1. INTRODUCTION

Life expectancy has doubled during the last century and the world population has witnessed sudden transition of deadly infectious diseases to chronic non-infectious diseases. Cardiovascular diseases (CVDs) viz. the diseases of the heart and blood vessels share a major burden due to these non-communicable diseases.[1,2] According to 2017 World Health Organization (WHO) data, CVDs are the most common causes of deaths in today's era with over 17 million deaths reported annually comprising of 31% overall deaths worldwide. The death toll is expected to rise to 25 million by 2030. These are mostly from low and middle income countries accounting US $ 863 billion global cost involved.[3,4] CVDs and cerebrovascular diseases including myocardial infarction (MI), ischemic stroke, deep vein thrombosis (DVT) and pulmonary embolism (PE) are the leading contributors of morbidity and mortality.[5,6] Thromboembolic disorders are the major underlying complications of CVDs and as per reports of International Society on Thrombosis and Heamostasis, 1 out of 4 patients are dying due to thrombosis-related conditions. About 900,000 people are affected due to DVT or PE in U.S. annually, out of them 100,000 die and nearly 30% patients would experience recurrence within a decade[7] while more than a million cases of venous thromboembolism (VTE) and 6 million cases of atrial fibrillation (AF) are reported in Europe causing half a million casualties each year.

1.1 Haemostasis and thrombosis

1.1.1 Haemostasis

Regulation of normal blood flow in a body is a highly complex process with several complementary and opposite events of control.[8] Blood transports nutrients and oxygen to tissues and takes away metabolic waste products. Haemostasis is a protective physiological process that involves blood clot formation to prevent uncontrolled blood loss due to injury to blood vessels and thus maintains vascular integrity and blood flow.[9] It mainly comprises two mechanisms, viz. primary haemostasis that involves local vasoconstriction which reduces blood flow at the site of injury followed by platelet aggregation where platelets are adhered and activated to form a primary hemostatic plug. The secondary hemostasis also called coagulation cascade causes initiation of complex series of enzymatic events to form fibrin strands that together with platelets

results into mechanically stable thrombus.[10] After repairment of the ruptured blood vessel due to vascular injury, fibrinolysis is initiated to dissolve or remove fibrin clot and maintain normal blood circulation.[11]

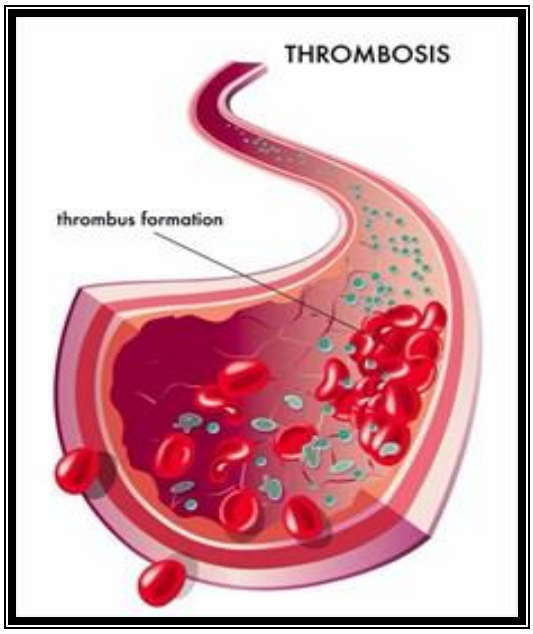


Figure 1.1: Thrombosis

Any dysregualtion in physiological response of hemostatic system leads to either bleeding or thrombotic complications in the cardiovascular system mainly in arteries, veins, capillaries and heart. Both these processes are complementary to each other under normal physiology.[12] During thrombosis normal hemostatic processes i.e. blood coagulation and platelet aggregation are activated inappropriately. Hence, inhibition of coagulation and prevention of platelet aggregation at different levels are the main strategies behind antithrombotic drugs therapy.[13]

1.1.2 Thrombosis

Thrombosis (**Figure 1.1**) is a pathological condition of unbalanced haemostasis which leads to formation of obstructive clot (thrombus) in the veins or arteries of blood circulation. The coagulation processes controlled irregularly may lead to occlusion of blood vessels. The

important factors contributing to thrombus formation are vessel wall injury, stasis of blood flow and hypercoagulability; all of these three collectively are called as Virchow's triad.[14-16]

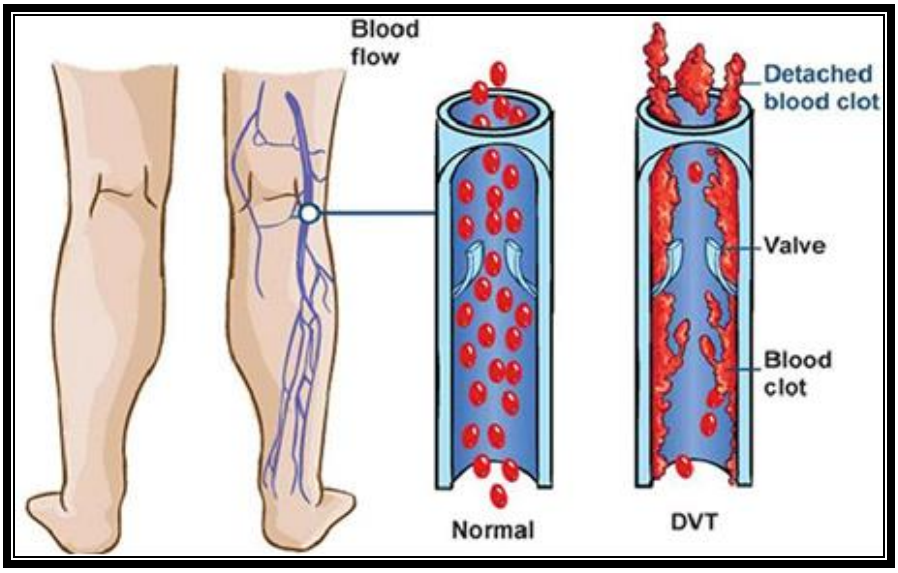


Figure 1.2: Deep vein thrombosis

Depending on its place of origin, thrombosis is either arterial or venous in nature. Both arterial and venous thrombi are made up of platelets, fibrin or red blood cells in varying proportions.[14] Arterial thrombi result from a ruptured atherosclerotic plaque and form in regions of turbulent blood flow and are responsible for fatal myocardial infarction (MI) and stroke. They are rich in platelets where platelets get activated and aggregated. Hence antiplatelet agents targeting platelet aggregation are useful in the prophylaxis and treatment of these conditions.[17] Venous thrombi are caused by excessive activation of coagulation or from stasis of blood flow. These thrombi are fibrin-rich with red blood cells trapped inside and often form in the large veins of legs, called deep vein thrombosis (DVT, **Figure 1.2**). If the thrombus fragment breaks free, it travels to the pulmonary artery blocking the blood flow, the condition called as pulmonary embolism (PE). PE associated with DVT is collectively known as venous thromboembolism (VTE). Anticoagulants are used in the prevention and treatment of DVT and PE.[18]

1.2. Coagulation cascade

The blood coagulation is a complex series of enzymatic biotransformations where each reaction involves conversion of inactive zymogens to active serine proteases, finally resulting into formation of fibrin. This coordinated process of interaction and activation of one enzyme to the other in an amplification manner is known as coagulation cascade (**Figure 1.3**).[18, 19]

