Biochemistry of Anticoagulant Drugs: A molecular approach

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Abstract —

In this review we want to explore the underlying biochemistry of blood coagulation and mechanism of action of widely used anticoagulant drugs which function by targeting the components of biochemical machinery of coagulation pathway. After a compact summary of biochemical incidents involved in blood coagulation, structure-function relationship of drug targets of the various classes of drugs have been discussed. Action of vitamin K dependent inhibitors is responsible for the production of incomplete clotting factors, thus, impairing blood clotting. Synthesis of gamma-carboxyl glutei acid of GLA domain of factors is mediated by the vitamin K peroxide reductive which is inhibited by warfare and other related molecules. Thrombin is the cardinal enzyme in blood coagulation process thus acting as a captivating drug target; PPACK and Argatroban are those drugs which inhibit the enzyme by covalent and non-covalent binding respectively. Another direct inhibitor of thrombin is hirudin, the anticoagulant weapon of leeches, recently being used in clinical treatments, binds in a bidental fashion with thrombin. The anticoagulant of human body, ant thrombin is the drug target for the drug heparin which provides an augmented anticoagulant activity just by functioning as a bridge between thrombin and antithrombin. Pharmacokinetic features of the representative drugs of every class have also been discussed in this review.

Keywords: anticoagulant, hemostatis, thrombomodulin, thrombosis, warfarin

I. Introduction

Management of proper blood flow is a complex and highly structured biological process, with synchronized action of many complementary and opposing mechanisms of influence.A delicate balance in the blood vessels is achieved for permitting unrestricted flow of blood and simultaneously facilitating instantaneous clot formation at the site of injury. Substances which promote coagulation of blood are known as procoagulants whereas substances which inhibit the coagulation of blood are called anticoagulants. In normal physiological condition, effect of anticoagulants suppresses the effect of procoagulants thereby preventing undesirable formation of blood clots.

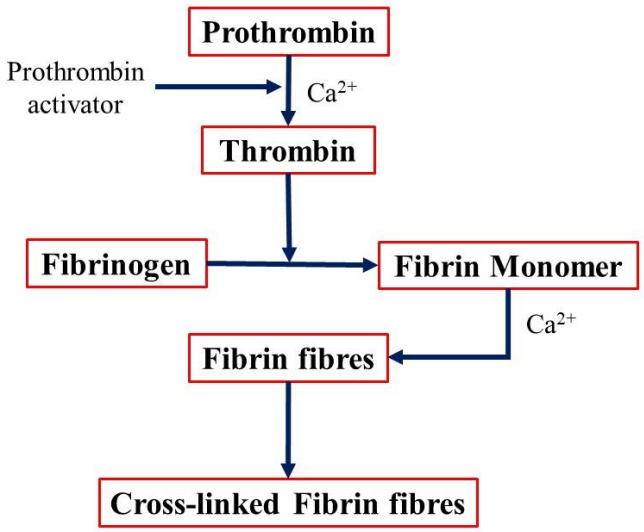


Figure 1: *Blood Coagulation Pathway; Damage to the blood vessel initiates a complex cascade of chemical reactions which produces prothrombin activator; prothrombin activator activates prothrombin by forming thrombin; thrombin acts on fibrinogen to from fibrin monomer; these monomers polymerise to form a cross linked mesh which ensnares blood cells and plasma to form the clot*

Prevention of blood loss, known as hemostasis, is accomplished in a ruptured blood vessel via multistage stage procedure [1]**(Fig. 1)**. Though blood coagulation evolved in humans and animals as a defensive response, some medical conditions and procedures

demand impediment of blood coagulation. There are several lifesaving surgeries like bypass surgery and heart transplant, where blood is passed through an external tubing and where it will eventually coagulate without any external retarding agent. Thrombophilia or hypercoagulability is an abnormal condition where blood in blood vessel is more likely to clot. This condition immensely increases the risk of fatality like Deep Vein Thrombosis (DVT) and pulmonary embolism. Under these circumstances, antithrombotic drugs play an indispensable role by counteracting threats to lethal blood clots.

Furthermore, the multifaceted regulatory framework of blood clotting naturally furnishes copious opportunities for intervention with drugs .Antithrombotic drugs, used for prevention of unwanted blood coagulation, can either act by slowing time of coagulation (anticoagulant drugs) or by preventing aggregation of platelets (ant platelet drugs). A list of currently marketed and clinically used anticoagulant drugs is introduced below **(Table 1).**

Table 1: List of clinically applied drugs[2]

Mechanism of action	Chemical Class	Generic Name	Trade Name
Activation of Antithrombin cofactor	polysaccharide Anionic	Heparin	Heparin
		Danaparoid	Orgaran
		Dalteparin	Fragmin
		Tinzaparin	Innohep
		Enoxaparin	Lovenox
		Fondaparinux	Arixtra
Vitamin K antagonist	Coumarin	Warfarin	Coumadin
Direct “bivalent” thrombin inhibitor	Polypeptide (Recombinant hirudin analogue)	Lepirudin	Refludan
		Bivalirudin	Angiomax
Direct active-site thrombin inhibitor	Small-molecule Arg-based substrate analogue	Argatroban	Novastan

II. Discussion

1. Biochemistryof blood coagulation process

Blood coagulation is one of the most complex biochemical processes in human body. For understanding the mechanism of action of antithrombotic drugs it is essential to discuss about the molecular basis of blood coagulation process. So,in this section we will provide a short insight to the molecular biology of blood clot formation and components required for the process.

1.1. Scheme of Blood coagulation pathway

Initiation of blood clotting, when a vessel is traumatized, can be carried out via two pathways- extrinsic pathway and intrinsic pathway**(Fig. 2)**.These pathways provide cascades of chemical reactions which comprise of proteins called factors (denoted by roman numerical). Many of these factors in their activated forms have proteolytic property which further activates another factor [3], [4]. These cascades ultimately result in to activation of thrombin from prothrombin.Intrinsic pathway is triggered by the trauma to the blood or exposure blood to the collagen, which activates factor XII whereas the extrinsic pathway is instigated when blood comes in contact with extra vascular tissues, leading to the release of tissue factor. Ultimately, both pathways act simultaneouslyinformationofbloodclot.

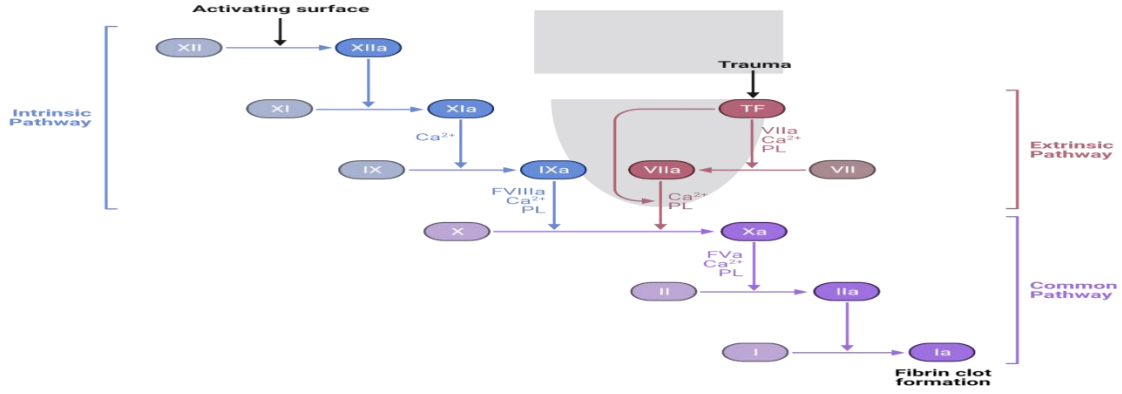


Figure 2*: Reactions involved in the blood coagulation cascade*

The following step of the coagulation pathway is fibrinogen activation. Thrombin is a proteolysis enzyme which hydrolyses fibrinogen to fibrin monomer. These monomers are associated by no covalent interaction between themselves and then covalently cross linked by fibrin stabilizing factor, followed by the attachment of platelets to the fibrin mesh to form blood clot.

