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ARTICLE *in* JOURNAL OF THROMBOSIS AND THROMBOLYSIS · MARCH 2012

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J Thromb Thrombolysis (2012) 34:251–259
DOI 10.1007/s11239-012-0712-z

Limitations of conventional anticoagulant therapy and the promises of non-heparin based conformational activators of antithrombin

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Published online: 28 March 2012

Abstract An elevated prothrombotic state is a major risk factor for venous thromboembolism, atrial fibrillation and cardiac strokes. The regulation of various coagulation cascade proteases plays an important role in determining a prothrombotic state. Clinically used anticoagulants are inhibitor of enzymes that are involved in the coagulation pathway, primarily thrombin and factor Xa. The conformational activation of antithrombin by heparin is a critical step in the inhibition of factor Xa by antithrombin. Despite heparin being the most potent physiological activator which enhances the otherwise very lethargic antithrombin inhibition of factor Xa by approximately 1,000-fold, the conventional heparin therapy poses serious complications because of heparin's polyanionic nature and its cross-reactivity. A number of attempts have been carried out in designing alternative non-heparin based conformational activators of antithrombin for factor Xa inhibition. Studies have demonstrated appreciable activation of antithrombin by small organic molecules, but not much is known about the specificity and effects of these molecules on structure and stability. It is assumed that these activators of antithrombin perform their function by binding to heparin binding site. A recently identified cavity which links the heparin binding site to the strand 2A for antithrombin activation also seems to be an ideal target apart the heparin binding site of antithrombin. There are opportunities in discovering more activators from naturally available organic scaffolds and also for modifying such scaffolds for designing better conformational activators with minimum associated complications. This review summarizes the current literature on the mainstay anticoagulants and non-heparin based antithrombin conformation modulators.

Keywords Serine protease inhibitor · Antithrombin · Heparin · Flavonoids · Coagulation

ATIII	Antithrombin
Serpin	Serine protease inhibitor
RCL	Reactive center loop
HIT	Heparin induced thrombocytopenia
LMWH	Low molecular weight heparin
INR	International normalized ratio
UFH	Unfractionated heparin
QTS	Quercetin 3,7,3′,4′-tetrasulfate
ATS	Quercetin 3-acetyl-7,3′,4′-trisulfate
aPTT	Activated partial thromboplastin time
PT	Prothrombin time
TT	Thrombin time
PAAs	Polyacrylic acids
HINT	Hydropathic interaction technique
ECS	Epicatechin sulfate
DHP	Dehydrogenation polymer
CDSO3	Sulfated dehydropolymer of caffeic acid

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Introduction

Coagulation cascade is a series of inactive enzymes that are converted into their active forms through limited proteolysis leading to the generation of thrombin, which further

converts fibrinogen into fibrin and activates platelets leading to the formation of clot [1, 2]. This expeditious outset of clotting is redeemed by a proportionate anticoagulation and fibrinolytic mechanism for quick and efficient response. Under pathological conditions the regulatory hemostasis is overwhelmed, which leads to thrombus formation where the clots are either inadvertently formed inside the vessels without an obvious injury or do not dissolve naturally once formed [3]. Thrombi can occur in veins or arteries and restrict the return of blood to the heart, and can result in pain and swelling as the blood amasses behind the clot. There is a significant risk of the thrombus embolizing and traveling to the lungs and causing pulmonary embolism [4]. The arterial circulation is a high flow, high-pressure environment [5], obstruction to blood flow to the heart or brain might lead to heart attack or stroke. Further, a clot formed in arteries usually originates as a consequence of an injured atherosclerotic plaque, and their formation involves the release of prothrombotic material (such as tissue factor), platelet aggregation and platelet adhesion to the vascular wall [6].