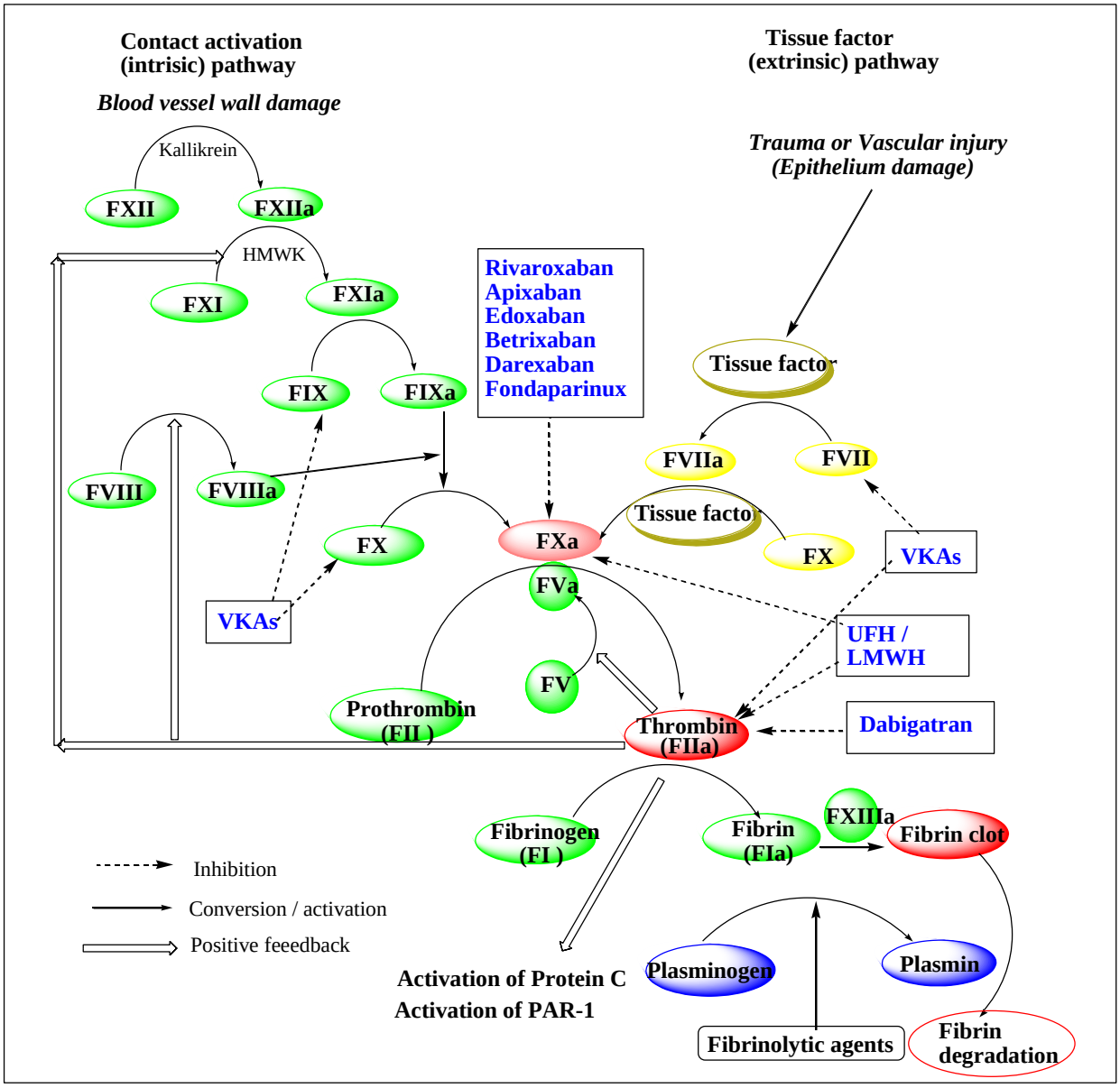


Figure 1.3: Classical Coagulation cascade and the targets for antithrombotic agents.[20, 31] F: factor, HMWK: High molecular weight kininogen, VKA: vitamin K antagonists, UFHs: Unfractionated heparins, LMWHs: Low molecular weight heparins, PAR-1: Protease activated receptor-1.

The classical coagulation cascade comprises of three major pathways, namely the intrinsic pathway, the extrinsic pathway and the common pathway. The extrinsic pathway is initiated through local vascular injury, by epithelium damage or hypoxia exposing tissue factor (TF), a surface glycoprotein released by injured cells or activated leukocytes to the blood components. Tissue factor by interaction with circulating factor VII (FVII) activates zymogen FVII to FVIIa (suffix 'a' notifies activated) and forms TF-FVII/FVIIa complex and facilitates activation of factor X to factor Xa. (FXa). The intrinsic pathway also called 'contact activation' pathway involves sequential activation of clotting factors e.g. factors XII, XI and IX, when blood comes in proximity with the glass surface, activated platelets or anionic surfaces. First, factor XII is activated into FXIIa by the action of kallikrein and subsequently FXIIa along with High Molecular Weight Kallikrein (HMWK) converts FXI to FXIa. Factor FXIa then catalyzes conversion of FIX to FIXa, which in combination with factor VIIIa forms tenase complex in presence of calcium ions and phospholipids. This complex then activates factor X to factor Xa.[16, 20, 21]

Finally, two pathways meet at a convergent point of activation of factor X by either way. The activated FXa with its cofactor FVa on phospholipid surface forms a 'prothrombinase complex' which activates prothrombin to thrombin. Thrombin in turn, converts fibrinogen to soluble fibrin which after the action of transglutaminase enzyme FXIIIa forms fibrin clots. Thrombin also mediates positive feedback by activation of factor XI, factor VIII and factor V in cascade to ensuring efficient production of thrombin and fibrin to maintain normal hemostasis. The classical coagulation cascade model is helpful in understanding of key mechanisms of *in vitro* coagulation but it was unable to explain *in vivo* observations. Hence, newly developed cell-based model of coagulation demonstrates the physiology of thrombogenesis and role of platelets and tissue-factor releasing cells.[16, 22]

According to cell-based model, coagulation process takes place in three major phases viz. initiation, amplification and propagation (**Figure 1.4**).[23] The initiation starts with the activation of a cell surface protein the tissue factor (TF) which activates factor VII. The TF-VIIa complex then activates factor FIX, FX and then FXa binds with factor Va forming FXa/Va complex which further produces small amount of thrombin. In amplification phase, the thrombin on TF-

bearing cell surface activates platelets, generates factors FVa and FXIa, and proteolysis of von-Willebrand factor (vWF) from FVIII causes activation of FVIII to FVIIIa. Lastly during the propagation phase, FXIa converts FIX to FIXa which in conjunction with FVIIIa forms a