Till now we have discussed about the factors which take part in the formation of blood clot or procoagulants. Discussing about the anticoagulants which are inherently present in the blood for prevention of coagulation in the vessel, we need to look up to the most crucial anticoagulant factors, which includes,

(1) Smoothness of the cell surface which prevents activation of intrinsic pathway;
(2) A layer of glycocalyx (a mucopolysaccharide adsorbed on the endothelial cell surface)which repels platelets and factors, thereby preventingclot activation;
(3) Thrombomodulin which is an endothelial membrane-bound protein, responsible for binding thrombin and hence minimising chances of coagulation.

1.2. Features of protein factors

Proteins which are involved in blood coagulation are known as factors. They are all heat labile and multi-domain proteins. Most of them are synthesized in liver in the form of inactivated zymogenand activated during coagulation process by proteolysis reactions. General features of these factors are stated in **Table 2**.

Table 2 General features of blood coagulation cofactors*[5], [6]

Component	Generic Names	Number of amino acids residues	Molecular Weight (kDa)	Function
Factor II	Prothrombin	579	72	Protease Zymogen
Factor III	Tissue Factor	219	37-40	cofactor
Factor V	Ac-globulin	2196	330	Cofactor
Factor VII	Proconvertin	406	50	Protease Zymogen
Factor VIII	Antihemophilic Factor A	2332	~300	Cofactor
Factor IX	Antihemophilic Factor B	415	57	Protease Zymogen
Factor X	Stuart Factor	448	59	Protease Zymogen
Factor XI	Antihemophilic Factor C	607	160	Protease Zymogen
Factor XII	Hageman Factor	596	80	Protease Zymogen
Factor XIII	Fibrin stabilizing Factor	731	325	Crosslinking Enzyme
Prekalikrein	Fletcher Factor	619	85	Protease Zymogen
High molecular weight kininogen	Fitzgerald factor	626	120	Cofactor

*Factor IV is Ca^{2+} ion and Factor I is Fibrinogen which are not included in this table.

Structural domains which are indispensable forGLA domain is especially important for the activity of factor-containing domain[7]. It anchors the proteins to the activated membrane**(Fig. 3)**. GLA domain can contain nine or more post-translationally modifiedg-carboxylic glutamic acid which is essential for activity of GLA domain as it helps the factors to interact with membrane, this interaction is mediated by Ca^{2+}[8].

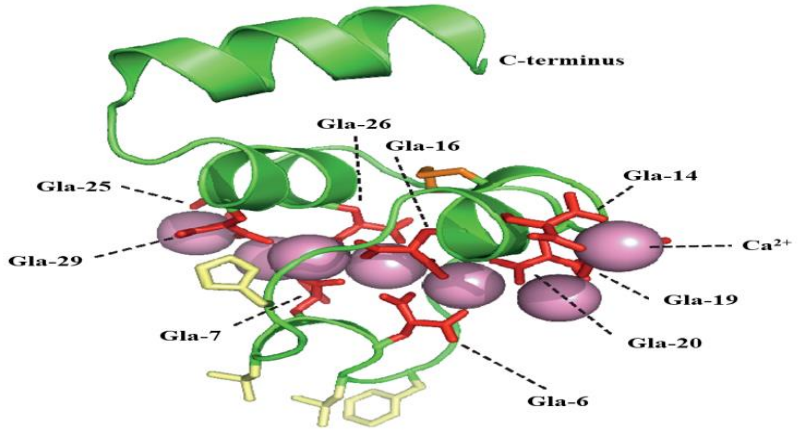


Figure 3: *Structure of GLA domain coordinated with calcium ions; nine gamma carboxy glutamic acids are marked in red and purple spheres represents the calcium ions*

Majority of these factors contain a protease as their main catalytic domain. These proteins belong to the serine protease superfamily which utilises serine, histidine, aspartate catalytic triad for cleavage of peptide bonds. All the proteolytic factors which are involved in the coagulation cascade cleaves the peptide bond next to the Arginine residue. Arginine has a positively

charged guanidine side chain which binds to the substrate specificity pocket of the enzyme and is then cleaved by the serine residue in charged relay mechanism[9].

1.3. Activation and assembly of factors

A factor is activated by peptide cleavage at one or more arginine sites which consequently liberates protease domain of that factor. This independent protease domain acts on the next factor of coagulation cascade to activate it and this sequential activation of factors continues. Family wise domain arrangements and cleavage site of these factors are specified in the following figure**(Fig. 4)**.

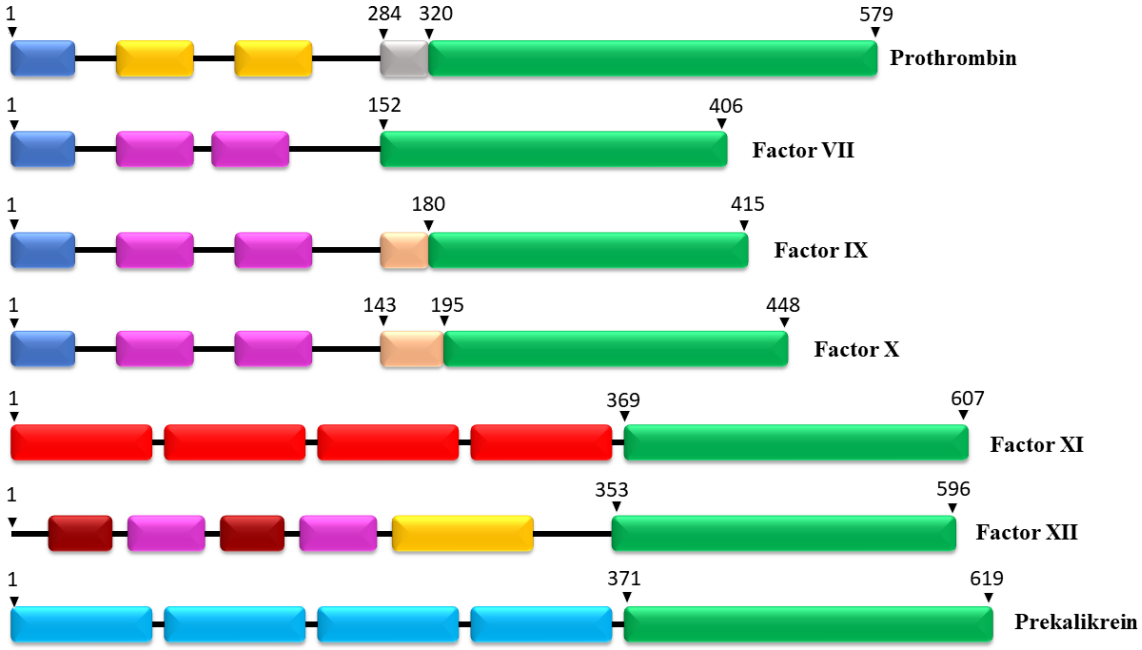


Figure 4: *Bar diagrams representing domains and cleavage site of different coagulation factors; the colours represent as follows:GLA domain- blue, Kringle domain- orange, protease -green, EGF domain-magenta, apple domain – red, fibronectin domain – cyan, activation peptide -brown. The grey bar in prothrombinis an activation peptide which remains as A chain in mature thrombin.*

Blood coagulation (in extrinsic pathway)is initiated by binding of activated FVIIa (presently at very low concentration, 1-2% of total FVII) to membrane bound protein Tissue factor (TF). This binding of the protein to the phospholipid membrane surface is accomplished by the GLA domain at the N-terminalend of the factor. This FVIIa/TF is an active"tenase" enzyme complex which can act upon FX. Activated FX,in the form of FXa, can act on small amount of prothrombin to form thrombin which further activates FV. This newly formed FVa interacts with previously membrane bound FXa and forms a highly effectiveprothrombinase enzyme complex which is 300,000 time more active than FXa alone[10]. Now as bulk amounts of thrombin areproduced, blood coagulation takes place in anincreased pace**(Fig. 5)**.Clotting is accelerated bythrombin,FXa and FVIIa/TF as they furtheractivate FVII and hence enhances local concentration of FVIIa which finally results into the formation of more FVIIa/TF complex andspeeding upthe process of coagulation.

