have been synthesized. These variants arise because the non-reducing end glucosamine residue D can be either *N*-sulfated (DEFGH) or *N*-acetylated (D′EFGH) (Fig. 11, Table II). The *N*-sulfated pentasaccharide (700 U/mg anti-fXa) is approximately twice as active as the *N*-acetylated variant. Although important information about the role of certain sulfate groups was available from chemically modified heparins and enzymatically derived heparin fragments, definitive conclusions regarding the importance of individual sulfate groups were obtained through synthetic derivatives of DEFGH. It was established that four sulfate groups, at the 6-position of residue D, 3- and 2-positions of residue F, and 2-position of residue H, are critical for high-affinity binding to antithrombin (Fig. 11). Of these, the 3-sulfate group on residue F was found to be most important for antithrombin activation. Although the crystal structure of pentasaccharide-antithrombin co-complex shows that each of these sulfate groups interact with either one or more Lys or Arg residues in the pentasaccharide binding

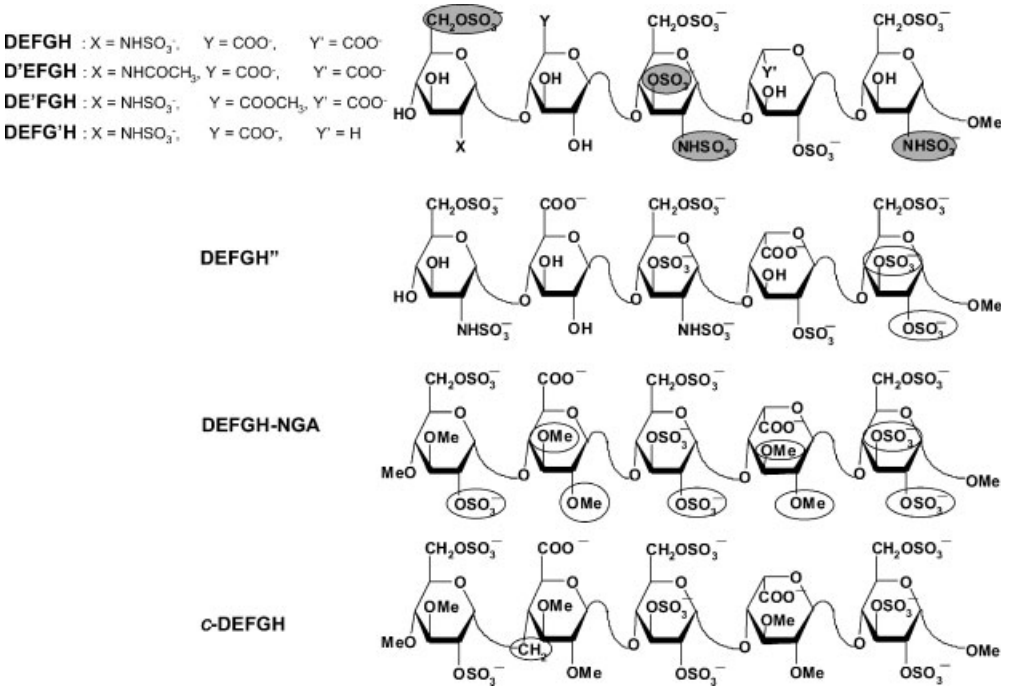

Figure 11. Structure of natural pentasaccharide DEFGH derivatives. Molecules with primes have a residue whose structure differs slightly from natural pentasaccharide DEFGH. Sulfate groups in DEFGH highlighted as filled ovals (⬭) are critical for high-affinity interaction with antithrombin. Pentasaccharide DEFGH″ has residue H containing sulfates at 2- and 3-positions (highlighted as empty ovals). DEFGH-NGA, the 'non-glycosamino' derivative of the natural pentasaccharide, has no *N*-sulfates and contains uronic acids that have 2-OMe groups. Note the number of changes introduced in DEFGH-NGA to ease the synthesis and enhance the anti-factor Xa activity. C-DEFGH contains a methylene (-CH_2) group replacing the interglycosidic oxygen atom between residues D and E.

site,[45] it is not immediately apparent why the 3-sulfate group of residue F plays the most important role. The carboxylate groups of GlcAp and IdoAp2S are also important as demonstrated by pentasaccharides DE′FGH and DEFG′H (Fig. 11) that exhibit less than 5% of the activity of the reference DEFGH.