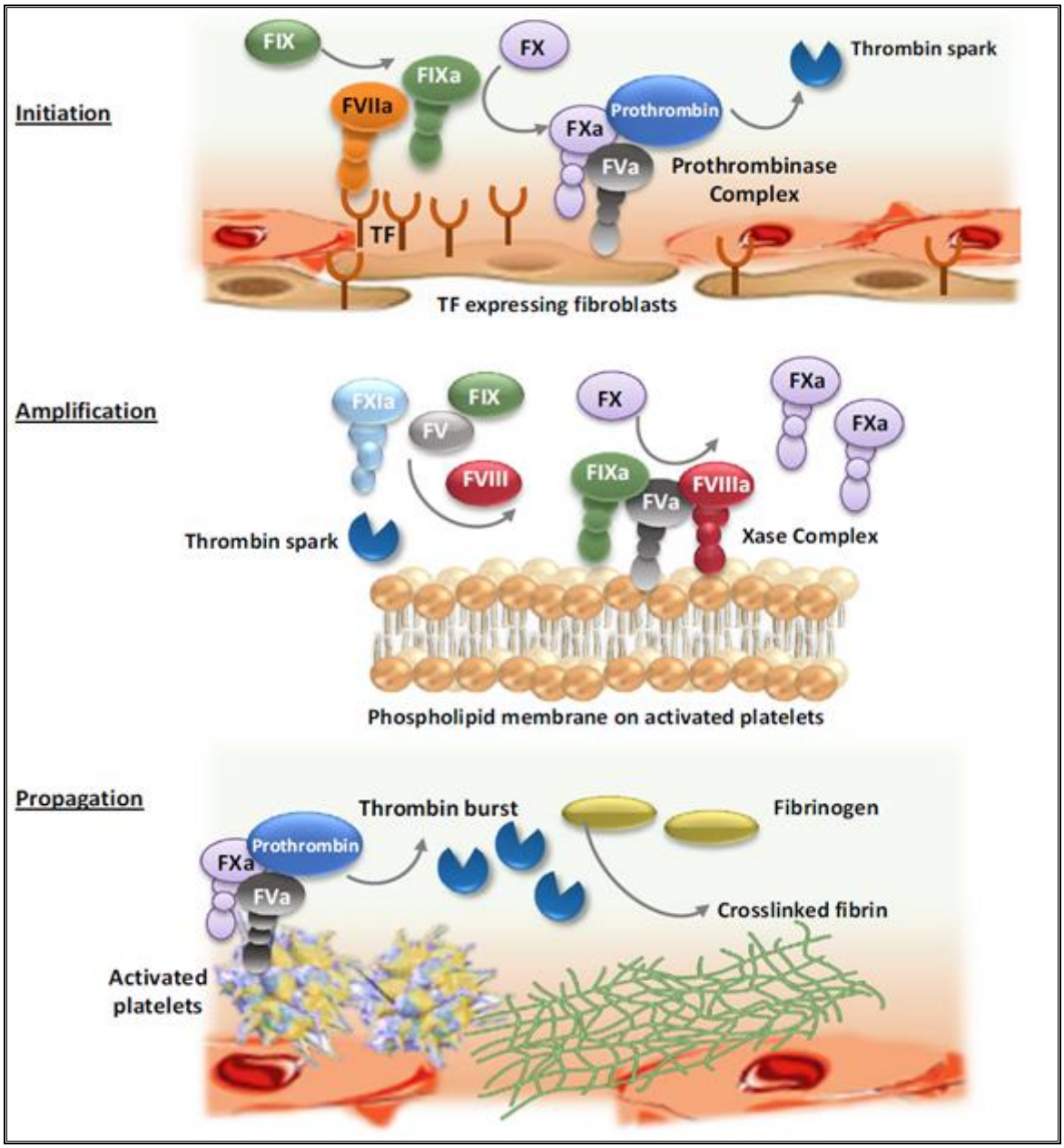


Figure 1.4: Cell based-model of coagulation cascade.[23]

complex (called 'tenase' complex) producing FXa. This FXa binds with FVa forming 'prothrombinase' complex which ultimately results in production of large quantities of thrombin

from prothrombin. The natural endogenous anticoagulants from the coagulation process regulate the enzymatic activity of procoagulants by inhibiting their actions e.g. antithrombin (AT) inhibit the activity of factors IIa (thrombin), FXa and FIXa, while tissue factor pathway inhibitor (TFPI) inactivates FXa as well as TF-FVIIa complex, and activated protein C degrades FVa and FVIIa. Thus, the clotting mechanism is restricted to the area of vessel injury maintaining normal process of hemostasis.[23-25]

1.3 Antithrombotic drug therapy

The quantity of an activated coagulation factor produced from its inactive form increases at every stage of cascade hence direct inhibition of any one specific enzyme at early stage would provide more effective way of anticoagulation.[26] In the last few decades enormous progress has been made in the development of antithrombotic drug therapies for the treatment of different cardiovascular diseases e.g MI, stroke, AF, and thrombotic disorders e.g. DVT, PE. These therapeutic agents are mainly classified into three classes as antiplatelet agents, anticoagulants and thrombolytic agents (**Figure 1.5**).[18]

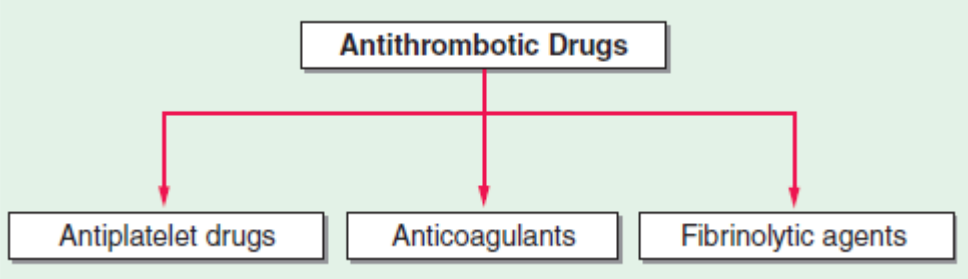


Figure 1.5: Classification of antithrombotic agents.[18]

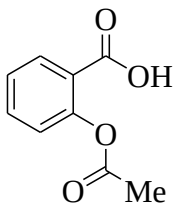
1.3.1. Antiplatelet agents

Platelets play crucial part in the activation of coagulation process and maintenance of hemostasis. Antiplatelet agents act by inhibition of platelet function and target platelet surface receptors which mediate their activation, adhesion to vessel wall and aggregation.[27] They are categorized into cyclooxygenase (COX) inhibitors, adenosine diphosphate (ADP) receptor antagonists, glycoprotein (GP) IIb/IIIa-receptor inhibitors and phosphodiesterase inhibitors. These agents are used for the prevention of arterial thrombi and in prophylaxis and treatment of

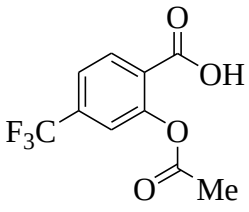
cerebrovascular and cardiovascular diseases like ischemic stroke, myocardial infarction and unstable angina.[28] Frequently they are used along with fibrinolytic agents to prevent reocclusion in post MI conditions.[29]

(a)Cyclooxygenase (COX) inhibitors

Aspirin (**1**) blocks biosynthesis of thromboxane A_2 which is a potent agonist of platelets, by irreversible inhibition of cyclooxygenase (COX-1) enzyme by covalent acetylation of serine residue in the active binding site. Antiaggregatory effects of 75-100 mg oral aspirin lasts for 7 to 10 days and it is indicated in the secondary prevention of acute MI and stroke. Aspirin may cause stomach ulcers and bleeding. Trifluoromethylated derivative of aspirin, triflusal (**2**) acts as antiplatelet agent by inhibiting COX-1 and inhibiting cAMP phosphodiesterase that leads to increased levels of cAMP, resulting into reduced platelet aggregation.[30, 31]



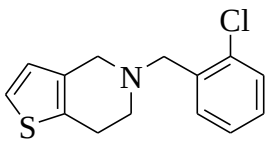
Aspirin (1)



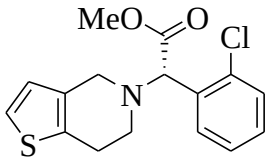
Triflusal (2)

(b) ADP-receptor antagonists

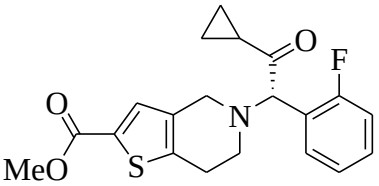
G-Protein coupled receptor $P2Y_{12}$ amplifies and facilitates platelet activation in response to ADP. Thienopyridine ring-containing drugs e.g. ticlopidine (**3**), clopidogrel (**4**) and prasugrel (**5**) selectively inhibit ADP-induced platelet aggregation by irreversibly blocking $P2Y_{12}$ receptors. Both clopidogrel and prasugrel are prodrugs that get metabolically activated.[32] They are used in the acute coronary syndromes (ACS) like unstable angina and percutaneous coronary intervention (PCI). Recently approved ADP-antagonists ticagrelor (**6**) and cangrelor (**7**) act reversibly on $P2Y_{12}$ receptor with less bleeding events and do not need metabolic activation.[29]