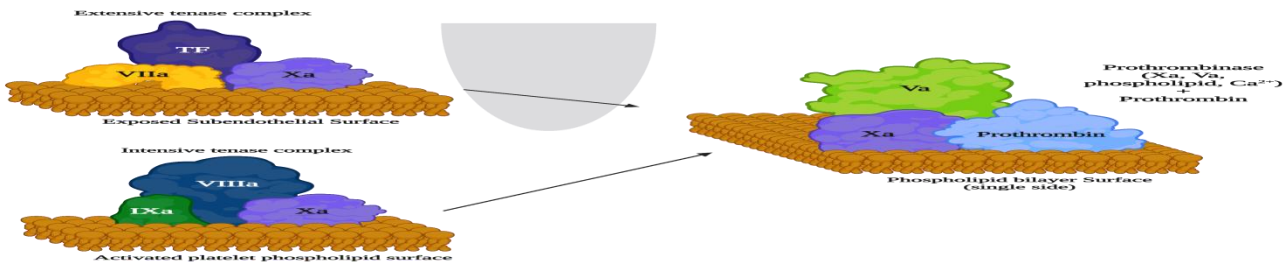


Figure 5: *Protein assemblies in coagulation cascade; The most important assemblies in coagulation cascade are 'tenase' complexes. Intrinsic tenase complex is made up of FVIIIa, IXa and extrinsic tenase complex is made up of TF and VIIa both of them activates FX to FXa. All of these assemblies are structured over phospholipid surfaces and utilise their GLA domain to attach non-covalently to the surface.*

The extrinsic pathway, is activated by contact of blood with negatively charged surface like silica or clay. FXII binds to these surfaces resulting into opening of an activated single chain which cleavesplasma prekallikrein. Now, plasma kallikrein forms

more FXIIa and hence this reciprocal activation elevates the extent of coagulation. FXIIa cleaves another substrate FXI which consecutively activates FXI.In the following step, FXIa along with previously activated FVIIIa forms another tenase complex **(Fig. 5)** which successively activates FX and blood coagulation takes place in the similar manner as stated in the case of intrinsic pathway[5].

1.4. Fibrin formation and crosslinking

Fibrinogen is a polymeric protein which consists of three sets of polypeptide chain, denoted by Aa, Bband g. Fibrinogen is a heterodimer as it is formed by a pair of each type of polypeptide chain, hence, atotal six chains.Completely assembled fibrinogen protein extendsupto 48 nm where six chains are linked together by total 29 disulphide bonds**(Fig 6)**. It should be noted that fibrinogen is N-glycosylated at total four sites of Bb and gchains. It is clear from the structure of fibrinogen, that there are two globular regions- central (E) and peripheral (D) which are connected by a long-coiled coil[11].

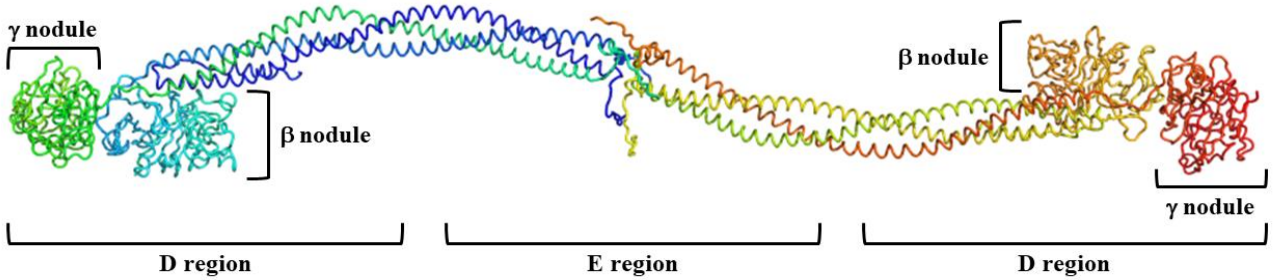


Figure 6*: Structure of fibrinogen (PDB ID- 1EI3); there are two globular D regions which are connected by elongated E region. Each D region consists of one beta chain and one gamma chain. E region is a coiled coil made up by two alpha chains. N-terminal of beta and alpha chains also known as fibrinopeptides, are projected outside from the central E region.*

The principal action of thrombin is to cleave the N-terminal peptide from the Aa and Bbchains. This peptide cleavage permits strong non-covalent interaction between fibrin monomer which ultimately leads to the formation of fibrin stands. This polymer attains further stabilization by transglutaminase (FXIIIa), which covalently links fibrin strands by creating isopeptide bonds by lysine and glutamine**(Fig. 7)**. The covalent cross linkage takes place between lysine-406 of one gamma chain and glutamine-398/399 of the gamma chain at another fibrin monomer[12].

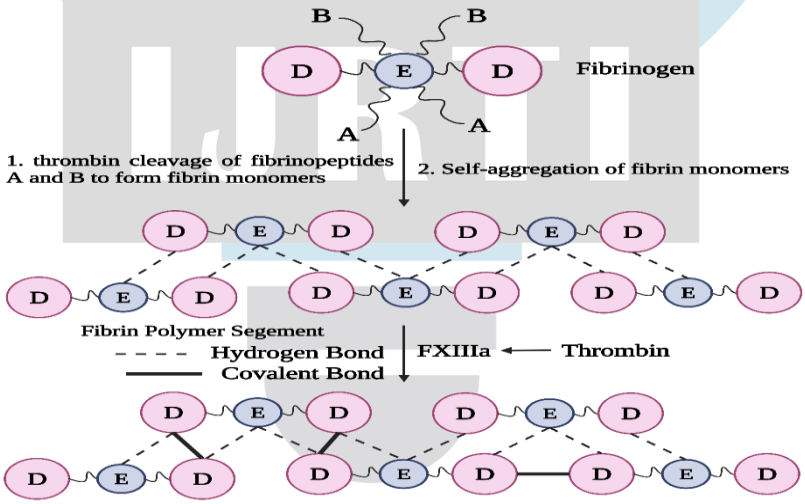


Figure 7:*Assembly offibrin monomer and crosslinking; fibrinopeptides are cleaved by thrombin resulting in the generation of active fibrin monomer which are aggregated by non-covalent interactions; Factor XIIa or transglutaminase which is activated by thrombin covalently links monomer to form a mesh.*

2. Anticoagulant Drugs

The first anticoagulant discovered was hirudin, in 1884 by Haycraft[13], from the saliva of medicinal leech but it was used clinically when it was produced by genetic engineering in 1986. So, first clinically used anticoagulant drug was heparin. Due to the outbreak of world war I, research was directed towards procoagulant studies than anticoagulant studies. In 1916, a medical student at John Hopkins university, McLean who was studying procoagulant activity of alcoholic extracts from brain, liver and heart of the dogs discovered a compound that could inhibit blood coagulation. Two years later, Howell isolated the active compound and named it “Heparin”[14]. In 1933, Charles and Scott purified and crystalized heparin and started to use it on

humans[15]. Another singular incident of discovery of anticoagulant started at North Dakota and Alberta when cattle suffered a mysterious haemorrhagic disease, later proved to be caused because of the presence of dicoumarol in the spoiled clover. In 1945, Link et. Al. decided to test coumarin derivatives as rodenticide and warfarin was introduced. It was considered extremely toxic for humans but unsuccessful suicide attempt by a navy inductee with 567 mg of warfarin proved that it was not as toxic as believed. This paved the path of commercialization of warfarin[16].

2.1. Vitamin K dependent Inhibitors

As stated previously, the formation of clot involves the interaction between factors and in most of the cases this interaction takes place on the cell surface. GLA domain facilitates theCa^{2+} mediated protein membrane interaction hence one can say GLA to be an essential feature for blood coagulation.GLA domain is produced in liver by post translational modification of glutamic acid which is carbonylated at the g position by the pathway known as Vitamin K dependent carboxylation system[7].

Carboxylation reaction is accomplished by the enzyme g-glutamyl carboxylase which needs CO_2as carboxyl source and O_2**(Fig. 8)**. It also requires Vitamin K as a cofactor which is successively converted into an epoxide. But the hydroquinone form needs to be regenerated for the catalytic activity of gamma glutamyl carboxylase, and this is achieved by the oxidoreductase enzyme Vitamin K epoxide reductase[17], this cyclic conversion is known as Vitamin K cycle[18]. Warfarin and other related molecules partially mimic the structure of Vitamin K and hence can bind with vitamin K epoxide reductase.However, the enzyme canno longer act upon those molecules, thereby, leading to the inhibition of the enzyme which further leads to the arrest of blood coagulation.

