1. Introduction

Cardiovascular diseases (CVDs) remain prime contributor to the burden caused by non-communicable diseases. CVDs are one of the primary causes of mortality worldwide, with more people dying every year from CVDs. Despite the fact that cardiovascular mortality rates have decreased in many developed countries over the last two decades, but have increased dramatically in developing countries. In 2017, 17.9 million individuals died from CVDs, comprising 31 percent of total deaths worldwide.[1] An approximate 7.4 million of these deaths were caused by coronary heart disease and 6.7 million were attributed to stroke.[2] CVDs are induced by heart and blood vessels disorders that include coronary heart disease (heart attacks), increased blood pressure (hypertension), stroke, peripheral arterial disease, rheumatic heart disease, congenital heart disease and heart failure. Thrombosis is the most commonly found basic pathology of these current major cardiovascular disorders.

Thrombosis is the development of a blood clot in a blood vessel that allows blood to occlude in the arterial and venous circulation.[3] Depending upon the site of formation of thrombus, thrombosis is classified as arterial or venous thrombosis. It is called as thromboembolism if the clot splits and moves through the bloodstream. Coronary and cerebral artery occlusions are correlated with platelet aggregation and coagulation induced by the rupture of atherosclerotic plaque, culminating in myocardial infarction and acute coronary syndrome. Obstruction of veins is associated with venous thrombotic diseases such as deep vein thrombosis (DVT) or pulmonary embolism (PE).

1.1 Hemostasis and thrombosis

Hemostasis is a normal physiological process in which bleeding is interrupted to minimize excessive blood loss. Hemostasis modulates the integrity of circulatory system but it sometimes becomes unbalanced, causing either thrombosis or hemorrhage. The balance between thrombosis and hemorrhagic condition is controlled in the body by the interactions between coagulation factors and fibrinolytic system as well as platelets and vascular membrane.[3,4] The normal hemostatic process includes three phases; primary

hemostasis, coagulation and fibrinolysis. All the three stages are highly interconnected with each other and regulate blood fluidity in the vessels by maintaining vascular integrity and their openness. Due to the overwhelming response of circulatory system, vascular endothelial injury, arresting of blood flow and hyper-coagulation take place which ultimately results into a pathological condition called thrombosis.

Primary hemostasis is initiated suddenly due to vascular endothelial injury or damage and is associated with vasoconstriction to reduce blood loss from the site of injury, and the aggregation and activation of platelets to form a platelet plug. Subsequently, secondary hemostasis begins with complex interactions of clotting factors and fibrinolytic system which results into formation of insoluble fibrin clot at the site of injury. Most of these clotting factors are trypsin-like serine proteases that are generated from their respective pro-enzymes or zymogens, synthesized by liver.[5]

1.2. Blood coagulation

Blood clotting is a complex process that has been well explained by two models:

- The cascade model
- The cell-based model

As per the cascade model, coagulation process is coordinated by two parallel pathways, the extrinsic and intrinsic pathways,[6] both intersecting at the point of activation of FXa as illustrated in **Figure 1.1**. The activated factor X (FXa) forms a complex with the FVa to bring about cleavage of prothrombin in order to generate thrombin, which then activates soluble fibrinogen in order to produce insoluble fibrin. The polymerized fibrin sheets and activated platelets at the injury site form a stable clot.[7]

The cell based model represents the *in vivo* mechanism of blood coagulation in more appropriate way that covers important interactions between cells, directly involved in hemostasis and coagulation factors. According to this model, coagulation process involves three phases; initiation, amplification and propagation as described in **Figure 1.2**.[8]