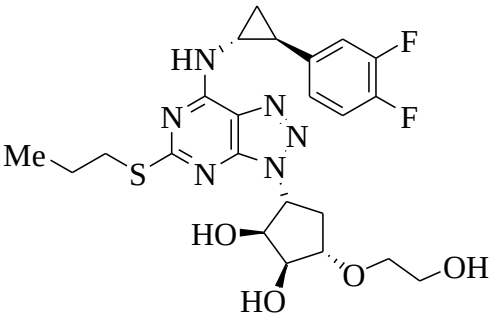
Ticlopidine (3)



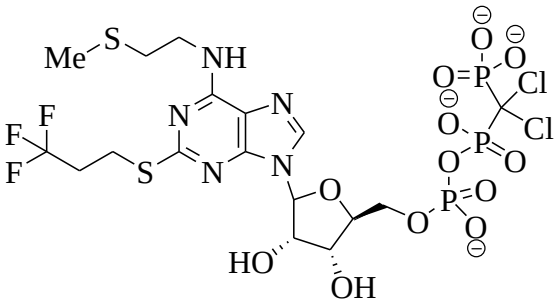
Clopidogrel(4)



Prasugrel(5)



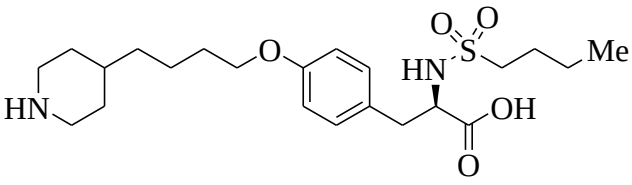
Ticagrelor (6)



Cangrelor (7)

(c) Glycoprotein IIb/IIIa-receptor antagonists

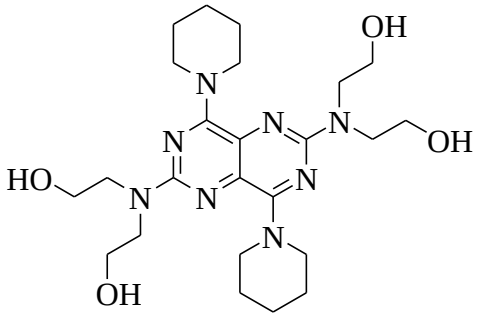
Binding of fibrinogen and vWF to the GPIIb/IIIa or $\alpha_{IIb}\beta 3$ integrin receptors on activated platelet surface is a final mechanism of platelet aggregation hence inhibition of these receptors is an effective way of antiplatelet properties of GP-antagonists. e.g. tirofiban (**8**), epfibatide and abciximab. Abciximab (a Fab fragment from human monoclonal antibody) and eptifibatide (a cyclic heptapeptide) are used in the treatment of ACS and PCI while tirofiban is indicated in unstable angina. They are parenterally administered and have adverse effects like severe bleeding and thrombocytopenia.[33, 34]



Tirofiban (8)

(d) Phosphodiesterase inhibitors

Phosphodiesterase-3 enzyme mediates hydrolysis of cAMP to AMP in platelets and blood vessels. A pyrimidopyrimidine analog dipyridamole (**9**) inhibits phosphodiesterase-3 enzyme thereby increasing cellular concentrations of cAMP and in turn inhibiting platelet aggregation.[27, 30] Because of its weak antiplatelet effects, dipyridamole in combination with aspirin or wafarin is recommended in thrombotic diseases.[35]



Dipyridamole (9)

1.3.2. Anticoagulants

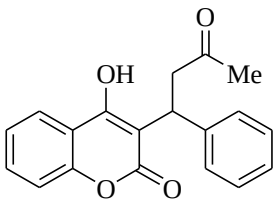
Anticoagulants are the drugs that target different clotting factors of the coagulation cascade to prevent formation of blood clot but unlike antiplatelet agents they are not effective against formed stable blood clots. They show their pharmacological action by direct or indirect inhibition mechanism. Anticoagulants are prescribed in the prophylaxis and management of various arterial and venous thrombotic diseases like DVT, PE, MI, AF and unstable angina. They are also given along with thrombolytics as adjuvants to prevent reocclusion.[16, 30]

Orally active Vitamin-K Antagonists (VKAs) e.g. warfarin and parenterally given heparins [Unfractionated Heparin (UFH) and Low Molecular Weight Heparins (LMWHs)] are the two agents of standard care in antithrombotic therapy for the last many decades. Though they possess potent antithrombotic activity, they have many serious adverse effects like bleeding, drug-drug interactions and other limitations like constant monitoring, and slow onset and offset of actions, which restrict their use in clinic.[36] Therefore there is a constant demand for safe and efficacious anticoagulants. Recently developed direct oral anticoagulants (DOACs) like selective

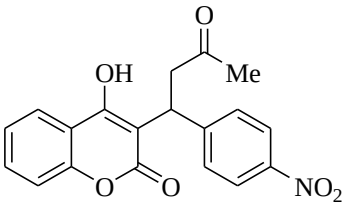
factor Xa inhibitors e.g. rivaroxaban, apixaban, darexaban, betrixaban and direct thrombin inhibitors e.g. argatroban and dabigatran etexilate are clinically approved for different thromboembolic conditions. They are most commonly used now for prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation (NVAF).[37, 38] Different anticoagulants with their mechanisms of action, side effects and other properties are described further as follows.

1.3.2.1. Vitamin-K antagonists

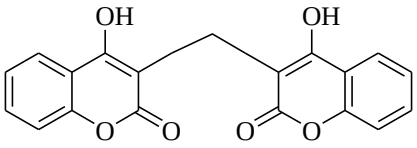
The importance of vitamin K for maintenance of normal hemostasis has been well known since 1920s. It is an essential cofactor in the γ-carboxylation of glutamate residues of coagulation factors like factor II, VII, IX and factor X. Vitamin K epoxide reductase enzyme catalyses γ-carboxylation which is necessary for the procoagulant activities of these factors.[14] Warfarin (**10**) and other coumarin derivatives e.g. acenocoumarol (**11**) and dicoumarol (**12**) inhibit vitamin K epoxide reductase enzyme and restricts regeneration of reduced form of vitamin K, and also block γ-carboxylation mechanism resulting into inactive or partially active clotting factors with diminished activity. VKAs also restrict the activity of anticoagulant proteins, protein C and protein S. This mechanism disturbs blood coagulation and is responsible for their serious bleeding effects.[30] Warfarin discovered in 1941, was first used as rat poison and later being used as anticoagulant for stroke prevention in patients with AF and for prophylaxis and long term treatment of VTE. Though associated with many adverse effects and limitations, till date warfarin is a first choice oral anticoagulant in patients with mechanical heart valves in AF and also in end-stage renal disease in AF patients.[39] Anticoagulant action of warfarin can be reversed by the use of vitamin K preparations, prothrombin-complex concentrates and fresh frozen plasma.[30]



Warfarin (**10**)



Acenocoumarol (**11**)