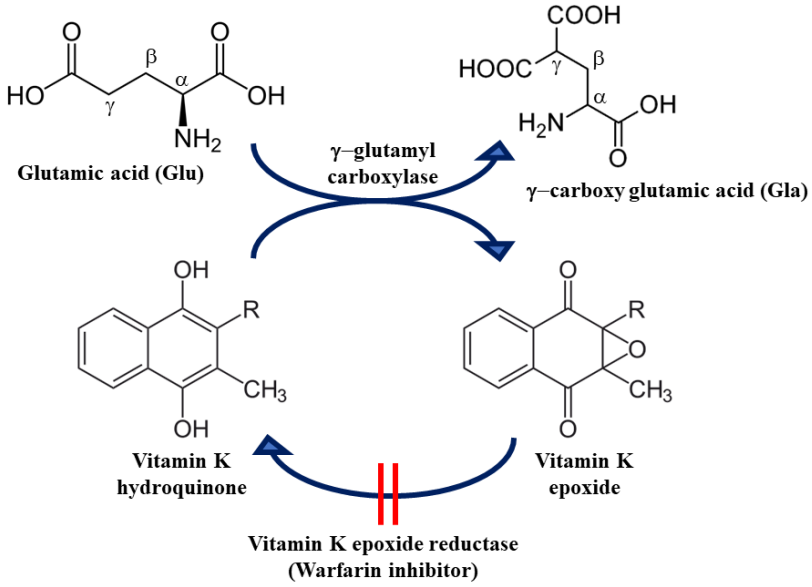


Figure 8: *Vitamin K Cycle; gamma position of glutamic acid is carboxylated during post-translational modification using gamma-glutamyl carboxylase enzyme which uses Vitamin K as a cofactor and oxidises it to the corresponding epoxide form. Active reduced form of vitamin K is regenerated by the enzyme Vitamin K Epoxide reductase and thus the catalytic cycle can be carried on.*

Vitamin K epoxide reductase (VKOR) is a type IIIintegral membrane protein[19], [20].It has 263 amino acid residues spanning across three transmembrane domains. Topology of the domains[21] are depicted in the following figure**(Fig. 9)**. Active site of this enzyme lies in the CXXC motif[22](which is the characteristic motif of thioredoxin enzyme family) inside the third transmembrane helix located near the lumen.

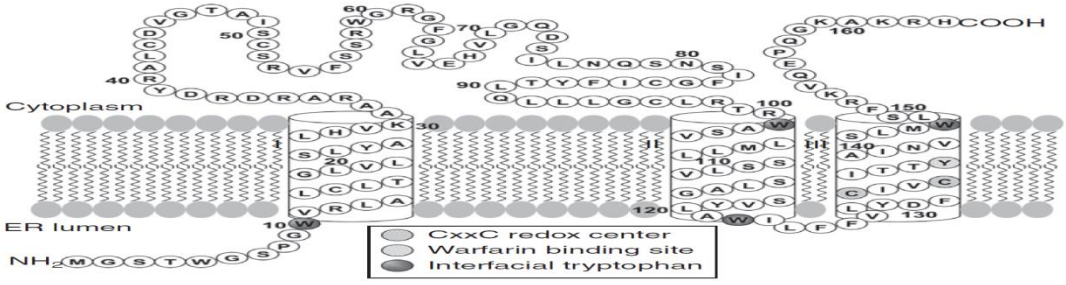


Figure 9*: Proposed topology diagram of Vitamin K Epoxide reductase; different sites are marked by hatch marks*

Mechanism of action of this enzyme was proposed by Silverman[23]. According tothis mechanism, reduction of disulphide bonds is the key for the activation of the enzyme. Further, the epoxide is protonated by a nearby residue which is probably Asp103. Now, due to the instability of the protonated three-member epoxide ring, the nearby sulphidesactupon the sterically less-

congested carbon ring and cleaves.After which, the alcohol group is re-protonated and ultimately leaves the system concerted reductive elimination reaction**(Fig. 10).**

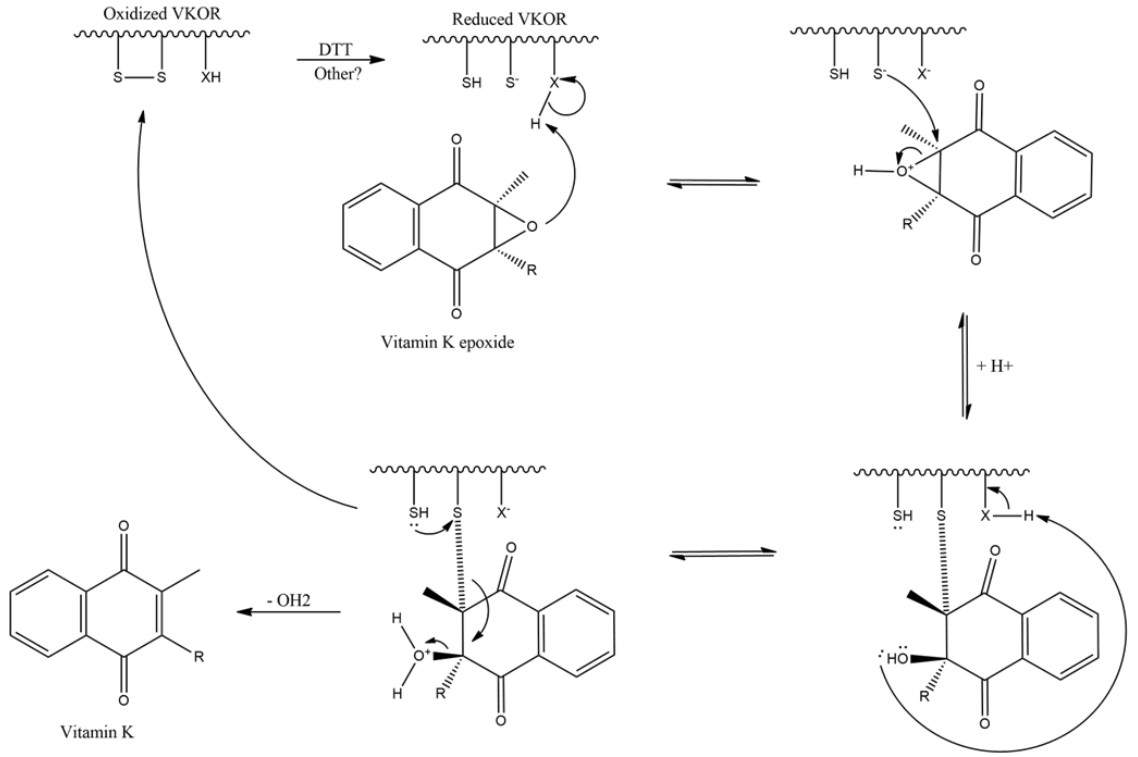
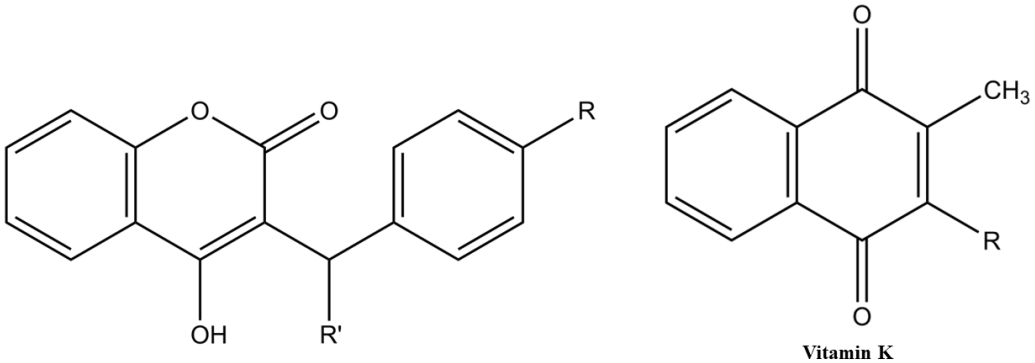


Figure10: *Mechanism of Vitamin K Epoxide reductase activity proposed by Silverman*

Warfarin (3-(a-acetonylbenzyl)-4-hydroxycoumarin), the most prescribed oral anticoagulant drug, contains a coumarin moiety within**(Fig. 11)**, which is essential for its activity. It inhibits the enzyme VKOR, thus,discontinuing the regeneration of KH_2 from KO,thus, leading tothe starvation of Vitamin K, thereby, causing thegeneration of under-developed factors that are biologically inoperative and consequentially blood coagulation ceases.