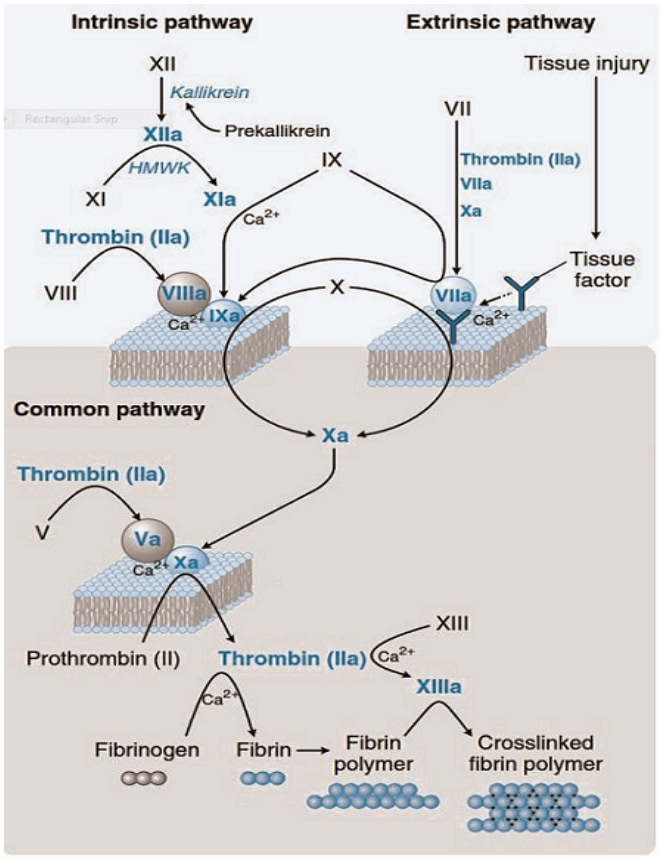


Figure 1.1. The coagulation cascade. The feedback mechanisms are removed for clarification. (HK = high molecular weight kininogen, PK = prekallikrein, PL = phospholipids)

Normally, the coagulation cascade is initiated when subendothelial tissue factor comes in contact with the blood flow due to either damage or activation of the endothelium. The tissue factor (TF)-factor VIIa complex of the classic 'extrinsic pathway' not only plays a role in factor X activation, but also activates factor IX of the classic 'intrinsic pathway' directly.[9] On the TF-exposed cells, FXa is responsible for generation of small amount of thrombin (FIIa) from prothrombin (FII).

In amplification phase, the surface bound thrombin activates platelets along with factor V, factor XI, and factor VIII. FXIa facilitates FIXa production on the surface of platelets, causing in a thrombin burst. The activated complexes FIXa-FVIIIa (known as 'Xase')[10] and FVa-FXa (known as 'prothrombinase') play important role in generation of FXa and thrombin respectively on the phospholipid surface of the activated platelets during the propagation phase. In addition, the surface bound FXIa activates FIX to form

more Xase and thus accelerates the generation of FXa which generates thrombin in association with FVa on the surface of platelets. Finally, thrombin leads to the proteolytic cleavage of fibrinogen to fibrin.[11,12]

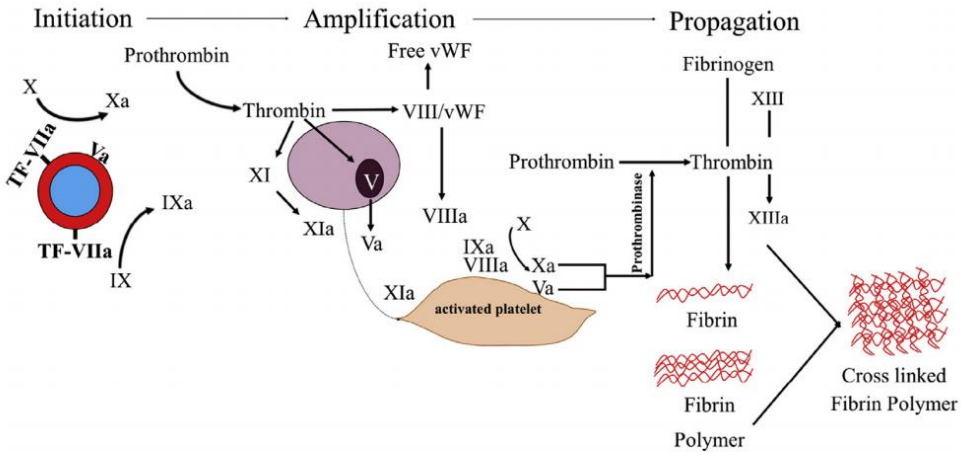


Figure 1.2. The coagulation cascade phases.

1.3. Conventional antithrombotic therapy

Various clinical and preclinical studies have proven the effectiveness of conventional anticoagulants such as vitamin K antagonists (VKAs), unfractionated heparin (UFH), low-molecular-weight heparins (LMWHs, fractionated heparin with decreased activity toward thrombin relative to UFH) in the prevention and treatment of a diverse set of thromboembolic arterial and venous diseases.[13]

Vitamin K antagonists (VKAs) such as warfarin (**1**), dicoumarol (**2**), pindone (**3**) are a category of substances that inhibit blood clotting process by suppressing the action of vitamin K. Warfarin (**1**) is the most commonly used vitamin K antagonist. Vitamin K is essential for the synthesis of certain proteins that are prerequisites for blood coagulation. These drugs work by inhibiting the enzyme called vitamin K epoxide reductase. The label "vitamin K antagonist"[14] is a misnomer because, in the pharmacological context, the drugs do not specifically antagonize the activity of vitamin K, but the recycling of vitamin K.