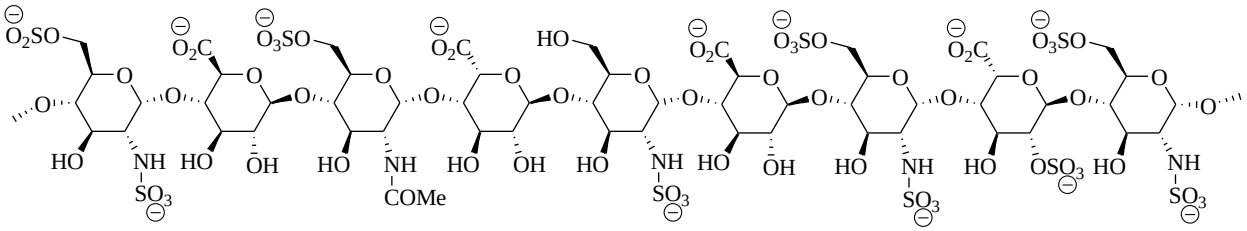
Dicoumarol (**12**)

1.3.2.2 Heparin and derivatives

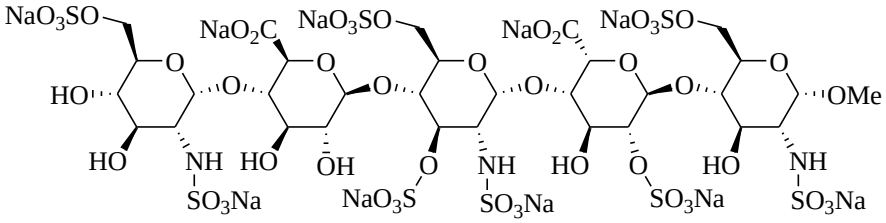
Heparin (**13**) or unfractionated heparin (UFH) is a natural anticoagulant isolated from mast cells of mammalian animals. Chemically it is heterogeneous mixture of highly charged, sulphated polysaccharide of molecular weight from 3000 to 30000 Daltons and made up of *D*-glucosamines and *D*-glucouronic acid units and mostly obtained from bovine lung or porcine intestinal tissues.[37,40] It acts as indirect anticoagulant by activation of endogenous anticoagulant antithrombin which after binding to heparin undergoes a conformational change that enhances its ability to inhibit clotting factors like thrombin, FXa, FIXa, FXIa and FXIIa.[41] The antithrombotic activity is directly associated with the number of sugar units present in the heparin chain. Thus for thrombin inhibition, a minimum of 18 saccharide units are required in UFH to bind to both thrombin and antithrombin while shorter chains than 18 residues still have the ability to inhibit FXa enzyme. UFH exhibits equal inhibitory activity on both thrombin and FXa in 1:1 ratio. Because of poor oral absorption they are given by intravenous or subcutaneous route and indicated in prophylaxis and short-term treatment of DVT. Rebound thrombosis after withdrawal and heparin induced thrombocytopenia (HIT: In HIT platelet count is low due to antibodies generation against heparin-platelet factor 4 binding complex) are serious side effects of UFH.[42]

Heparin is generally divided into two types, UFH and LMWH. LMWHs due to their low molecular weight and small chain lengths have greater FXa inhibitory action but lower anti-thrombin properties. LMWHs are comparatively safe and effective agents than UFH and are produced by controlled chemical depolymerization of UFH. LMWHs e.g. enoxaparin, dalteparin and tinzaparin are approved for antithrombotic activity.[14] Synthetic pentasaccharides fondaparinux (**14**) and idraparinux are selective anti-FXa agents as they do not have anti-

thrombin effect due to lack of longer chains needed for thrombin binding and they are approved in postoperative prevention of DVT in patients with hip or knee replacement. [30, 43]



Heparin (**13**)



Fondaparinux sodium (**14**)

1.3.2.3 Factor Xa inhibitors

Coagulation cascade is a complex and co-ordinated process triggered by endothelial damage and comprises of a number of serine proteases with specific roles. Hence inhibition of a specific enzyme provides agents with wider therapeutic window. Two enzymes, factor Xa and thrombin play critical role in clot formation. Therefore, direct inhibition of either of these two is one of the most effective strategies of anticoagulation.[44]

Factor Xa a key serine protease is positioned at the junction of intrinsic and extrinsic pathways and plays a central role in thrombin generation in coagulation cascade. It possesses γ-carboxylglutamic acid part which facilitates in calcium and phospholipid binding and two epidermal growth factor–like domains, one of which is associated with factor VIIa/Va complex binding. FXa is a dimeric glycoprotein with molecular weight of 59000 Da and consists of two chains of amino acids, one heavy chain of 303-residues and other a light chain of 139-residues

connected by disulphide linkage. The heavy chain accommodates trypsin-like domain in closed anti-parallel β-sheets having a catalytic triad and substrate binding active site. The triad is made up of His57, Ser195 and Asp102 residues while the FXa binding site is defined by S1 and S4 subsites (**Figure 1.6**).[14, 45, 46]

Schechter and Berger have assigned a nomenclature (**Figure 1.7**) to describe the numbering of subsites on the protease enzyme and amino acids on the substrate peptide. According to this system, the protein cleavage takes place between subsites S1 and S1' and between P1 and P1' related amino acids of substrate. The numbering of subsites increases in both sides from the cleavage point as S1, S2, S3….Sn towards N-terminus and as S1', S2', S3',…..Sn' towards C-terminus. Each enzyme subsite e.g. named as S1 binds with the corresponding amino acid substrate residue, P1.[36, 47]

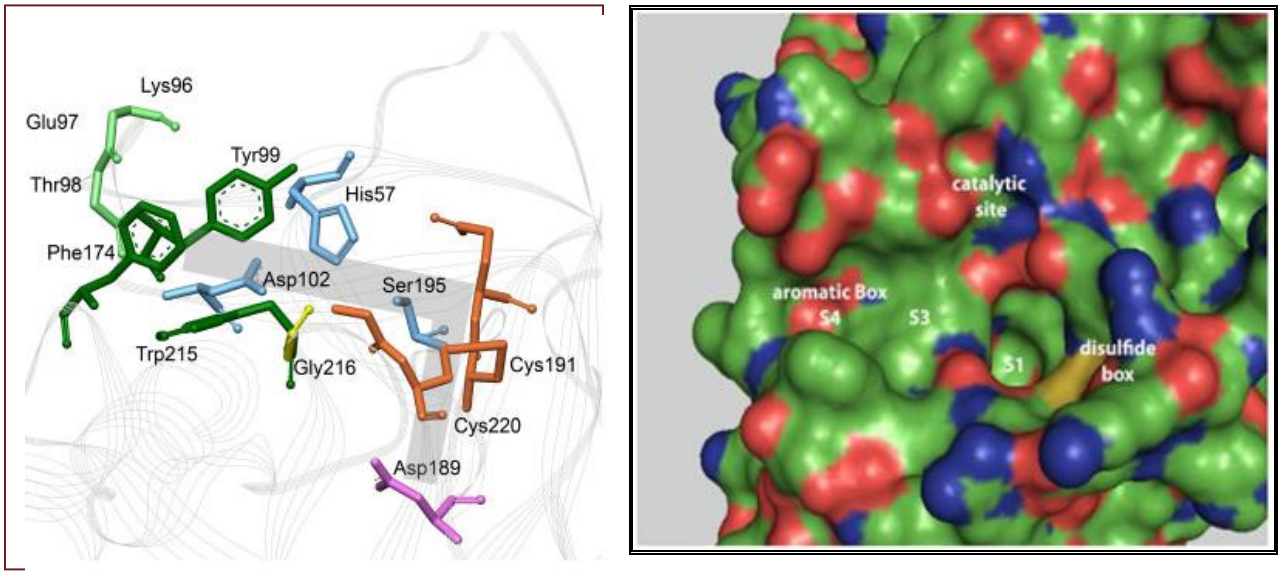


Figure 1.6: Factor Xa enzyme with binding sites, subsites and residues.[14]