Following extensive study of derivatives, including DEFGH′ and DEFGH-NGA (Fig. 11), two major advances were achieved by Petitou and co-workers.[161] Replacement of $NHSO_3^-$ group in all

Table II. Anti-Factor Xa Activities of Natural Pentasaccharide and its Derivatives*

Pentasaccharide[a]	*Anti-fXa activity (U/mg)*
DEFGH	700
D′EFGH	350
DE′FGH	35
DEFG′H	~0
DEFGH″	1,250
DEFGH-NGA	1,323
C-DEFGH	880

*Compiled from van Boeckel and Petitou[161] and Petitou et al.[183]

[a]See Figure 11 for structures.

three glucosamine residues with OSO_3^- and introduction of alkyl ethers at the available free hydroxyl groups not only retained anti-fXa activity, but enhanced it ~200% (Table II).[161] The new ''non-glycosamino'' pentasaccharide DEFGH-NGA contains glucose residues instead of glucosamines. This together with *O*-methylated residues, especially GlcAp2OMe and IdoAp2OMe, made the total synthesis much easier than that of heparin-like fragments. The ''non-glycosamino'' pentasaccharide preserves the distribution of critical sulfate and carboxylate groups of the natural pentasaccharide sequence. Further, it is likely that the new pentasaccharide retains the conformational preference and flexibility of the parent molecule. The total synthesis of the advanced pentasaccharide has been further improved following the discovery of higher activity.[182]

Recently a *C*-pentasaccharide, consisting of a carbon (CH_2)-based interglycosidic bond between residues D and E (Fig. 11), was shown to hardly affect the biological properties of the natural pentasaccharide.[183] This *C*-pentasaccharide shows an anti-factor Xa activity ~34% better than DEFGH and represents the first member of new anti-factor Xa pentasaccharides (Table II). It is likely that this discovery spurs the search for molecules that have a ''pseudo-saccharide'' skeleton.

B. Synthetic Oligosaccharides for Thrombin and Factor Xa Inhibition

While the natural pentasaccharide sequence DEFGH is essential for factor Xa inhibition through conformational activation of antithrombin, its presence is also required for accelerated thrombin inhibition through the bridging mechanism (Fig. 5). Whereas the structure of antithrombin-binding domain (ABD), i.e., DEFGH, could not be altered greatly, the structure of thrombin-binding domain (TBD) in heparin was thought to be relatively non-specific.[184] The TBD was predicted to compose of ~5–6 negative charges distributed on either two or three saccharides, most probably representing the dominant disaccharide sequence, → 4) IdoAp2S (1 → 4) GlcNp2S,6S (1 → , of heparin. Thus, linking the ABD (or DEFGH) with the TBD through a spacer would engineer a full-length heparin mimic possessing both factor Xa and thrombin inhibitory activity. Previous molecular modeling result,[185] later confirmed by a crystallography study,[45] had suggested that TBD was located on the non-reducing end of the DEFGH sequence. Thus, the relative orientation of the two domains could be fixed. However, questions remained on the constitution and length of spacer, as well as the fine structure of TBD.

Chemical synthesis of oligosaccharides, deca- to eicosasaccharides, containing the heparin pentasaccharide at the reducing-end and heparin disaccharide at the non-reducing end of the chain, established that for accelerated thrombin inhibition a minimum chain length of either 15 or 16 residues is needed.[186,187] This was consistent with results obtained previously with purified low molecular weight heparin fragments.[38] Several different spacer structures were utilized. Comparison of a flexible polyethylene glycol type spacer with a rigid polyglucose type spacer suggested that the rigid spacer gave higher anti-factor IIa activity.[188] More importantly, these anticoagulants elicited much lower platelet factor 4 binding activity, thus suggesting that these heparin mimics are likely to reduce heparin-induced thrombocytopenic adverse effects.