Venous thrombosis is caused either by environmental (acquired) factors or genetic factors. It is a complex disease with an annual incidence of 1–3 per 1,000 per year and is present in all major vascular pathologies encompassing deep-vein thrombosis, myocardial infarction, cerebrovascular disease and peripheral arterial disease [7, 8]. Although it occurs infrequently in young individuals, it is still a major cause of morbidity and mortality during pregnancy and child birth, and is a common cause of disease in young women using oral contraceptives. In addition to appreciable acute morbidity, thrombosis is usually succeeded by a post-thrombotic syndrome with disabling pain and ulceration. The environmental risk factors include surgery, prolonged bed rest, air travel, cancer and use of oral contraceptives and hormone replacement therapy [7].

Mainstay of anticoagulant therapy

The leading treatment of venous thrombosis is the use of anticoagulants, many of which work by inhibiting blood clotting enzymes, primarily thrombin and activated factor X (factor Xa). The clinically used anticoagulants for example are either direct or indirect inhibitors of clotting enzymes. The direct inhibitors interact with the procoagulant enzyme's active site or an exosite and block its activity including thrombin and factor Xa inhibitors [9]. On the other hand the indirect inhibitors enhance the antiproteinase activity of natural anticoagulants; antithrombin and heparin cofactor II. Among the indirect inhibitors the unfractionated heparin, low molecular weight heparins and heparin pentasaccharide are the most widely used [10]. Factor Xa plays a central role in the coagulation cascade as it occupies a point where the intrinsic and extrinsic pathways converge (Fig. 1). Factor Xa with activated factor V (factor Va) as a cofactor converts prothrombin (factor II) to thrombin (factor IIa) [11]. One molecule of factor Xa catalyses the formation of approximately 1,000 thrombin molecules, thus factor Xa remains the primary site of amplification [12]. Because of its pivotal role in thrombogenesis, various thrombin inhibitors are being used to regulate it for the treatment of thrombotic diseases [13]. For this reason, development of medications that inhibit factor Xa is an active and promising area of pharmaceutical research [14].

Limitations of current anticoagulation therapy

The heparins and vitamin K antagonists (e.g., warfarin) have been the most prescribed anticoagulants for over the last 50 years. In spite of its substantial and unparalleled success the current anticoagulation therapy is associated with serious bleeding complications. There is reciprocity between the degree of anticoagulation and severity of bleeding. Further, each anticoagulant is associated with specific complications [15]. The anticoagulant therapy during thrombotic diseases has been seen to cause erratic changes in the vascular wall leading to vascular wall injuries, possibly leading to thrombocytopenic purpura, a condition where the platelets are present but they don′t function properly to agglutinate and stop bleeding [16]. Warfarin carries the risk of serious bleeding and its activity is affected by food and drug interactions. It has a narrow therapeutic window and unpredictable pharmacokinetics with marked inter and intra-individual variability [17]. Warfarin can also induce skin necrosis as a specific adverse reaction [18]. As a consequence a continuous monitoring for accurate dosing is required to ensure that the international normalized ratio is within the therapeutic range since both over anticoagulation and under anticoagulation are associated with an increased risk of hemorrhage and thrombosis respectively. While such a monitoring is inconvenient for both the patients and physicians, it is also costly for the healthcare system. These drawbacks of warfarin and other vitamin K agonists have limited their use for stroke prevention in patients with atrial fibrillation [19].

Heparins are the universal anticoagulants that are associated with several adverse effects and suffer a number of limitations due to heparin's polyanionic and heterogenous nature [20, 21]. The use of heparin is limited to intravenous application and can also lead to hemorrhage, liver toxicity, heparin induced thrombocytopenia (HIT), osteoporosis and variable patient response, besides demanding regular