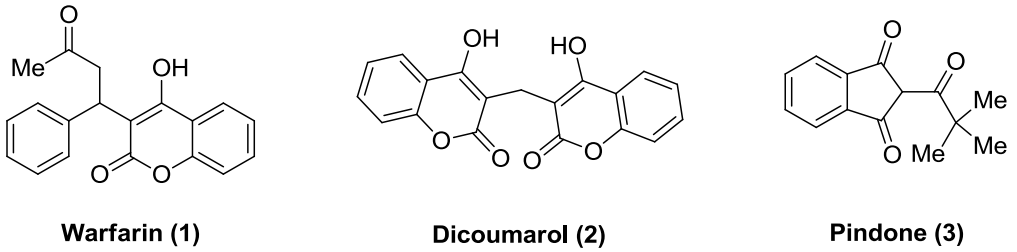


Figure 1.3. Examples of vitamin K antagonists.

The anticoagulant actions of unfractionated heparin (UFH) were first discovered in 1916. Heparin binds to antithrombin and greatly improves the thrombin and FXa inhibition potential of this protein. Low molecular weight heparin (LMWH) also acts by the same antithrombin-mediated mechanism but it has undergone fractionation for the purpose of making its pharmacodynamic profile more predictable. The administration of LMWH results in lower bleeding risks than that of UFH.[14–16]

Direct thrombin inhibitors (e.g. dabigatran, argatroban and hirudin derivatives) bind directly to thrombin and do not need any cofactor such as antithrombin to exert their action. They are competitive, reversible direct thrombin inhibitors. They prevent clot formation by inhibiting the active site of the free thrombin as well as fibrin-bound thrombin and thrombin-induced platelet aggregation. Dabigatran (**4**) is given orally as the prodrug, which is hydrolysed to active form by carboxyesterase in plasma and in the liver. Argatroban (**5**), a highly selective thrombin inhibitor is given intravenously.[17,18]

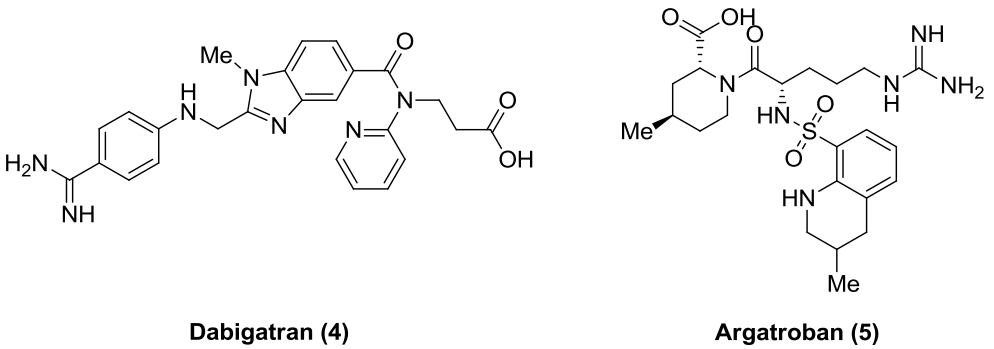
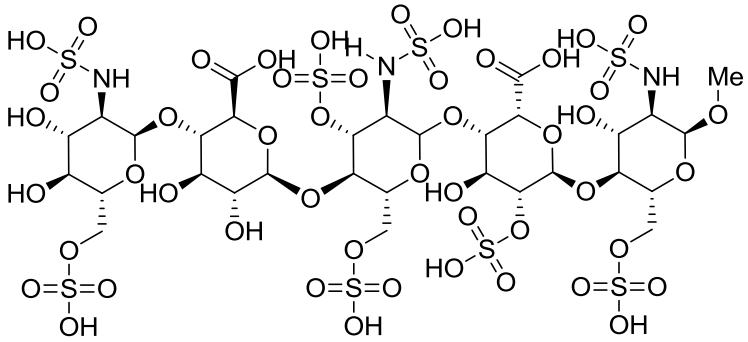


Figure 1.4. Examples of direct thrombin inhibitors.