Synthetic oligosaccharides, comprised of an ABD prolonged at the non-reducing end by multiple TBDs, have been studied.[189] These molecules have numerous negative charges positioned along the entire saccharide chain, as in full-length heparin. As expected, each of these saccharides enhance the antithrombin inhibition of both factor Xa and thrombin, with non a-decasaccharide being better than full-length heparin. Yet, the anticoagulant activity of the oligosaccharides could be neutralized by PF4. In a final move, a heparin mimic was engineered that has the ABD connected to TBD through an uncharged hexasaccharide spacer. To ease the synthesis, the TBD was constructed of a disulfated glucose-based hexasaccharide (Fig. 12).[44,190,191] This heptadecasaccharide is an extremely potent anti-factor Xa and anti-factor IIa molecule and cannot be neutralized by PF4. It is likely that this molecule has negligible heparin-induced thrombocytopenic adverse effect. Recently, a heparin mimic containing TBD, which is perphosphorylated rather than persulfated, has been found to exhibit an

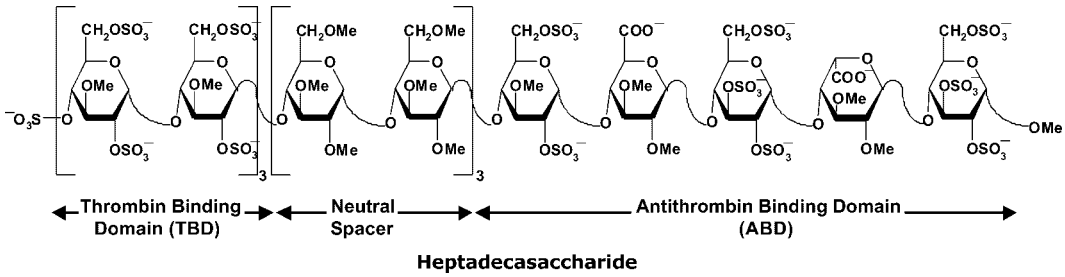

Figure 12. Structure of a novel heptadecasaccharide showing high anti-factor Xa and anti-factor IIa activity. The heptasaccharide consists of high-activity antithrombin-binding domain (ABD), i.e. DEFGH-NGA, joined at its non-reducing end to a thrombin-binding domain (TBD), a six residue disulfated monosaccharide sequence, through a neutral spacer consisting of six methyl-protected glucose residues.

increase in antithrombin activity.[192] These full-length heparin mimics show tremendous promise of moving into clinical trials.

11. SYNTHETIC NON-SUGAR ANTITHROMBIN ACTIVATORS

A fundamental tenet of the studies described above has been the assumed requirement of (i) a saccharide skeleton and (ii) a minimum size corresponding to the five residue sequence for a high-affinity interaction with antithrombin. These were apparently supported by the dramatic loss in accelerated inhibition following a saccharide ring disruption in the pentasaccharide framework.[161] A major concern with the saccharide-based approach is the difficulty of synthesis that leads to much-reduced cost effectiveness. Recently, synthetic non-sugar molecules have been designed that exhibit antithrombin activation for accelerated inhibition of factor Xa.[193,194] These molecules represent the first in the class of small, non-sugar activators and possibly are future anticoagulants.

To rationally design non-sugar activators, a robust modeling tool was required that accurately simulates the interactions of antithrombin at a molecular level. First, hydropathic interaction (HINT) analysis was used to quantify the interactions of antithrombin.[195] HINT is molecular modeling tool that provides quantitative information regarding inter-molecular interactions at an atomic level.[196,197] For antithrombin–pentasaccharide interaction, HINT map showed favorable interactions between positively charged residues of helix A, helix D, and the polypeptide N-terminus and the sulfate and carboxylate groups of the pentasaccharide.[195] Further, HINT predicted reasonably well the interaction of DEFGH with heparin-binding mutants of antithrombin.[193] Using this as a basis, the interaction profile of designed *de novo* ligands was studied.[193] The small ligands were selected to mimic trisaccharide DEF, given its equivalence with pentasaccharide DEFGH in inducing acceleration in antithrombin inhibition of factor Xa. After a series of structural screens, a small non-sugar skeleton, (−)-epicatechin sulfate (ECS, Figure 13A), was designed (Fig. 13B). ECS was found to interact with antithrombin with an affinity comparable to DEF. More interestingly, it accelerated the inhibition of factor Xa nearly eightfold, the first small non-sugar molecule known to activate antithrombin. Several other molecules were designed in a similar manner (Fig. 13A), however the activation of antithrombin did not increase beyond ~20-fold (Table III), in comparison to that known for DEF (~300-fold).[194,198] Detailed competitive binding and molecular modeling studies have indicated that these designed small molecules prefer to bind in an electropositive domain adjacent to the pentasaccharide binding site and formed by residues Arg132, Arg133, and Arg136, a site called the extended heparin-binding site (EHBS), explaining the weaker activation potential.[194]