FXa S1 binding site is more open, deep groove-like cavity and hydrophobic in nature, at the base, lined by Asp189 and Tyr228 residues. Asp189 fraction forms ionic and hydrogen-bonding interactions with positively charged arginine moiety of the substrate. The S2 pocket of the enzyme is limited to small residues e.g. glycine because of blockage by large side-chain Tyr99. S3 site also known as esteric site is made up of Glu192 amino acid and exposed to the

solvent. Tyr99, Phe174 and Trp215 side-chains make up the hydrophobic aromatic box at the S4-binding region which also encompasses carbonyl moieties of amino acids forming electronegative cavity called as 'cation hole' present at the back of the aromatic site.[47] This S4 site of FXa enzyme is widely explored for design of direct factor Xa inhibitors because of the distinguishing feature of FXa from over other serine proteases in the cascade.[12]

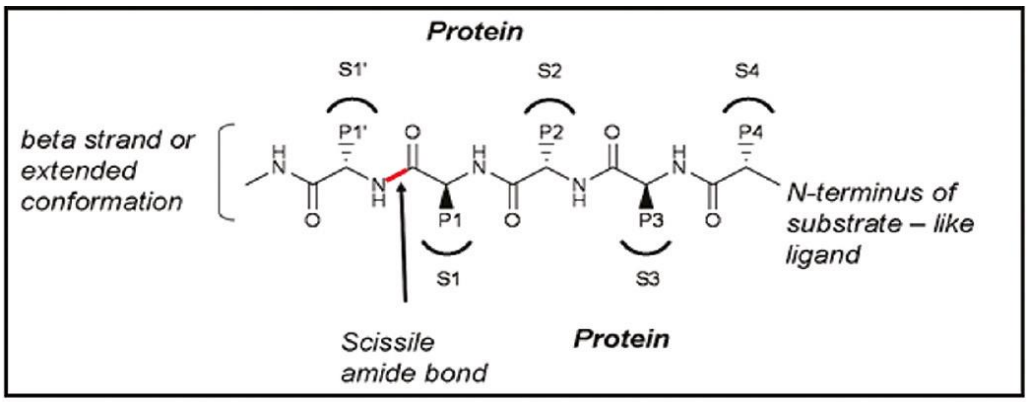


Figure 1.7: Schechter and Berger nomenclature for numbering on serine protease enzyme and peptide substrate. [36, 47]

b. Functional role of FXa enzyme

The vital role played by thrombin and factor Xa in coagulation make them the targets of choice in the development of new anticoagulants. FXa along with FVa, in the presence of calcium ions form prothrombinase complex which converts prothrombin to thrombin. FXa regulates the thrombin production by catalyzing formation of 1000 thrombin molecules from one activation and hence, activation of FX enzyme is a primary site of amplification where thrombin burst takes place at the injury site followed by clot formation and wound closure.[48]

Direct inhibition of FXa is comparatively more effective strategy to control thrombogenesis than inhibition of thrombin downstream since the amount of activated factor produced from its inactive form increases at every step of cascade. Also inhibition of FXa prevents formation of new thrombin molecules and does not affect normal basal thrombin level and platelet function needed to maintain normal hemostasis and thus shows minimum bleeding effects. Factor Xa displayed activation of clotting over a wider range of concentrations compared

to thrombin in animal model and *in vitro* studies and thus exhibited wider therapeutic window. All these reasons have promoted factor Xa enzyme as an attractive target for anticoagulation therapy. Direct, low molecular weight FXa inhibitors inhibit free and prothrombinase-bound as well as clot-bound FXa molecules and thus prevent the prothrombin to thrombin activation and propagation of thrombosis.[20, 26, 36]

c. Design of FXa inhibitors

Early efforts in the development of antithrombotic agents were directed for small, low molecular weight thrombin inhibitors. But it was evidenced that inhibition of FXa at upstream position in the cascade provided better antithrombotic potency.[49] Hence most of the field research then shifted towards small, direct FXa inhibitors. Preclinical studies with tick anticoagulant peptide (tAP, obtained from soft tick, *Ornithodorous Moubata*) and antistatin have supported factor Xa as a valid target for anticoagulation.[50] Failure to show oral bioavailability by these two direct FXa inhibitors tAP and antistatin, encouraged scientists to develop small, orally active enzyme inhibitors.[47, 51] Though FXa possesses resemblance with other serine proteases like thrombin and trypsin, some unique structural differences among FXa, thrombin and trypsin were helpful in structure-based design of direct FXa inhibitors. Selective inhibition of a particular enzyme is a big problem in development of enzyme inhibitors as all serine proteases play different regulatory roles in the coagulation cascade.[52]

The initially designed antithrombotic molecules were mostly dibasic peptide derivatives, mainly amidine and non-amidine types. Several X-ray crystallographic studies on enzyme-inhibitor complexes have been used for identification of different S1 and S4 binding moieties and it was reported that amidine moiety binds with Asp189 residue via salt-bridge interactions in the S1 pocket while non-amidine type molecules show chloro-binding mode with Tyr228 residue at the bottom of S1 pocket.[12,53,54] It was observed that all selective FXa inhibitors adopt L-shaped binding conformations within the active enzyme site.[53] Bioavailability issues with amidines have prompted replacement of amidines by nonbasic and nonpolar neutral moieties. Discovery and development of these agents is chronologically evolved from early symmetric dibasic peptide inhibitors to asymmetric, highly basic, poor orally bioavailable, nonselective agents progressing to mildly basic, more selective with improved orally bioavailable derivatives finally leading to

nonbasic, highly selective and potent orally active, small molecular weight direct FXa inhibitors.[10,14]

A general structure (graphical representation) for FXa inhibitors is depicted in **Figure 1.8**. It has three common structural features, the P1 and P4 motifs and the linker between the two motifs. The chemical groupings used to represent them are shown in **Figure 1.9-1.11**. Three structural components make up the structure of factor Xa inhibitors viz. P1 residue, P4 residue and the central linker. The preferred nonbasic P1 moieties include 5-chlorothiophene (rivaroxaban,**15**), 4-methoxybenzene (apixaban,**16** and darexaban,**20**), 5-chloropyridine (edoxaban,**17** and betrixaban, **18**), 4-chlorobenzene (eribaxaban), and chloroindole while the hydrophobic P4 groups frequently used are morpholinones, lactams, pyridones, piperidinones, tetrahydrothiazolopyridines, aminoimidazole, amidines, guanidines etc. The reported central linkers or scaffolds connecting both P1 and P4 moieties are oxazolidinones, anthranilamides, cyclic diamines, piperazinones, indoles and indazoles.[47, 52] Factor Xa inhibitors are mainly divided into two main categories as indirect and direct FXa inhibitors. Heparin analogs e.g. UFH, LMWH, enoxaparin, danaparoid sodium, fondaparinux and idraparinux are all antithrombin-dependent derivatives acting as indirect FXa inhibitors. Direct FXa inhibitors again are divided into naturally derived and synthetic inhibitors. Most of the new oral anticoagulants are direct synthetic, reversible, selective FXa inhibitors. e.g. rivaroxaban, apixaban, edoxaban, betrixaban, razaxaban (**19**) and darexaban.[21,55] Some currently approved oral FXa inhibitors with their pharmacological properties are described in **Table 1.1**.[20, 56] Both razaxaban and darexaban have been discontinued after toxicity issues reported during clinical trials.[57]

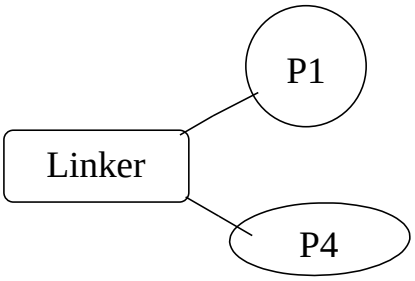


Figure 1.8: Graphical representation of general structure of FXa inhibitors.