Fondaparinux (**6**) is a synthetic indirect inhibitor of FXa. It was the first to be discovered in a new class of selective antithrombin-dependent FXa inhibitors. Fondaparinux (**6**) interacts with antithrombin without affecting other plasma proteins, resulting in predictable pharmacokinetics that makes it unnecessary to monitor and adjust the dose. Its chemical structure is based on the natural pentasaccharide contained within heparin and LMWHs.[19]



Fondaparinux (6)

Figure 1.5. Example of indirect FXa inhibitor.

Although these anticoagulant therapies are the existing standards of care for their proven effectiveness, it has been noticed that these anticoagulants have several problems (**Figure 1.6**) that limit their therapeutic utility and have generated the need for better treatments.[20]

The use of warfarin (**1**) and other VKAs is particularly troubling, although they offer the convenience of their oral administration. Unfortunately, warfarin (**1**) has been found to be involved with numerous drug and food interactions, slow recovery with vitamin K antidote, unpredictable pharmacokinetic (PK) and pharmacodynamic (PD) profile and considerable intra/inter-patient variability in drug response. As a direct consequence, the appropriate therapeutic dose differs, requiring constant monitoring and regular adjustment of the dose. Regular supervision of warfarin (**1**) therapy is important due to this uncertainty in response and its relatively narrow therapeutic index, which often results in subtherapeutic anticoagulant action and a higher risk of thromboembolism[21] or prolonged anticoagulant action and greater risk of bleeding. Furthermore, warfarin's delayed onset of action often prevents its clinical use in emergency situations when a rapid-acting,

parenteral anticoagulant is needed to initiate therapy.[22] Immediate surgical or invasive treatments may also be reinforced by the fact that the anticoagulant actions of warfarin (**1**) are continued for many days after medication has been stopped.[23]

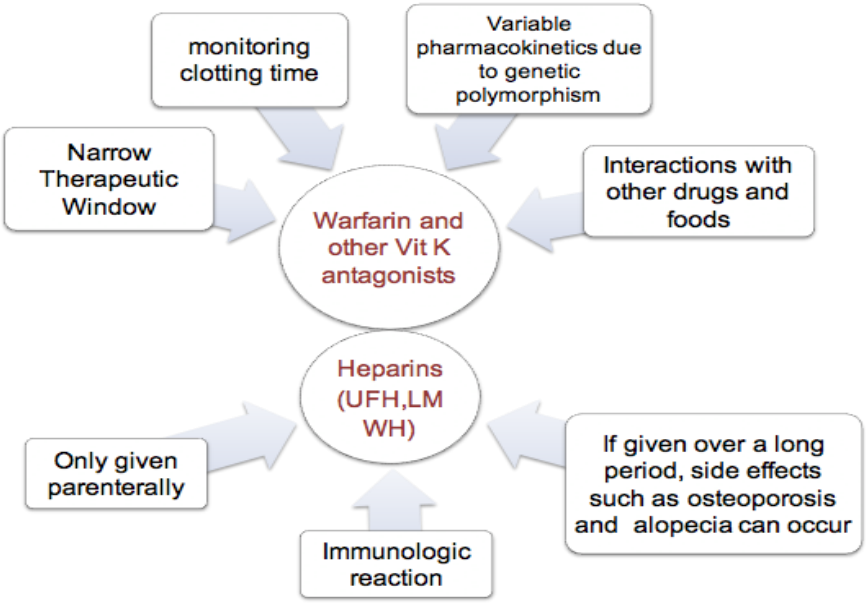


Figure 1.6. Limitations of conventional antithrombotic therapy.

Short-term anticoagulant agents usually involve UFH, LMWHs, fondaparinux (**6**, as indirect FXa inhibitor), argatroban (**5**), bivalirudin and hirudin (as direct thrombin inhibitors).[24] These medications need intravenous administration that creates a problem for their use outside the hospital and causes clot formation at the site of injection. Heparin analogs like UFH and LMWHs bear the risk of thrombocytopenia and as they have originated from animal tissue, they sometimes exhibit serious complications like immunological reactions and uncontrolled bleeding. Besides this, UFH has an uncertain PK profile and anticoagulant reactions that require monitoring.[25] All such drawbacks of parenteral anticoagulants, especially the need for injection render warfarin (**1**) and other VKAs more convenient in many countries as the only orally bioavailable anticoagulants representing the sole feasible alternative for long-term anticoagulation.[26] Dabigatran (**4**), an oral thrombin inhibitor has been found to cause uncontrolled bleeding which could prove fatal at high doses.[27] These clinical limitations in the existing drugs prompt

researchers to develop new orally bioavailable antithrombotic drugs with a better safety profile.