Names	R	R'
Warfarin	H	CH_2COCH_3
Phenprocouman	H	CH_2CH_3
Acenocoumarol	NO_2	CH_2COCH_3

Vitamin K

Names	R
Vitamin K_1	C_{20} phytyl
Vitamin K_2	polyprenyl

Figure 11: *Structure of Warfarin and Vitamin K derivatives*

Binding of warfarin to VKOR is very tight and thus thought to be irreversible. Silverman, based on the structural similarity between Vitamin K and warfarin as well as accounting for the mechanism of VKOR activity, proposed a mechanism for irreversible inactivation by warfarin[24]. In his hypothesis, he proposed the binding of deprotonated warfarin to the reduced form of enzyme. Further experimental data suggested that warfarin attaches to the oxidised form rather than the reduced one. It was reported that inhibition of VKOR by warfarin was highest and happens more rapidly, when the enzyme was subjected to warfarin prior to its contact to DTT. However, preincubation of VKOR with DTT, before warfarin incubation, reduces warfarin inhibition of VKOR activity[25].

Most currently, various derivatives of warfarin have been synthesized and their activity on VKOR are being surveyed[26]. It has been observed that, when the -OH in the 4th position is replaced by -SH the activity of enzyme is decreased by 8-fold. Substituents

at position 3 have been shown to be important for binding, for example, as natural compound ferulenol, is almost 22 times more inactivating than that of warfarin. Hence, the following features about the mechanism of action of warfarin and related drugs can be inferred. Firstly, warfarin binds to the active site of enzyme VKOR. Secondly, inhibition by warfarin is noncovalent and most importantly, warfarin binds to the enzyme because it mimics the transition state of the reductive elimination step **(Fig. 12)**[26].

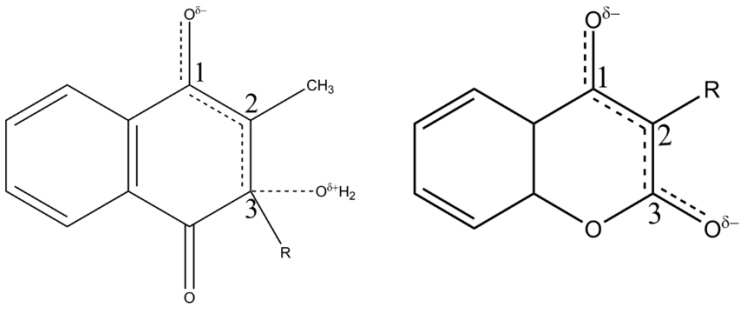


Figure 12:*Structural similarity between transition state of reductive elimination (right) and deprotonated form of warfarin*

Detection of VKOR gene[27], [28] enables in order to further elucidate the binding of warfarin to the enzyme from the molecular perspective. It has been observed that three mutations in the recombinant protein VKORY139C, Y139S, or Y139F,are warfarin resistant[29], [30]. Mutation of Tyr-139 toPhe, makes the VKOR resistant to warfarin, but still retains the actual enzyme activity. Again, it is clear from the topology diagram that, Tyr-139 lies as the same side of the helix that of active cystine residues. So, these observation leads to the conclusion that Tyr-139 or specifically hydroxyl group of tyrosine plays a key role in warfarin binding[30].

The arena of orally administered anticoagulant drug is dominated by warfarin. It is used as a racemic mixture and nobenefit is observed by administrating only one enantiomer. Because of its mechanism of action, the complete pharmacodynamic effect to a dose, takes almost 2-5 days. The main enzyme responsible for the metabolism of warfarin is cytochrome P450 (CYP2C9). It converts (S)-warfarin to its inactivated oxidised form. Warfarin can also be metabolised by reductases which produces compounds with minimum anticoagulant activity. Thus, an entire dose of warfarin can be excreted through urine.

2.2. Synthetic Thrombin Inhibitor

Thrombin is the central enzyme in the blood coagulation cascadebecause its converts factor XI, VII, V, XIII to their respective activated form and most importantly thrombin generates fibrin monomer form fibrinogen[31]. Therefore, the development of novel therapeutically-effective drugswere an alluring prospect in targeting thrombin. Drugs, using both covalent and non-covalent mechanism of action, has been designed for inhibition of thrombin.

Thrombin is constituted of two polypeptide chains:A chain (36 residues) and B chain (259 residues). These two chains are attached by disulphide bonds between Cys-1 and Cys-122[32]. Previous studies on A chain received little interest in the field of research as it was thought to be an additional appendage to the catalytic B chain. Later it was revealed that A chain plays a vital role for stabilization of B chain as mutations in A chain resulted into severe bleeding[33], [34]. B chain is comprised of two asymmetrically associated six stranded b-barrels which comes together to accommodatethe catalytic triad of the enzyme i.e.,His-57, Asp-102, Ser-195. Thrombin is normally represented in Bode orientation **(Fig. 13)**where small A chain is placed behind the B chain[35]. Here,the active site hasbeen viewed in the centre, apart from two distinctive Exosites. These sites are rich in positively charged residues like Arg/Lys. Exosite I is situated at the right-hand lower side and interacts with substrates like fibrinogen and thrombomodulin. Exosite II is located at left hand upper side and binds the molecules of heparin, dermatan etc. There are other substrate specific pockets responsible for accommodation of specific ligand groups, thus contributing to the overall specificity of thrombin (sites are designated by S1, S2, S3 etc)[36].

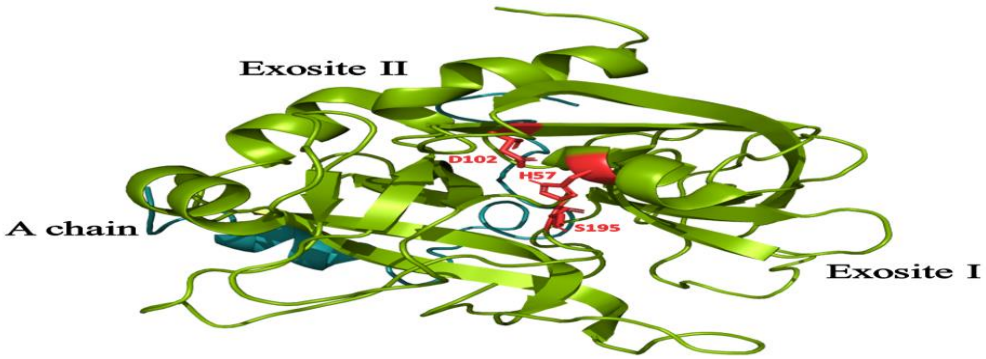
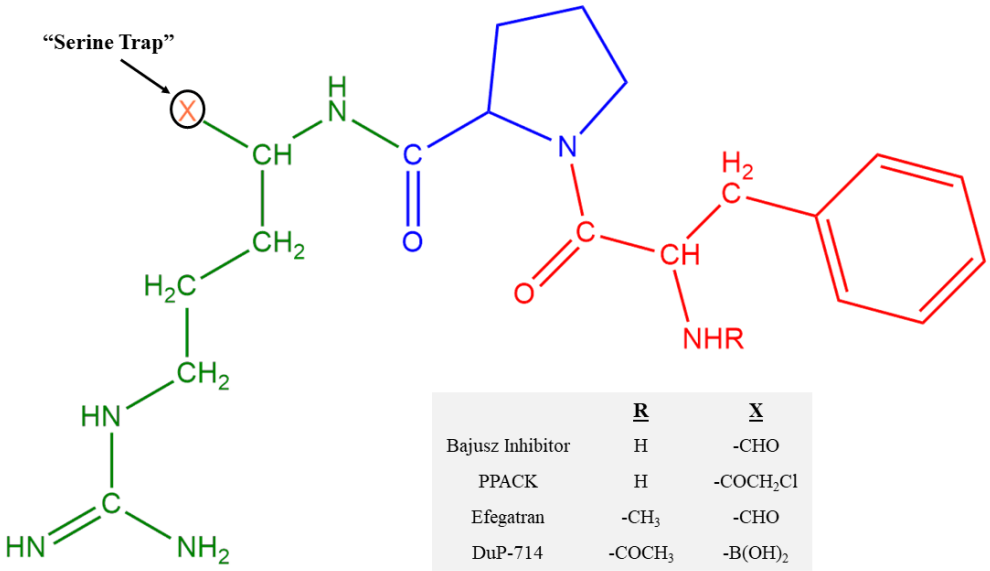


Figure 13:*Structure and important sites of thrombin; thrombin is viewed in standard Bode orientation where A chain (blue) is placed behind the B chain (green); active site residues (red) are situated in the centre between two beta barrels (PDB ID 1ETT)* [37].