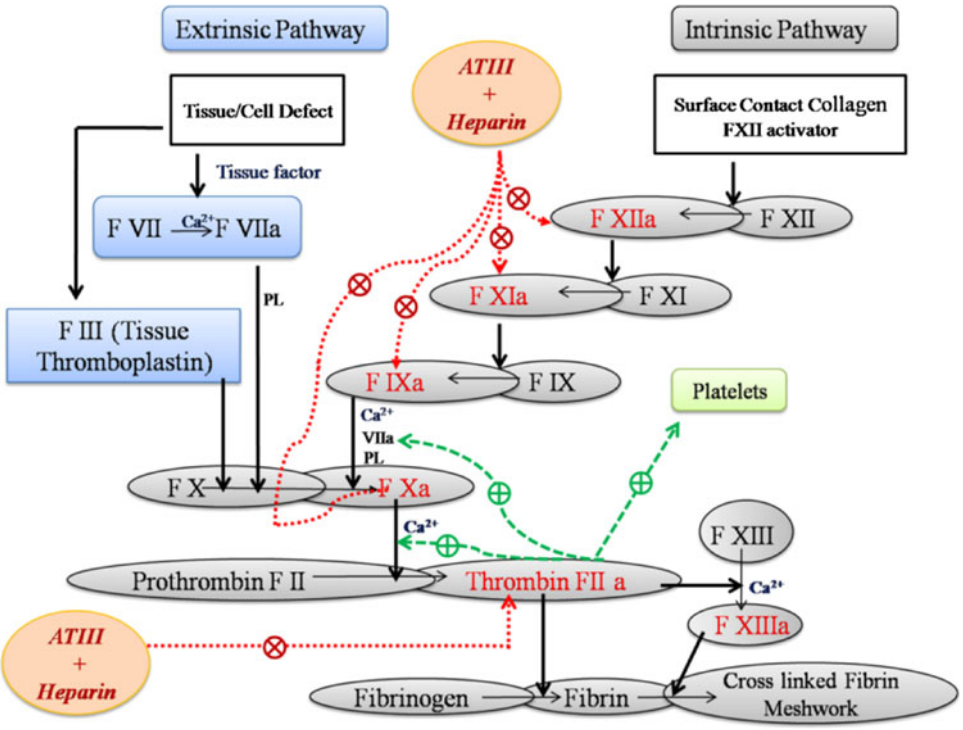

Fig. 1 Coagulation cascade: effect of antithrombin on procoagulant proteins like factor IXa, factor Xa, factor XIa, factor XIIa and thrombin is critical anticoagulant step to maintain regulatory hemostasis

laboratory monitoring for dose adjustment [22, 23]. In addition, the animal origin of the drug is also a cause of concern as recently certain batches of heparin have been associated with anaphylactoid-type reactions, with some leading to hypotension and death. Such reactions were known to occur due to contamination of semi synthetic oversulfated chondroitin sulfate in unfractionated heparin (UFH) preparations [24]. Some of the problems associated with heparin have been rectified with the development of low molecular weight heparins (LMWH) and a minimal antithrombin binding pentasaccharide sequence, fondaparinux. LMWHs are superior to UFH in terms of therapeutic complications since they induce less bleeding complications and have an improved bioavailability. However, like heparin, LMWHs are heterogenous and polydisperse and are associated with HIT and the iatrogenic bleeding risk is not completely eliminated. Even though fondaparinux is completely free of HIT, its synthesis is a complex, multi-step and low yielding procedure [25, 26] and is also associated with bleeding and lacks an effective antidote to reverse excessive anticoagulation [27, 28]. Other shortcomings involved when these are used as the sole agents in coronary angioplasty include their likelihood of accumulation in patients with renal impairment and the risk of catheter thrombosis. Yet, the daily requirement for subcutaneous administration limits the long term use of LMWH and fondaparinux [29]. The rationale behind these limitations of UFH and LMWHs is the profuse negative charge on each polymeric chain generating a massive polyanion [20, 21], which is capable of interacting with a large number of plasma proteins and proteins present on cells lining the vasculature, which likely induce many of the UFH and LMWH complications [30, 31]. Heparin therapy has also been known to decrease plasma antithrombin levels by increasing the hepatic clearance of antithrombin [32, 33].