1.4. FXa as a promising target for the development of antithrombotic drugs

To regulate blood coagulation, various enzymes involved in blood coagulation process have gained great attention as potential targets by medicinal chemists for the development of new antithrombotic agents (**Figure 1.7**). Efforts have been made for developing strategies for selective inhibition of target specific enzymes within the coagulation cascade.

Two serine proteases, factor Xa (FXa) and factor IIa (FIIa or thrombin) have particularly emerged as promising targets. Between these two targets, direct inhibition of FXa has appeared as the most effective strategy to achieve anticoagulation with minimal bleeding risks by maintaining normal hemostasis. Due to its upstream position in the amplification cascade and limited function outside the cascade, direct inhibition of FXa is more effective than direct inhibition of thrombin. Selective inhibition of FXa displays less bleeding risks because it does not affect the normal existing thrombin level, and activation and aggregation of platelets. Several preclinical studies revealed that FXa inhibitors displayed a larger therapeutic window than direct thrombin inhibitors.[28,29] FXa has received great attention of the researchers to develop novel antithrombotic drugs due to its vital role in coagulation cascade.

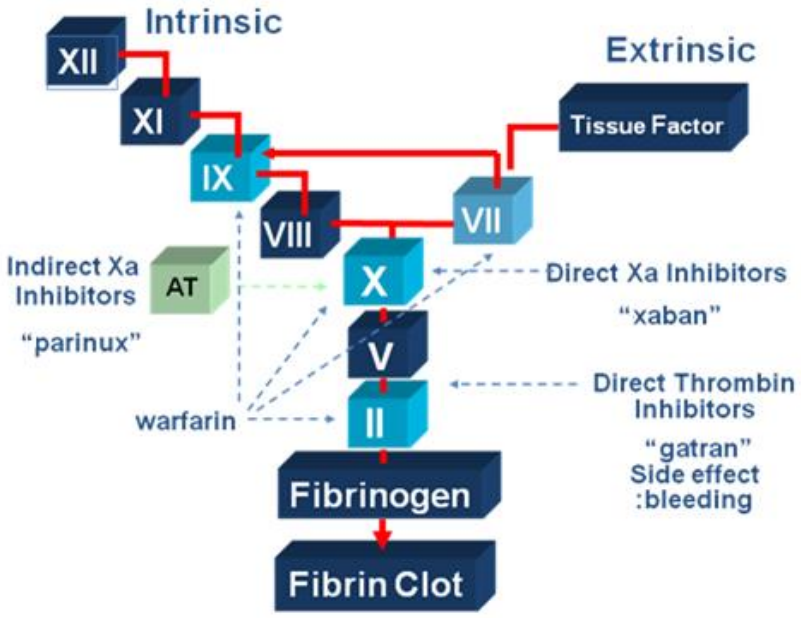


Figure 1.7. Sites of action of new antithrombotic agents.

Factor Xa, a vitamin K-dependent serine protease is activated in blood from its inactive form by factor IXa-VIIIa complex through the intrinsic pathway or by the factor TF-VIIa complex through the extrinsic pathway. FXa is a key enzyme in coagulation pathway which is essential for conversion of prothrombin to thrombin.[30] FXa catalyzes this conversion with the help of calcium ions and phospholipid membrane of activated platelets by forming a complex with prothrombinase and Factor Va. Thus, FXa plays a crucial role in the enzymatic activation of blood clotting cascade and controls normal hemostasis by modulating thrombin generation and fibrin formation subsequently. Factor Xa is also responsible for the propagation of coagulation network by converting prothrombin (factor II) to thrombin (factor IIa). One molecule of FXa is responsible for the formation of more than thousand thrombin molecules. Thus, FXa plays an important amplifying role in the coagulation process.[31]

Extensive research efforts in the development of novel orally active, selective and small molecular weight FXa inhibitors resulted in approval of four clinical candidates such as rivaroxaban[32] (**7**), apixaban[33] (**8**), edoxaban[34] (**9**) and betrixaban[35] (**10**) (**Figure 1.8**).