Thrombin is a chymotrypsin-like serin protease enzyme, its mechanism of action has been reviewed in many resources[38], [39]. Thrombin selectively cleaves the Arg residue (P1 residue) and it is often found that the next residue is Pro (P2 residue). Early anticoagulants based on inhibition of thrombin were designed by imitating endogenous ligand fibrinogen A. Bajusz designed a series of tripeptide aldehyde developed after fibrinogen A peptide cleavage site. One class of these tripeptide i.e., D-Phe-Pro-Arg-CHO**(Fig. 14)**has been found out to be the most efficient in terms of clotting time[40]. This molecule can bind to the active site as it has same structure as that of endogenous substrate, Arg at P1 and Pro next to it. The active serine residue attacks at the aldehyde and the reaction can be said to be arrested at that stage by forming a hemiacetal linkage**(Fig. 15)**, here the aldehydic group acts a "Serine trap".



	R	X
Bajusz Inhibitor	H	-CHO
PPACK	H	$-COCH_2Cl$
Efegatran	$-CH_3$	-CHO
DuP-714	$-COCH_3$	$-B(OH)_2$

Figure 14: *Structures of covalent thrombin inhibitors; most of them utilises the D-Phe-Pro-Arg sequence to bind with the active site*

Further investigation on this class of drugs led to development of more potent thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (PPACK)**(Fig. 14)**. When PPACKsare attached to the thrombin Ser-195 and His-57, both are linked covalently to the drug. His-57 residue is appended to the chloromethylketone trap and Pro, D-Phe are stabilised in the S2 and S4 sites, respectively. In case of boronic acid mediated inhibitors, boronic ester linkage is formed at the active Ser-195 residue[41]. Many drugs of this class portray high anticoagulant activity which is principally attributed to their covalent bond forming mechanism of binding[42].

However, it must be mentioned that serine trap concept has many potential drawbacks. For example, these drugs fail to achieve necessary efficacy as they exhibit slow binding kinetics. Further studies have revealed that slow binding inhibitors are less successful than fast binding inhibitors. Again, nonspecific covalent binding might lead to immunological responses and other side effects.

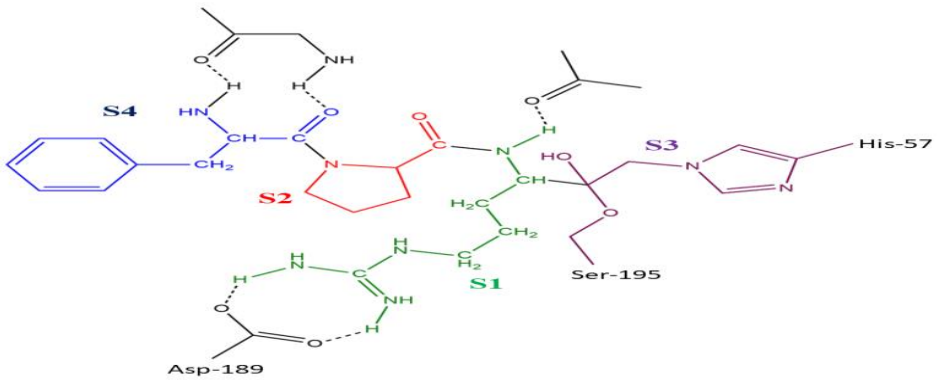


Figure 15:*Covalent binding mode of thrombin inhibitor PPACK; phenyl side chain occupies the S4 cleft whereas the proline side chain occupies the S2 cleft. Ser195 and His-57 both are trapped covalently within the molecule*

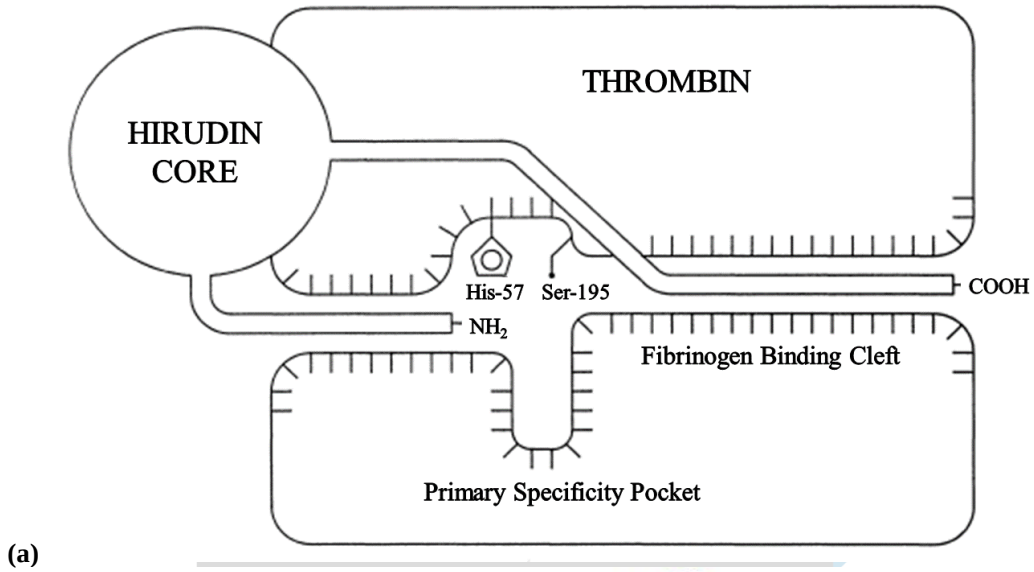
Parallel to the 'serin trap approach', another class of drug was developed which are not dependent on covalent bond formation with active serine residues[43]. Two initial examples of this class are NPAP (naphthylsulfonyl-glycyl-4-AmidinoPhenylAlaninePiperidide) and argatroban[44]. Both of these drugshave been designed earlier after the prototype, N-

tosyl-arginine methyl ester (TAME). Argatroban is a type of TAME where methyl ester is substituted by amide. Toxicity due to sulfonyl group is counteracted with carboxylic acid group. X-ray structure of argatroban bound thrombin showed that the S1 pocket is occupied by the guanidine side chain, an orientation different from that of serine trap inhibitors or PPACK and consequently guanidine can make an ionic bond with Asp-189[45]. Furthermore, a part of piperidine ring along with methyl appendage is inserted into the S2 pocket tightly and the carboxylic group is pointed towards the oxyanion hole forming hydrogen bonds with Ser-195. Another unique feature of argratroban is that it can bind to both soluble thrombin and clot-bound thrombin[46].