Antithrombin–heparin activation mechanism and structural features

Antithrombin (ATIII) is the main plasma protein inhibitor of blood coagulation proteinases like thrombin and factor Xa. It belongs to the family of serine protease inhibitors (serpins) which have a common mechanism of inhibition [34–36]. Antithrombin is different than most of the serpins in that its anticoagulant function arises due to high affinity interaction with heparin. Heparin accelerates the antithrombin inhibition of these enzymes to several 1,000-fold by inducing large scale conformational changes. For the induction of conformational change in antithrombin, the minimum necessary heparin species have been determined to be a specific pentasaccharide unit called the DEFGH that is involved in high affinity binding [37, 38]. Although the pentasaccharide is sufficient to accelerate the inhibition of factor Xa; the inhibition of thrombin requires a bridging contribution from heparin for the formation of a ternary complex among antithrombin, thrombin and heparin

species [39]. Heparin binding to antithrombin is a two-step process consisting of an initial weak interaction that induces a protein conformational change leading to the formation of a high affinity binary complex with the cofactor (AT*H) and antithrombin activation [36, 40]. Heparin binding produces a series of conformational change in antithrombin like extension of helix D by forming a 2 turn helix (P-helix) at the N-terminal end and a 1.5 turn extension of D-helix towards the C-terminal end [41, 42]. Moving of strand 3A and strand 5A and expulsion of reactive center loop leads to activated antithrombin [43].

Mechanism of allosteric activation of antithrombin follows suicide substrate inhibition mechanism [41, 44]. The reactive center loop (RCL) (P1–P17) in antithrombin (Fig. 2a) includes a P1–P1′ (Arg393–Ser394) bond which resembles the substrate for factor Xa, thus acting as a "bait" for target enzymes. After docking, the protease cleaves the Arg-Ser bond. Once the bond is cleaved, the protease is covalently linked to the P1 residue and the reactive loop peptide becomes mobile [45, 46]. The reactive loop peptide then hinges on residues P15–P10 and incorporates as a sixth strand into the central A β sheet. Figure 2b shows the region of conformational change on account of heparin binding and the residues involved. The heparin binding domain of antithrombin is formed by positively charged residues of helices A and D, and the polypeptide N-terminus. DEFGH binds in the pentasaccharide binding site (PBS) comprising of residues Arg47, Lys114, Lys125, Phe121, Phe122 and Arg129. On the other hand the full length heparin in addition to PBS binds to the extended heparin binding site, EHBS. The interaction between sulfate and carboxylate groups of DEFGH with positively charged residue of the PBS leads to the transmission of the binding energy to the RCL (as shown in Fig. 2c, d) [47–49]. The studies with truncated saccharides of DEFGH pentasaccharide viz., DEF trisaccharide and the