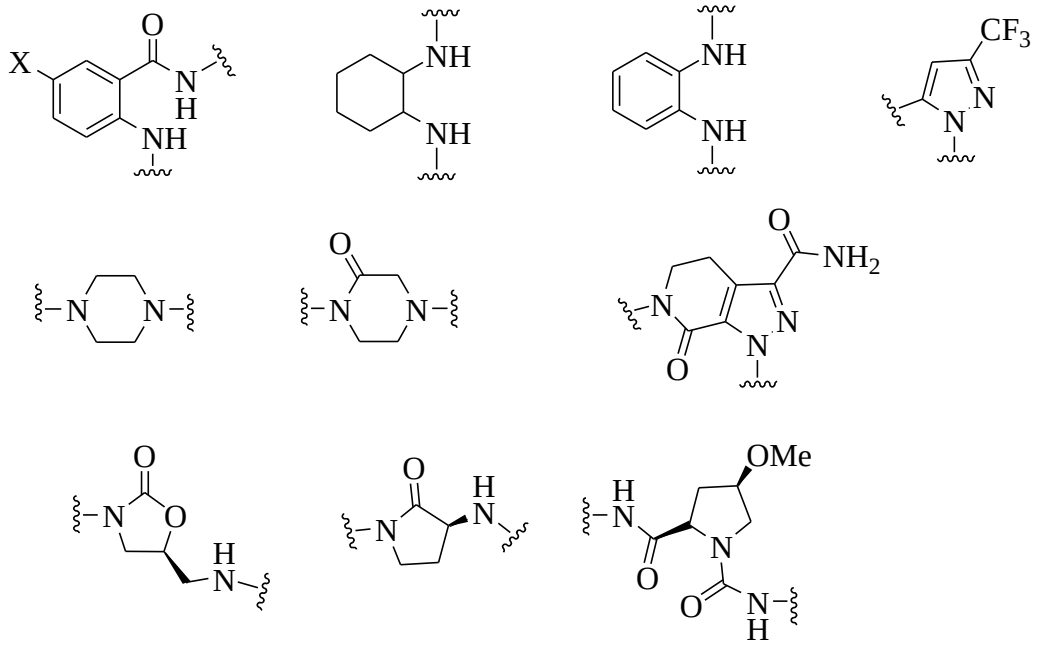


Figure 1.9: Commonly used central scaffolds or linkers in FXa inhibitors.

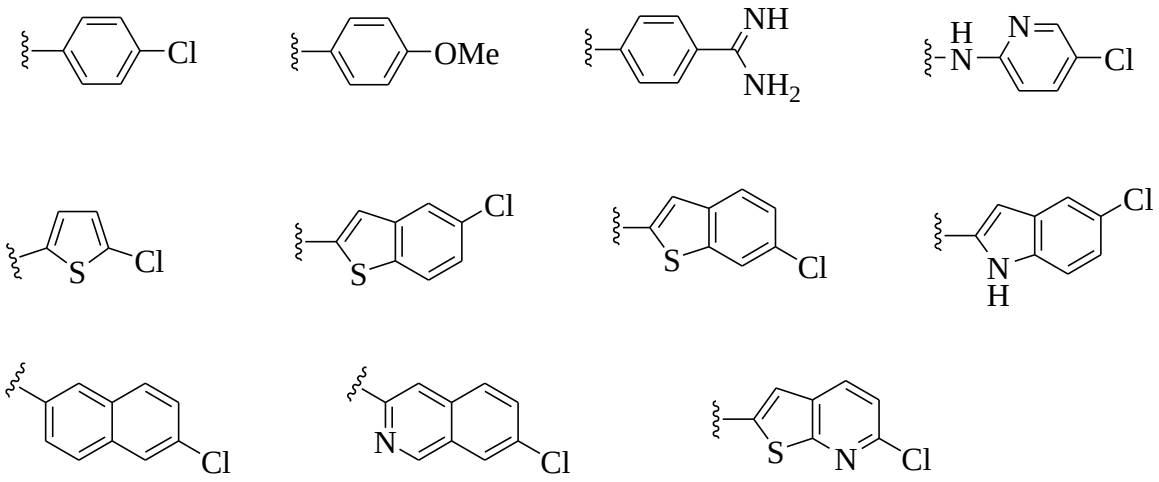


Figure 1.10: Commonly used P1 moieties in FXa inhibitors.

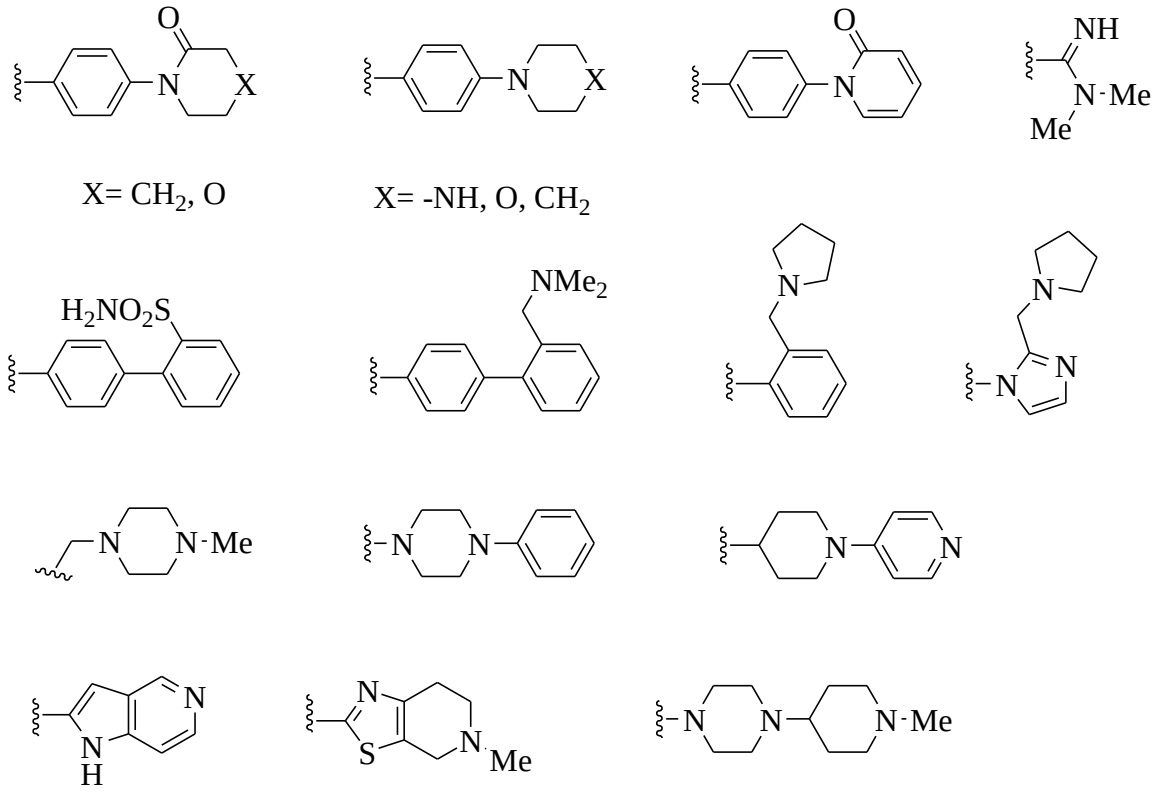
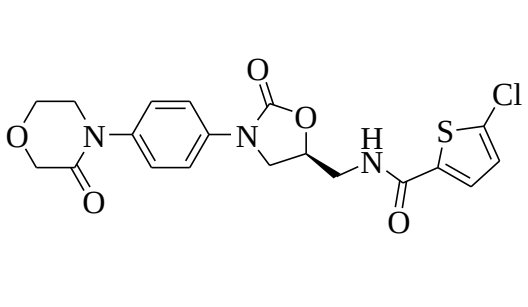
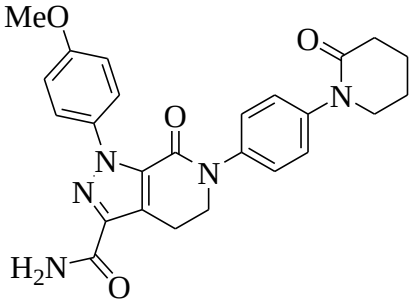