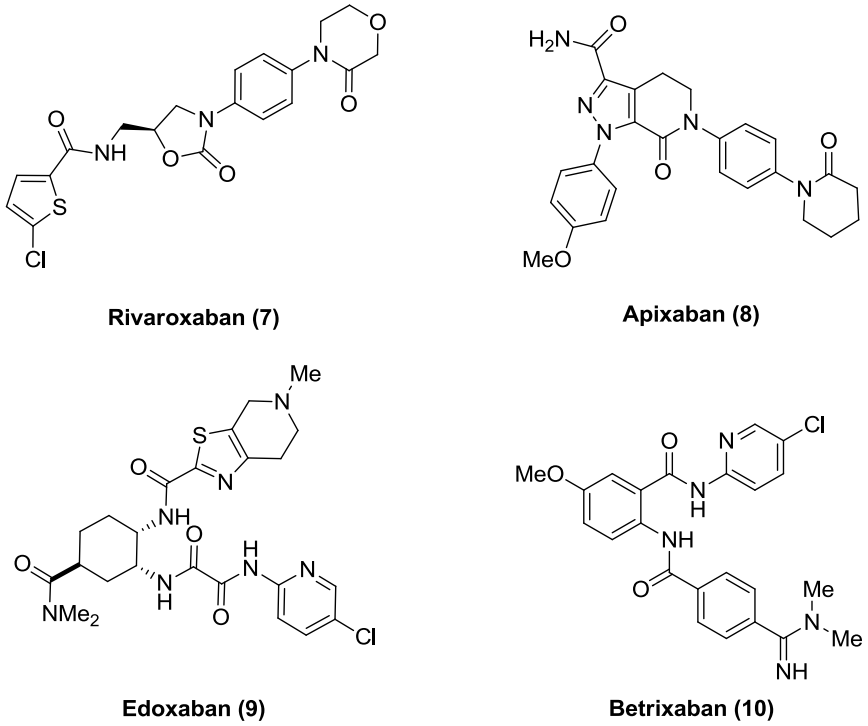


Figure 1.8. Structures of currently available FDA-approved oral direct FXa inhibitors.

Compared with the conventional anticoagulant agents, all these approved FXa inhibitors demonstrated higher specificity, lesser food and drug interactions, and better oral bioavailability.[36] However, they still possess many drawbacks like drug-drug interactions[37] and lack of a specific antidote for preventing bleeding.[38,39] These inhibitors also displayed narrow clinical indications as they cannot be recommended for use in patients with renal and hepatic diseases and patients having mechanical heart valves. Recently it has been found that discontinuation of rivaroxaban and apixaban therapy may cause rebound thrombosis.[40] Hence, there is a demand to further develop novel and safer FXa inhibitors with suitable profile to overcome these issues and to advance their clinical use.

1.5. FXa: structural analysis

FXa, a vitamin K-dependent serine protease has two chains of amino acids linked by a disulfide bridge. The heavy chain comprises of 303 amino acids and the light one of 139 amino acids. The catalytic triad in FXa is present in the heavy chain and comprises of Ser195, His57 and Asp102.[41] The

active site of FXa is identified as S1 and S4 subsites and the surrounding residues. The S1 subsite is a narrow pocket formed by Trp215-Gly216 on one side of the wall and Ala190-Cys191-Gln192 on the other side. The bottom of S1 pocket is lined by Asp189 and the side chain of Tyr228.[42] Prothrombin, the natural substrate of FXa, interacts through ionic hydrogen bonding of the side chain of Arg to the Asp189 in S1 pocket **(Figure 1.9**). In contrast to other serine proteases, access to the S2 pocket in FXa is blocked by Tyr99. The S4 binding pocket is an aromatic box formed by the side chains of Tyr99, Phe174 and Trp215. This active site provides noticeable difference in FXa from the binding sites of other serine proteases. Selective inhibition of FXa can be achieved by targeting the S4 pocket.[43]