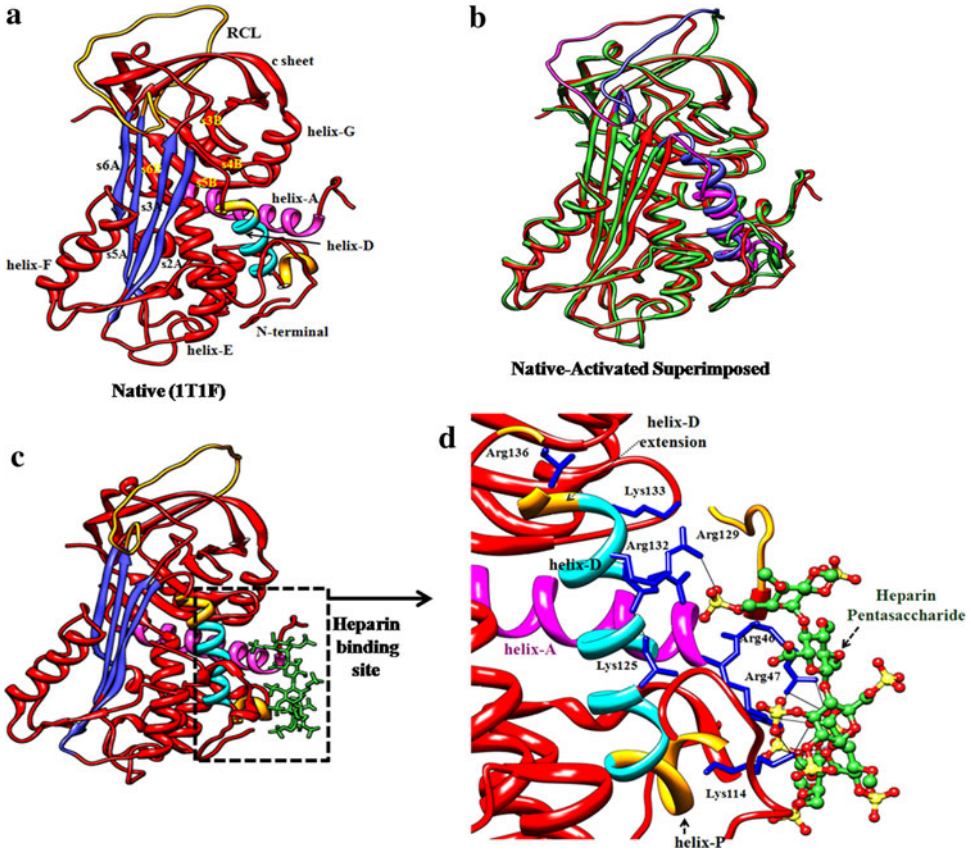

Fig. 2 Conformational changes in cofactor (heparin) bound antithrombin and residues involved in cofactor interaction. **a** represents the native circulating antithrombin in blood, showing several regions that are important in controlling and modulating conformational changes. The reactive centre loop (RCL) is involved in protease recognition and conformational transformation as strand 4A after inhibition. **b** shows the overlap of the native (red) and activated (green) conformation which shows the key structural differences between these states. It illustrates the heparin dependent conformational changes in antithrombin; like extension of helix D by forming a 2 turn helix (P-helix) at the N-terminal end, a 1.5 turn extension of D-helix towards the C-terminal end. Movement of strand 3A and strand 5A and expulsion of RCL. **c** and **d** shows the basic residues in the heparin binding site that interact with the pentasaccharide: Lys-11 and Arg-13 in the N-terminal end; Arg-46 and Arg-47 in the A-helix; and Lys-114, Phe-121, Phe-122, Lys-125 and Arg-129 in the region of the D-helix. The figures were made and superimposed using Chimera

EFGH have revealed that the DEF sequence is crucial for both initial recognition and conformational activation [50].

Search for alternative antithrombotic agents

Out of the numerous attempts to design or discover new molecules as anticoagulants, designing molecules that may activate antithrombin is quite promising [15, 51]. Although much of the trials have been based on the assumption that a saccharide scaffold is critical to induce antithrombin activation. Raghuraman et al. [26] and Correia-da-Silva et al. [52] have designed non-saccharide activators of antithrombin which are likely advantageous over saccharide based heparin mimics. They showed greater non-ionic binding energy in antithrombin recognition, greater hydrophobicity for oral delivery, greater synthetic accessibility and higher specificity of action [26, 52]. Desai et al. [50] have reported the synthesis of sulfated flavonoids acting as activators of antithrombin for accelerated inhibition of factor Xa [26, 53, 54].

Sulfated organic molecules as clotting inhibitors

Sulfated organic molecules are increasingly gaining importance as inhibitors of clotting [15, 52–57]. With the increase in their structural diversity, better modulators are expected from such scaffolds. Various efforts in this direction have resulted in the synthesis of several heparin and heparan sulfate mimetics which mimic the binding of heparin to several coagulation proteins like antithrombin, IIa and factor Xa [58]. These molecules include sulfated flavanoids [53–56], benzofurans [59], isoquinolines [26], sulfated dehydrogenation polymers (DHPs) of lignin type [60] and xanthones [25] (some of them are shown in Fig. 3).

Flavonoids in antithrombotic therapy

Flavonoids represent an array of diverse polyphenol scaffolds that possess structures which are different from heparin and heparan sulfate [61]. In various epidemiological studies it has been reported that the intake of dietary flavonols and flavones is inversely associated with the risk for cardiovascular diseases. This is possibly due to their effect on hemostasis as flavonoids have been reported to inhibit platelet aggregation in vitro. A study by Janssen et al. [62] reports the concentration dependence collagen induced platelet aggregation potential of quercetin, apigenin and catechin.