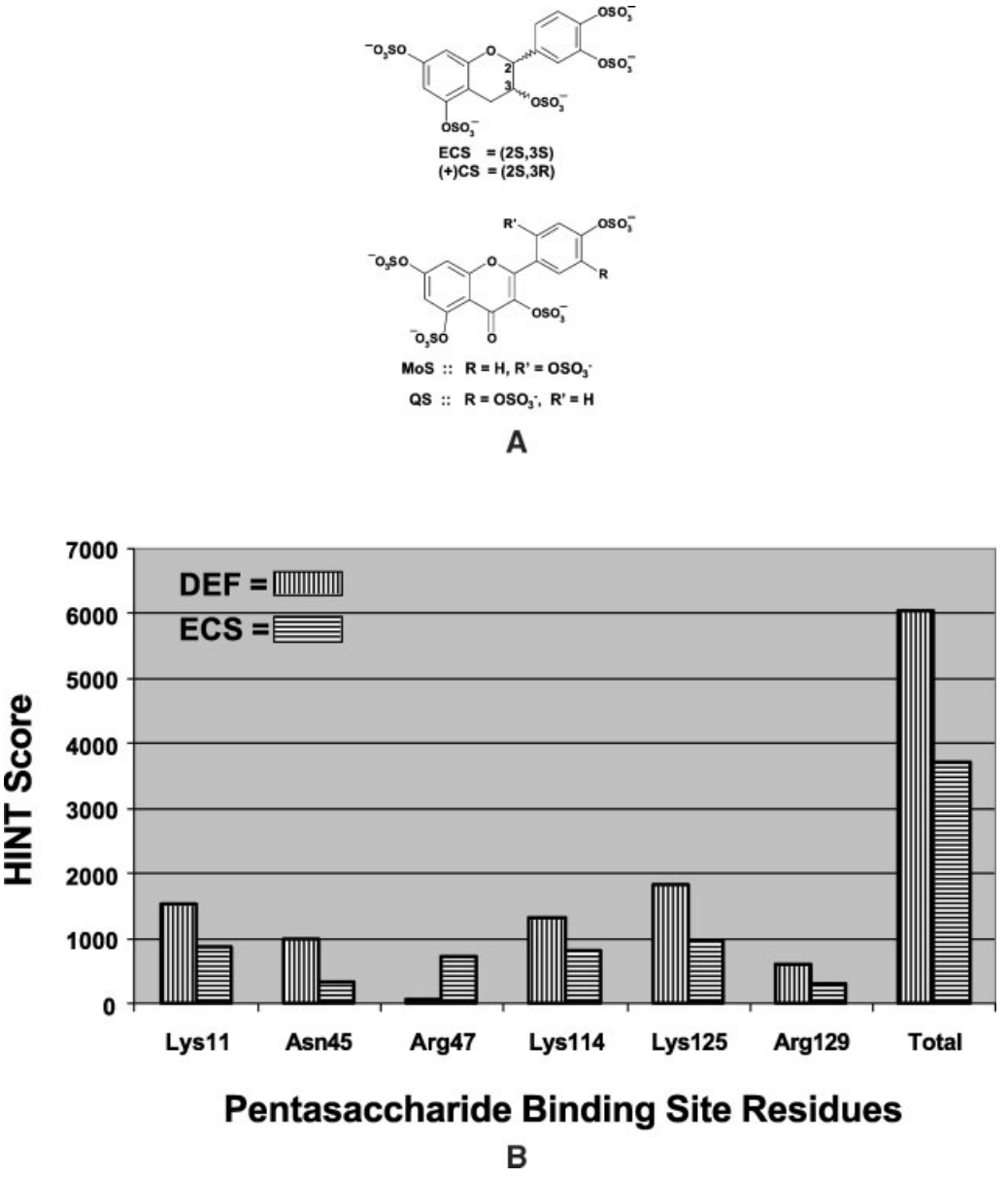

Figure 13. Novel small, non-sugar sulfated activators of antithrombin. **A**: Structure of flavanoids and flavonoids shown to interact with plasma antithrombin with μM affinity and inducing an 8–20-fold acceleration in inhibition of factor Xa. ECS ((−)-epicatechin sulfate) and (+)-CS ((+)-catechin sulfate) are diastereomeric flavanoids. Morin sulfate (MoS) and quercetin sulfate (QS) are flavonoids. **B**: Hydropathic Interaction (HINT) profile of trisaccharide DEF and ECS interacting with antithrombin. HINT is a computer interaction analyzer that quantifies atomic level interactions on the basis of their hydrophobicity (log P values). HINT was used to rationally design ECS, the first organic non-sugar sulfated activator of antithrombin. The residue level scores for ECS were comparable to DEF suggesting similarity in their interaction profiles.

Using these initial studies, it is likely that better activators that recognize the pentasaccharide-binding site and result in higher antithrombin activation will be rationally designed. The success of this endeavor highlights the unique opportunities of designing small organic activators of antithrombin.

Table III. Acceleration in Antithrombin Inhibition of Factor Xa by Non-Sugar Sulfated Flavanoids, Rationally Designed Trisaccharide DEF Mimics*

	Acceleration
Sulfated non-sugar molecule[a]	$k_{activator}/k_{UNCAT}$
ECS	10.4 ± 1.5
(+)-CS	20.8 ± 3.1
MoS	21.8 ± 2.9
QS	17.5 ± 3.2
DEF	320 ± 20

*Compiled from Gunnarsson and Desai.[193,198]
[a]See Figure 13 for structures.

12. CONCLUSIONS