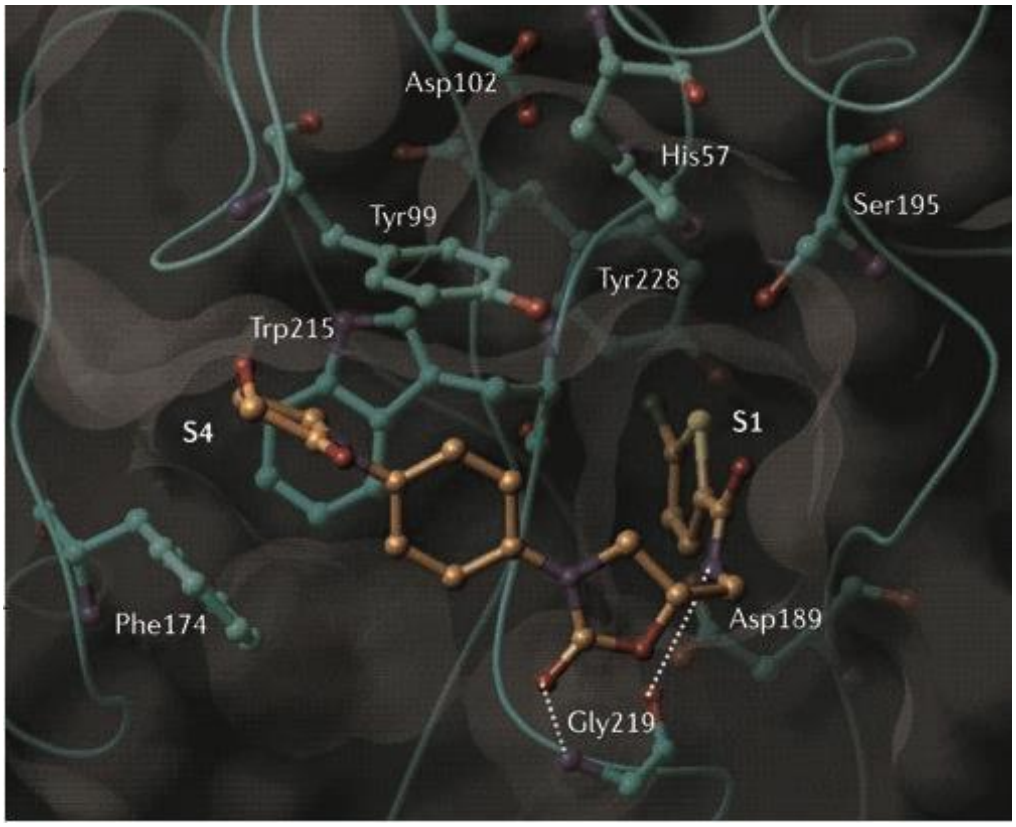


Figure 1.9. X-ray crystal structure of FXa (PDB Code: 2W26) with a focus on active subsites showing key interactions between rivaroxaban (**7**) and active site amino acid residues (S1: Tyr228; S4: Tyr99, Phe174 and Trp215). White dotted lines indicate hydrogen bonds.[42]

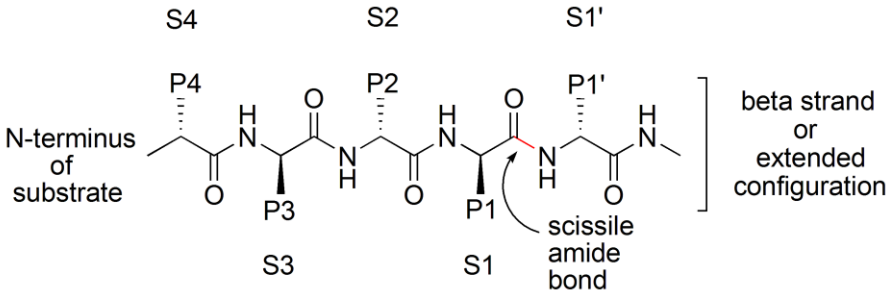


Figure 1.10. Nomenclature for substrate/inhibitor residues and the corresponding binding sites in the enzyme.

The nomenclature of binding pockets (S1-S4, S1′) of a serine protease has been given by Schechter and Berger to simplify identification of the sites. As per this nomenclature, a protein subsite indicated as S1 binds to the corresponding substrate amino acid indicated as P1. The cleavage point for the enzyme subsite is between S1′ and S1 and for the peptide substrate it is between P1′ and P1. In both the directions, the numbering of subsites is given from the cleavage point in ascending order (S1, S2,...., Sn increasing towards the N-terminus and S1′, S2′,....., Sn′ increasing towards the C-terminus) as shown in **Figure 1.10**.[44]

1.6. Structural difference between FXa and thrombin

Selectivity is an important issue for the development of FXa inhibitors. Due to structural similarities between both the enzymes, FXa inhibitors may bind to thrombin. Hence, there is a need to address the selectivity issue for the development of newer FXa inhibitors by understanding structural differences between FXa and thrombin.[45]