Flavonoids are also known to inhibit the procoagulant activity of adherent human monocytes. Among a number of flavonoids from different classes tested by Lale et al. [63] 18 compounds showed inhibition of interleukin-1-B induced expression of tissue factor on human monocytes.

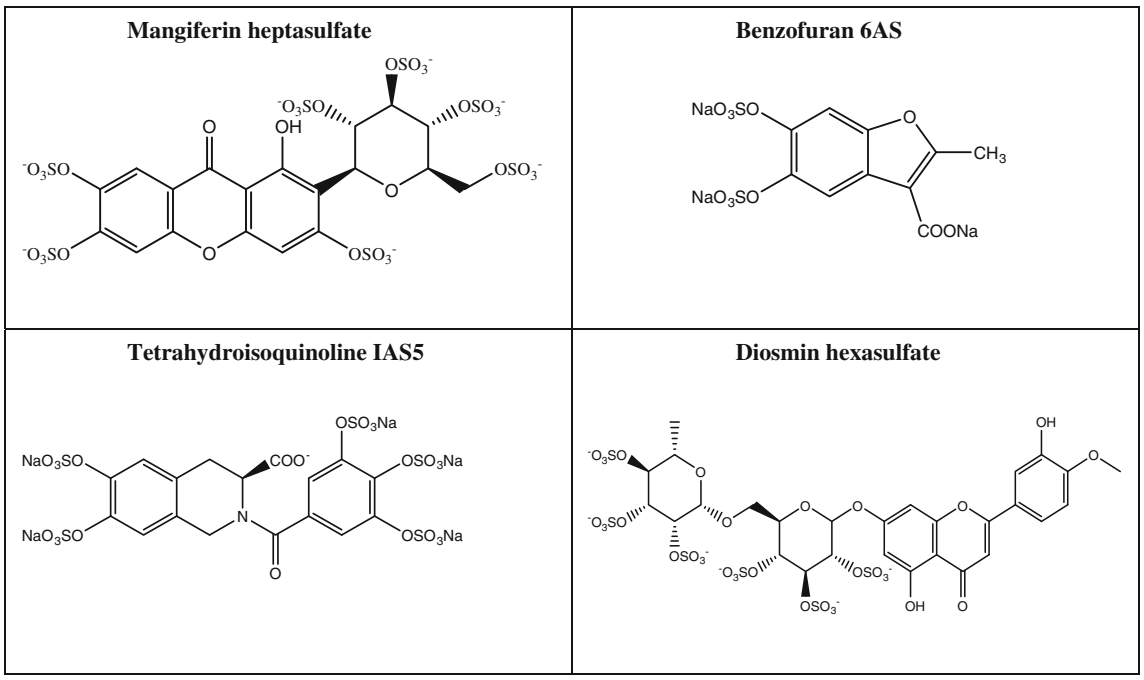

Fig. 3 Structures of some of the sulfated organic scaffolds that have shown promise in non-heparin based activation of antithrombin. The sulfated ligands in the figure are: Mangiferin 2,3,3′,4′,6,6′,7-*O*-heptasulfate, 2-methyl-5,6-*O*-disulfate benzofuran 3-carboxylic acid, tetrahydroisoquinoline 2-benzoyl 3′,4′,6,7-*O*-tetrasulfate 3-carboxylate and diosmin 2″,2‴,3″,3‴,4″,4‴-*O*-hexasulfate

Thus flavonoids inhibit various aspects of blood vessel wall interactions involved in thrombosis, which includes their inhibitory effects on platelets and leukocyte function along with their protective effect on endothelial cells. It suggests that they are of potential interest in the development of inhibitors of such interactions. Flavonoids; taxifolin-7-*O*-rhamnoside, isoquercitrin, quercitrin, quercetin and kaempferol from *Hypericum japonicum* Thunb (Tianjihuang, TJH) have been reported to exhibit anticoagulatory effects. For example, quercitrin and isoquercitrin have been reported to show anticoagulation of activated partial thromboplastin time (aPTT) reagent; taxifolin-7-*O*-rhamnoside and quercetin have been shown to promote coagulation in prothrombin time [PT] assay [64].