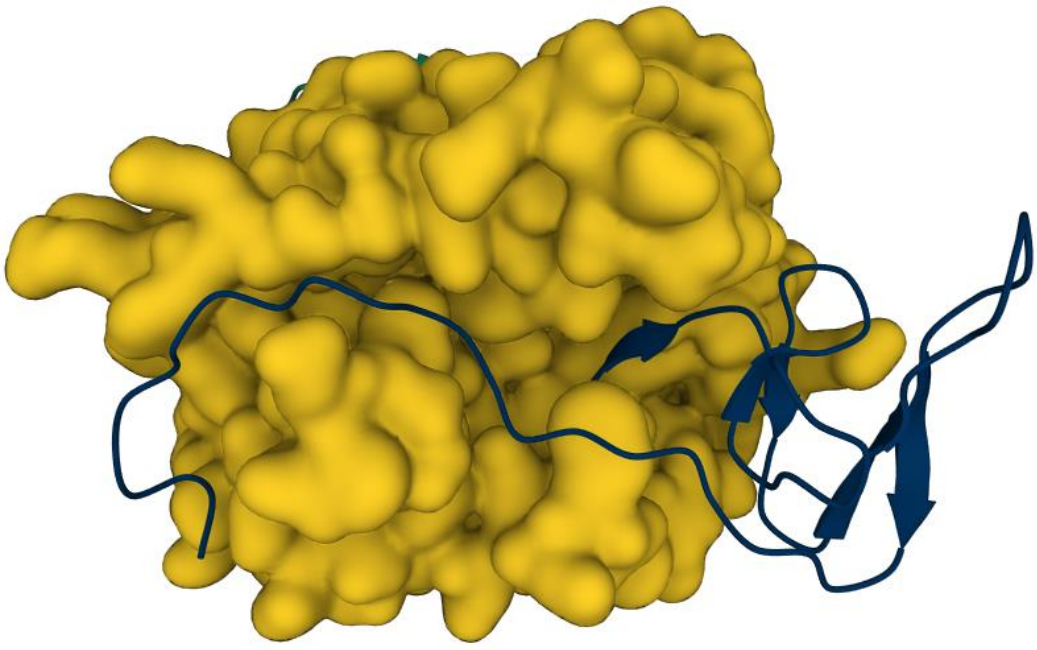
2.3. Hirudin and related derivatives

Hirudin is an anticoagulant[47], extracted from medicinal leech*Hirudo medicinalis*. It prevents coagulation of blood during blood extraction and even after the removal of the leech, it continues to bleed due to the effect of hirudin. It is another example of an inhibitor, which directly targets the active site of thrombin for its anticoagulant activity.

Hirudin is a family of around 20 related small proteins or polypeptides having 65 to 66 residues (molecular mass around 7kDa), where the tyrosine at 63 position is O-sulphated. The structure of hirudin[48] is stabilised by three disulphide bridges. There are three distinctive regions in the three-dimensional structure of hirudin, first one is a central core made of 3-30 residues (in other isoforms 37-46, 56-57), second there is a "finger" of 31-36 residues and a disordered C-terminus loop. Therapeutically used hirudin are produced by recombinant technology using yeast so they lack the sulphate group at Tyr-63 but nevertheless they are highly selective and efficacious towards thrombin[49].



(a)



(b)

Figure 16: *Binding of hirudin with thrombin;* ***(a)*** *schematic diagram showing binding of C-terminal of hirudin at the fibrinogen binding cleft and binding of N-terminal of hirudin at catalytic active site;* ***(b)*** *structure showing the binding of hirudin (blue) at fibrinogen binding site of thrombin (yellow) (PDB ID 1HRT)*[50].

Binding of hirudin to thrombin is elucidated by x-ray crystallography**(Fig. 16)**. This binding is very tight as it is a result of interaction of hirudin with active site of thrombin as well as fibrinogen binding Exosite I so hirudin can be thought to be a bivalent ligand[51].The long chain of C-terminus of hirudin attaches to the positively charged fibrinogen binding site whereas the N-terminus simultaneously occupies the main active site of the thrombin[52]. Val-1 and Tyr-3 side chains plays an important role in N-terminus binding as they occupy S2 and S3 pockets respectively by making several hydrophobic interactions. The primary substrate-specificity site S1 remains vacant on hirudin binding[53]. Desirudin is used in hip or knee surgery for prevention of DVT. Lepirudin, another variant of hirudin where Val-Val is substituted by Leu-Thr, is used as a substitute of heparin in HIT patients[54].

Major structural alterations in hirudin lead to the discovery of hirudin like molecules namely, "Hirugens" and "Hirulogs"[55]. The Hirugens are peptide fragments containing only the C-terminal fibrinogen binding domain. "Hirulogs"[56] are the peptide analogues of hirudin where the nonbinding core sequence is eliminated and thrombin binding sequence are attached by Poly-Gly linker. One of the most important examples of hirugen is Bivalirudin.

As hirudin is a non-human protein it can trigger the formation of antibodies in body[57]. Another drawback of the hirudin is its strong pharmacokinetics dependency on renal functionwhich makes it difficult in determining the dose for elderly and patients with renal impairments. If a patient surfers minor bleeding due to drug effects, then pausing the drug is suffice but if the bleeding is fatal or the patient has suffered from renal failure, hemofiltration[58] is required to reduce the level of plasma lepirudin[59].

2.4. Heparin and other related drugs

The final class of drugs that will be described over here is heparin. Heparin is the choice of anticoagulant when a rapid therapeutic effect is required. Heparin utilises an indigenous anticoagulant factor antithrombin for this activity. Antithrombin is a glycoprotein present in blood plasma (0.12 mg/ml) and has a life time of 3 days. It contains 432 amino acid residues andits structure is stabilized by three disulphide bonds. Antithrombin also contains four glycosylation sites[60], each of which contains four similar biantennary oligosaccharide chain. Three-dimensional structure of antithrombin[61] shows two noteworthy features**(Fig 17)**.First, it contains a five stranded beta sheet which dominates the structure. Second feature is the presence of a large loop containing the active residues (Arg-393 and Ser-394). This loop is the characteristic of a serine protease inhibitor or serpin and is also present proteins, e.g., antichymotrypsin etc[62], [63].

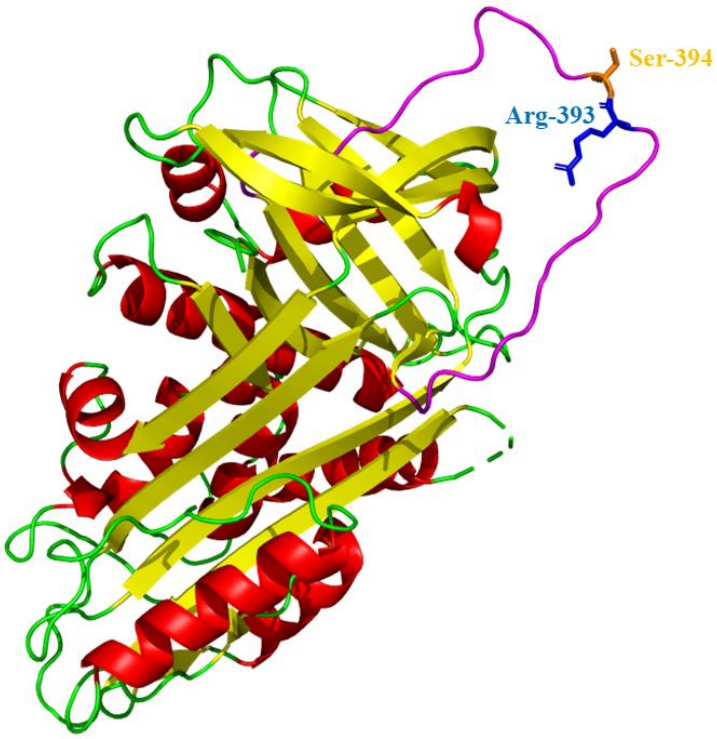


Figure 17: *Structure of Antithrombin (PDB ID-1ATH); reactive bond loop is coloured pink and active residuesare highlighted*

Antithrombin acts on various type of factors involved in coagulation cascade but its principal effect is discerned by its action on thrombin and Factor Xa[64]. Inactivation by antithrombin is achieved by trapping of thrombin and other protease in an equimolar amount. The process of inactivation is initiated by the recognition of reactive bonds by protease enzymes. Arg-393 is critical for its recognition as it the P1 residue of the enzyme, the proteinase can bind to the extended loop of the antithrombin[65]. Hence the process of cleavage is ceased in an intermediate stage. Thrombin can cleave the bond in greater than three days followed by the dissociation of thrombin-antithrombin complex, henceforth, setting free the thrombin for action[66].

Heparin, a polysaccharide or precisely a glycosaminoglycan**(Fig. 18)**, is composed of the alternating arrangement of D-Glucosaminesand uronic acids. This disaccharide repeats can be 5 units long (molecular weight ~3000) or can go upto 50 units in highest cases (molecular weight ~30,000). The anomer of uronic acid, iduronic acid is present in sulphated form at position 2. Its other anomer glucuronic acid is also present in non-sulphated form but as minor constituent. Glucosamine can be N-sulphated at the position 3, or can be present in N-acylated form in position 6, or both acylated sulphated modifications can be present in a

single glucosamine unit[67]. Numerous structural combinations are possible for formation of heparin by the above-mentioned units but fortunately, when a cell synthesizes, it only manufactures a limited number of features necessary for its biological activity. The commercial preparation of heparin can alter the overall composition of biological heparin but still can retain the activity.