Some research groups have resorted to molecular modeling techniques to obtain supportive results to understand the structural differences between both the enzymes and to sort out the selectivity issue. There are different schools of thoughts as per literature regarding the selective binding of various inhibitors to FXa and thrombin.[46,47–49] However, a more clear picture regarding the selectivity issue was given by Bhunia et al.[50] with the help of ligand and structure based modeling approaches. The most noticeable difference from FXa (**Figure 1.11B**) is the presence of D-pocket in thrombin (**Figure 1.11**), composed of residues Tyr60A-Pro60B-Pro60C-Trp60D. This

pocket is considered as an important recognition site in thrombin for the development of thrombin-selective inhibitors. In both FXa and thrombin, the S1 pocket is highly similar for each other having two residues, Asp189 and Tyr228, and Cys191-Cys220 residues located at the bottom of the site. S1 pocket could be playing an important role toward potential inhibition of both the enzymes. The S1 pocket is about 12 Å deep, made up of two antiparallel ß-sheets which form a partial roof by undergoing U-turn at the entry of S1-pocket.[51,52] This partial roof was named as S2-pocket where the two amino acid residues namely Gly216 and Gly218 were found to have a critical role for potential inhibition of FXa whereas three amino acid residues (Gly216, Gly219 and Ser214) were found to have the same role in thrombin.[50]

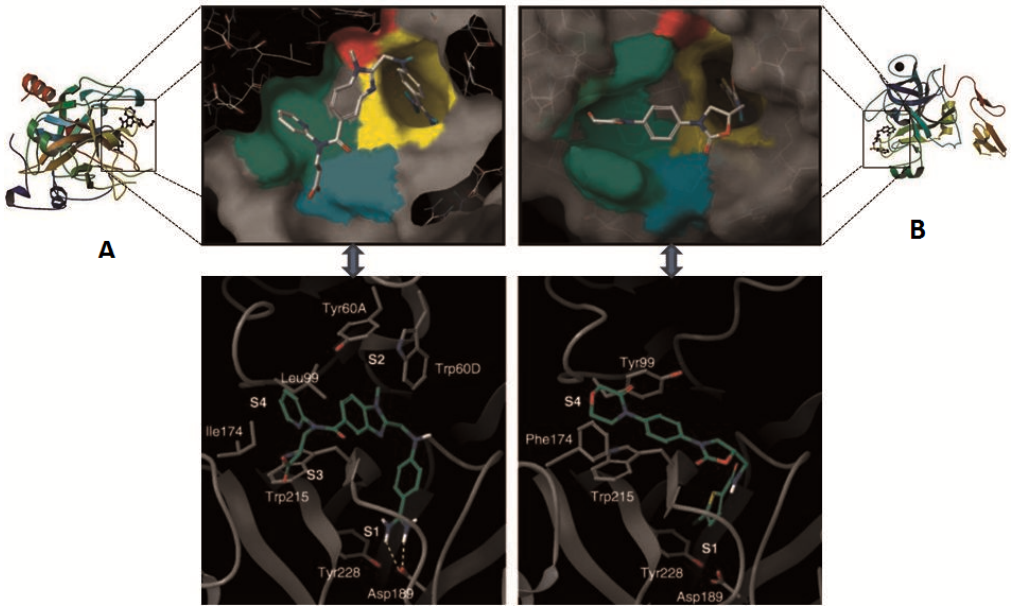


Figure 1.11. Comparison of active sites of (A) thrombin and (B) FXa. Top left: Thrombin complexed with dabigatran (PDB code 1KTS) and Top right: FXa complexed with rivaroxaban (PDB code 2W26).

Co-crystallization experiments have established the fact that these residues of S2 pocket are actually involved in forming H-bonds with the bound molecules. As far as amidine-based inhibitors are concerned, it has been reported that NH group of amidine formed strong H-bond with residual water located near Tyr228.[51] In case of non-amidine based inhibitors, instead of water residue, chloro group of the inhibitor has been responsible to make

strong bonding with Tyr228 in this pocket through Van der Waals interactions. Additionally another molecular recognition site i.e. S3 pocket made up of Glu192 in thrombin and Gln192 in FXa, called as esteric site, is present. Carboxylate group containing compounds interact more favorably with glutamine in FXa than with glutamate in thrombin. Differences in S4 pockets are more prominent between both of them. In thrombin this subsite is formed by Leu99, Ile174 and Trp215 at the bottom while it is comparatively more symmetrically formed by the corresponding Tyr99 and Phe174 in FXa, which make an "aromatic box" along with Trp215.[51]