The inhibitory effects of sodium quercetin mono and disulfate on pig platelet aggregation induced by thrombin have been reported, the quercetin 7-*O*-sulfate and quercetin 4′,7-*O*-disulfate sodium salts have been shown to execute the inhibitory effects on thrombin-induced platelet aggregation through a mechanism involving Ca^{2+} influx inhibition [65, 66]. It has been further documented that biflavonoids along with ascorbic acid are promising to counteract the tendency to capillary bleeding during the anticoagulant therapy [16].

Anticoagulant potency of natural and synthetic polysulfated flavonoids

A number of studies have been conducted to investigate the anticoagulant activity of flavonoids. Several synthetic sulfated flavonoids with measurable antithrombin activation properties have been identified so far. Gugliemone et al. [55] have investigated quercetin 3-acetyl-7,3′,4′-trisulphate (ATS) and quercetin 3,7,3′,4′-tetrasulphate (QTS) for in vitro anticoagulant activity. Both ATS and QTS have been shown to exhibit anticoagulant action with prolongation of aPTT through heparin cofactor II dependent thrombin inhibition. ATS and QTS have further been evaluated for their effects on human platelet aggregation and it was found that QTS markedly inhibited agonist induced platelet aggregation whereas ATS showed only slight antiplatelet effect [67]. Sulfated flavonoids are hydrophilic in nature with less charge density compared to heparin which fairly suffices the structural requirements of an anticoagulant; however, they exhibit limited anticoagulant potency. In order to augment the anticoagulant potency of flavonoids, several polysulfated oligoflavonoids have been synthesized. These polysulfated oligoflavonoids exhibited a much higher in vitro and in vivo anticoagulant activity compared to their corresponding aglycones. Among the synthesized sulfated polysaccharide-sulfated flavonoid hybrids, 3-*O*-rutinosides: 3″,4″-bis(2-*O*-sulfate ethoxy)-7-(2-*O*-sulfate ethoxy)-rutin and rutin 2″,2‴,3′,3″,3‴,4′,4″,4‴,5,7-*O*-decasulfate were reported inhibiting factor Xa directly and 7-*O*-rutinosides: diosmin 2″,2‴,3″,3‴,4″,4‴-*O*-hexasulfate and hesperidin 2″,2‴,3″,3‴,4″,4‴-*O*-hexasulfate inhibited coagulation by enhancing the inhibition of factor Xa via antithrombin activation [52]. While several synthetic sulfated flavonoids have been shown as direct and antithrombin activated inhibitors of factor Xa, the activation of antithrombin achieved with these molecules is weak.

It has been predicted by hydropathic interaction technique (HINT), a quantitative computerized tool for analysis of molecular interactions that the binding affinity of a flavonoid with antithrombin correlates with its molecular size, wherein an increase in molecular size is associated with an improvement of the binding affinity, with the rationale that a dimeric compound engages both the extended heparin binding site and pentasaccharide binding site of antithrombin simultaneously [68, 69].

Epicatechin sulfate (ECS), designed using HINT analysis by Gunnarsson et al. has been shown to accelerate antithrombin inhibition of factor Xa nearly by tenfold and hence it is the first small non-sugar molecule to activate antithrombin. Further, morin sulfate has been shown to bind to antithrombin nearly fivefold tighter than ECS, and hence represents an interesting lead scaffold for the design of better activators [54]. In addition to flavonoids and their sulfated derivatives, the effects of rutin and hesperidin and their metal chelates Al(III) and Cu(II) complexes on in vitro plasma coagulation assays have been investigated. It was found that all investigated complexes prolonged only aPTT and had no effects on PT and thrombin time (TT) [70].

Benzofurans—as allosteric inhibitors of factor Xa

Chemo-enzymatic preparation of various small organic molecules based on sulfated DHP scaffold has been reported [59]. These synthetic B-5 like monomeric benzofuran derivatives were screened against thrombin and factor Xa. Seven out of seventeen of these molecules were found to be inhibitory although with a weak potency. In addition, the interaction pattern exhibited selectivity of recognition with thrombin and factor Xa recognizing different structural features. These CDSO3 (sulfated dehydropolymer of caffeic acid) based synthetic molecules could be potent functional mimics of heparin [53].