On the new molecule front, exciting sugar-based molecules have been designed for selective inhibition of factor Xa. Oligosaccharides have also been developed for effective simultaneous inhibition of factor Xa and thrombin. Low molecular weight heparins have been rationally designed to minimize their adverse effects and enhance their anti-fXa activity at the expense of anti-IIa activity. Finally, novel small, rationally designed, non-sugar molecules have shown early promise of antithrombin activation. On the biochemical front, detailed knowledge on the mechanism of antithrombin activation, the structure of heparin-binding site in antithrombin, and the structure-function relationships in the heparin pentasaccharide sequence has been obtained. In combination, these advances bode well for rationally designed, high-affinity, high-activity indirect anticoagulants with minimal adverse effects. One can expect that anticoagulation therapy will likely undergo major changes in the near future.

ABBREVIATIONS

α_1-PI	α_1-proteinase inhibitor
ABD	Antithrombin-binding domain
AT	Antithrombin
DEFGH	Natural pentasaccharide sequence DEFGH of the natural octasaccharide ABCDEFGH
EHBS	Extended heparin-binding site
fIIa	Factor IIa (thrombin)
fXa	Factor Xa
GAG	Glycosaminoglycan
GlcAp	β-D-glucuronic acid
H_5	Heparin pentasaccharide
HAH	High-affinity heparin
HINT	Hydropathic interaction
HIT	Heparin-induced thrombocytopenia
IdoAp	α-L-iduronic acid
LAH	Low-affinity heparin
LMWHs	Low-molecular weight heparins
PBS	Pentasaccharide-binding site
RCL	Reactive center loop
Serpin	Serine proteinase inhibitor
TBD	Thrombin-binding domain
UFH	Unfractionated heparin

ACKNOWLEDGMENT

The author thanks Dr. Kier and Dr. Gunnarsson of VCU for their critical reading of the manuscript.

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