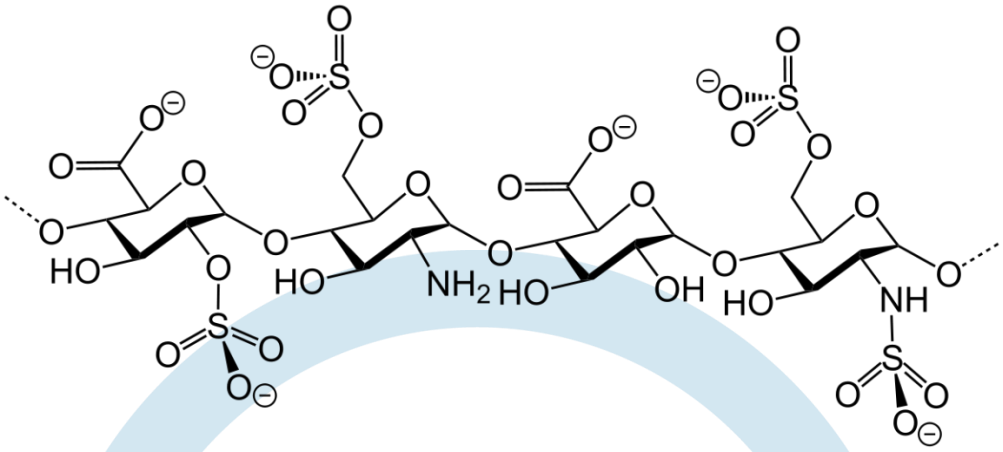
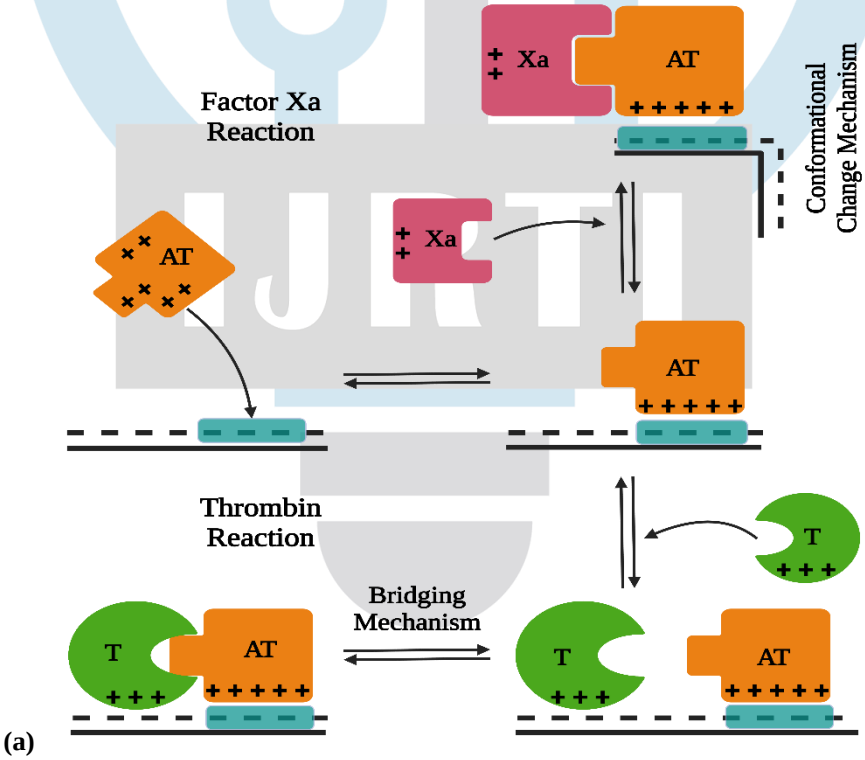
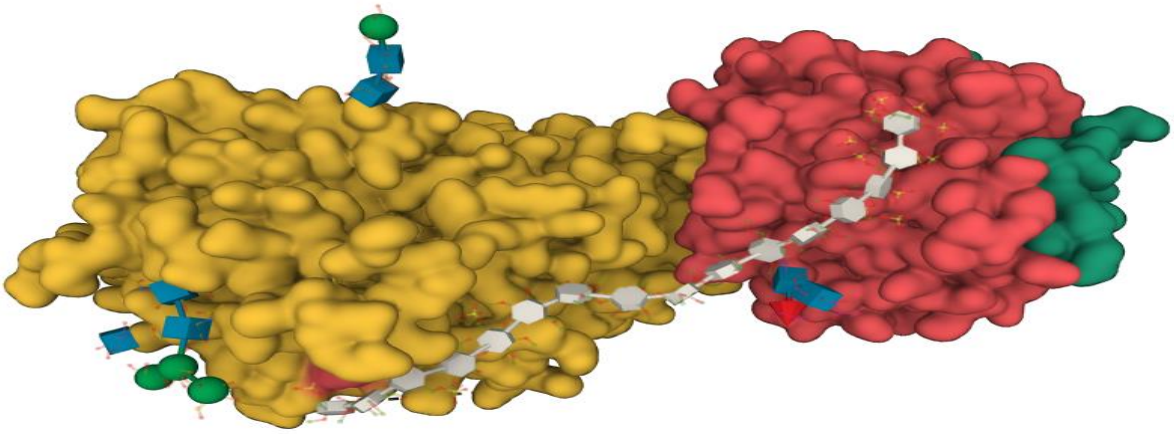


Figure 18:*Structure of Heparin; only a small portion of the large polymeric chain has been depicted here*

The rate of antithrombin-thrombin reaction is 1.5-4*10^7 $M^{-1}s^{-1}$ but in presence of heparin it can be increased by 2000 to 4000-fold[68], [69]. The reaction between Factor Xa and antithrombin is accelerated by heparin up to 500 to 1000-fold. Mechanism of this extraordinary acceleration starts with formation of heparin-antithrombin complex. Heparin binds to the antithrombin using first five pentasaccharide which induced the conformational changes[70]. It has been proposed that this conformational change leads to the partial insertion of reactive bond loop into the beta sheet and thereby bringing the reactive loop into active conformation[71]. Now thrombin binds to this newly formed heparin-antithrombin complex**(Fig 19)** through Exosite-2 by some non-specific interactions between positively charged Lys/Arg residue and strong negatively charged heparin. That is how thrombin comes in the contact with reactive bond loop of antithrombin through the assistance of bridging mechanism of heparin. Then, heparin rapidly dissociates from the overall complex and inhibits another protease, hence we can shed light on the catalytic mechanism of action of heparin inhibition[72].



(a)



(b)

Figure 19: *Mechanism of action of Heparin;* ***(a)*** *Heparin antithrombin (AT) complex is formed by the binding of antithrombin to the heparin chain; then thrombin (T) binds to the heparin chain of the heparin antithrombin complex and consequently the reactive bind is inserted into the active site of thrombin thus it is inhibited. Binding of FXa proceeds in a similar fashion but does not involve binding of FXa to the heparin chain****(b)*** *Structure of antithrombin(yellow)-Heparin(pink)-heparin chain(white) complex; small chain of thrombin is depicted in green (PDB ID 1TB6)*[73].

Due to the large size and charge on heparin, it cannot be orally administered and has to be injected in the form of intermittent IV. Heparin canbe eliminatedvia two mechanisms, first it can bind to the macrophages and endothelial cells where it can undergo degradative metabolism and second pathway is through renal clearance. First process is saturable whereas the latter process is unsaturable and slow. Pharmacokinetics of heparin is non-linear; therefore,heparin can only be administered in hospital set-up, under strict invigilation of patients.

III. Conclusion

Blood coagulation is one of the most complex procedures in mammalian physiology resulting from a joint performance of many well-orchestrated smaller processes. Further research in the field of blood coagulation will give an insight of the process from the molecular level. But the development of anticoagulant drugs is restricted with targeting small number of enzymes. Surely, there are many factorseffect of whose inhibitions are currently unknown and further investigation in this field may lead to development of safe and smoothly administrable anticoagulant compounds.

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