Tetrahydroisoquinolines—as allosteric activators of antithrombin

A pharmacophore based screening employed by Raghuraman et al. [26] has led to the designing of allosteric

activators of antithrombin. A tetrahydroisoquinoline based scaffold was designed to mimic four anionic groups of DEF of pentasaccharide. IAS5 was found to bind antithrombin with an affinity comparable to DEF and also activated antithrombin for factor Xa inhibition nearly 30-fold.

Dehydrogenation polymers or synthetic lignins

Although having no resemblance to heparin scaffold the carboxylic acid based polymers, poly acrylic acids (PAAs) have shown quite a high acceleration of antithrombin inhibition of thrombin and factor Xa, owing to their high charge density comparable to that of heparin [71, 72]. Despite the high activating potency of PAAs they are not useful in vivo [70]. Yet, another report has advanced this concept by preparing sulfated DHPs, wherein sulfated derivatives have shown a further two to three-fold greater potency than the unsulfated parent [60].

Polysulfated Xanthones—an emerging class of compounds with anticoagulant and antiplatelet properties

A new synthetic class of small sulfated molecules has been evaluated for in vivo and in vitro antithrombotic effect. The polysulfated xanthonosides have shown strong anticoagulation potential both in vitro and in vivo. The structure–activity relationship of the tested polysulfated xanthonosides has revealed that mangiferin heptasulfate inhibited factor Xa directly, while persulfated 3,6-(*O*-β-glucopyranosyl) xanthone activated coagulation by a dual mechanism, inhibiting the factor Xa directly and via antithrombin III activation. In addition to anticoagulant effect, two of these xanthonosides are also endowed with antiplatelet effects. Their antiplatelet activity was attributed to the inhibition of arachidonic acid and ADP-induced platelet aggregation. While this family of compounds has a well defined composition, they are merited with a feasible synthesis protocol. These compounds are likely to overcome the bleeding complications and hepatic toxicity owing to their minimal charge density [25].

Conclusion

Despite their proven efficacy, the anticoagulant drugs that directly or indirectly inhibit thrombin and factor Xa, share several limitations. Also most heparin based products are isolated from pig intestine or cattle lungs and are prone to contamination. Fondaparinux is the most widely used synthetic anticoagulant for treatment of deep vein thrombosis which has non animal origin but it is also associated with inappropriate dose–response, excessive bleeding and high cost. There is a pressing need for cost effective oral anticoagulant preferably from natural source. The energy of antithrombin–heparin interaction indicates that ionic (5–6 ionic interactions) and non-ionic interactions contribute 40 and 60 % of the binding free energy respectively [49]. Extensive data is available for the contribution of positively charged arginine and lysine residues in the heparin binding site that are involved in initial heparin binding and conformational activation [73]. Despite contributing more to the binding energy non-ionic interaction involving non-polar groups continue to remain largely unknown. The fundamental basis of the design of non-heparin mimics as activators of antithrombin is based on the assumed requirement of a saccharide skeleton and a minimum size corresponding to five residue sequence for a high affinity interaction with antithrombin. The synthesis of non-sugar molecules exhibiting antithrombin activation for accelerated inhibition of factor Xa is a promising and growing field in anticoagulant research. Flavonoids, xanthones, dihydroxybenzofurans and tetrahydroisoquinoline scaffolds show promise when sulfated at specific locations. Appropriate placement of sulfate and carboxylate groups in similar type of structural scaffold and a comprehensive screening strategy to target antithrombin and to compare the activation rates and binding energies seems to be the way forward. However the molecular basis of the predominant non-ionic contribution in heparin interaction need to be determined for more appropriate design considerations.

Acknowledgments The research in the lab is supported by grants from Department of Biotechnology, University Grant Commission and Indian Council of Medical Research, Government of India. QR is supported by CSIR Senior Research Fellowship. PS is supported by a grant from Rajiv Gandhi National Fellowship.

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