Figure 1.11: Commonly used P4 motifs in FXa inhibitors

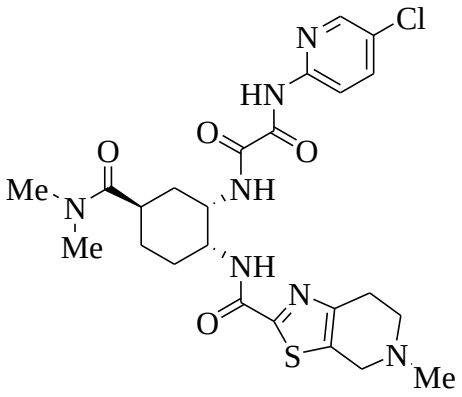
First oral FXa inhibitor, rivaroxaban was approved in 2008 and used in prophylaxis of stroke and systemic embolism in AF patients, VTE after knee or hip surgery, recurrent VTE and in patients with ACS having atherothrombotic events. It is also used in treatment of DVT and PE. Apixaban and edoxaban are also approved for similar indications.[10,14] Betrixaban is approved very recently in 2017 by USFDA for prevention of VTE in hospitalized patients.[58]



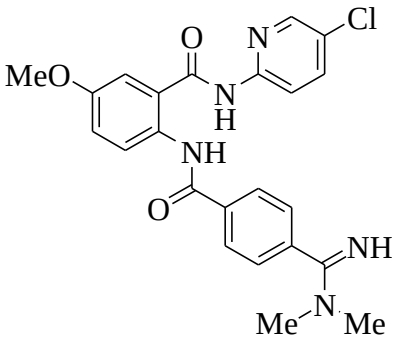
Rivaroxaban (**15**)



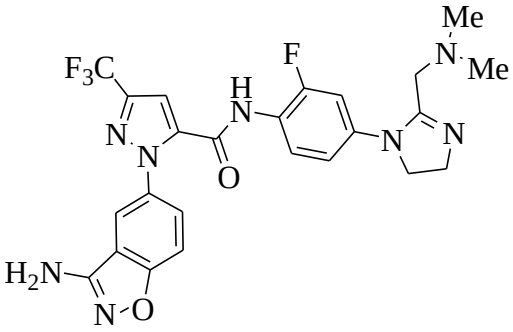
Apixaban (**16**)



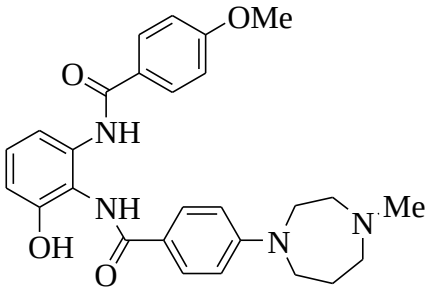
Edoxaban (**17**)



Betrixaban (**18**)



Razaxaban (**19**)



Darexaban (**20**)

Table 1.1: Currently approved oral FXa inhibitors with their pharmacological properties.[20, 55, 56]

Properties	Rivaroxaban (15)	Apixaban (16)	Edoxaban (17)	Betrixaban (18)
Manufacturer	Bayer Healthcare/ Janssen	Bristol-Myers Squibb	Daiichi-Sankyo	Portola
FXa K_i (nM)	0.4	0.08	0.56	0.11
(%) Oral bioavailability	80 %	66 %	50 %	35 %
Half life (hr)	7-11	12	5-11	37
T_{max}	2-4 hr	3-4 hr	1-2 h	3-4 h
Monitoring	Not necessary	Not necessary	Not necessary	Not necessary
Plasma protein binding	90 %	87 %	40-59 %	60 %

Renal excretion	66 %	25 %	35 %	7 %
Antidote	Andexanet alfa	Andexanet alfa	--	--
Clinically Approved Indications	-Postoperative prophylaxis of DVT in US, EU -Prevention of stroke and systemic embolism in non-valvular AF patients - Treatment of acute DVT & PE in US, EU for long-term recurrence. -Reduction in the risk of DVT and PE recurrence	- Prophylaxis of DVT after hip or knee replacement surgery - Treatment of DVT and PE - For reduction of risk of stroke and systemic emboli in non-valvular AF in US, EU, JP.	- VTE prophylaxis in JP -For treatment of non-valvular AF in patients with CrCL, 50-95 ml/min in US, EU & JP. - In patients with CrCL, 15-50 ml/min in JP for treatment of DVT and PE	-Prophylaxis of VTE in hospitalized patients in US, EU.

INR: International normalized ratio; VTE: Venous thromboembolism; AF: Atrial fibrillation; MI: Myocardial infarction; DVT: Deep vein thrombosis; PE: Pulmonary embolism; ACS: Acute coronary syndrome. EU: European Union, US: United States, JP: Japan, CrCL: Creatinine clearance.

1.3.2.4 Thrombin Inhibitors

a. Thrombin: Multifunctional role in coagulation cascade

Thrombin, a serine protease is a multifunctional enzyme playing a pivotal role in the regulation of coagulation and fibrinolysis processes and maintenance of hemostasis. It possesses both procoagulant and anticoagulant properties.[59] Thrombin or factor IIa catalyzes the final step of coagulation cascade viz. conversion of insoluble fibrinogen to soluble fibrin and activates factor XIII forming cross-linked fibrin strands and stable clot. It also facilitates activation of clotting factors FXI, FIX, FX and cofactors FV and FVIII through positive feedback mechanism and thus regulates its own production. It is responsible for down-regulation of fibrinolysis by activation of thrombin-activatable fibrinolysis inhibitor (TAFI).[47, 60] Thrombin also acts as a potent platelet activator by interacting with platelet surface glycoproteins and cleavage of protease-activated receptors (PAR-1 and 4) inducing platelet aggregation. Additionally, thrombin also displays anticoagulant activities. It binds with thrombomodulin forming a complex which activates natural anticoagulant serine protease protein C that along with protein S causes degradation of factors Va and VIIIa leading to feedback inhibition of thrombin.[61,62] Many

research findings have revealed that thrombin also exhibits physiological effects on smooth muscles and endothelial cells, leucocytes, heart and neurons.[63]

Human thrombin is made up of two polypeptide chains, one light chain of 36 amino acid residues and another heavy chain of 259 amino acid residues, both joined by disulfide bridges.[64] Various enzyme inhibitor complexes reported earlier have revealed valuable data regarding distinct active sites of thrombin and binding of different substrates.[65] Being a serine protease, thrombin consists of catalytic triad Ser195, Asp102, His57 and an oxyanion hole. The S1 active site also known as 'recognition site' or specificity pocket is made up of Ala190 residue that differentiates it from trypsin, and Asp189 at the bottom forms hydrogen-bond and ionic interactions with the substrates containing basic P1 moieties e.g. arginine, lysine.

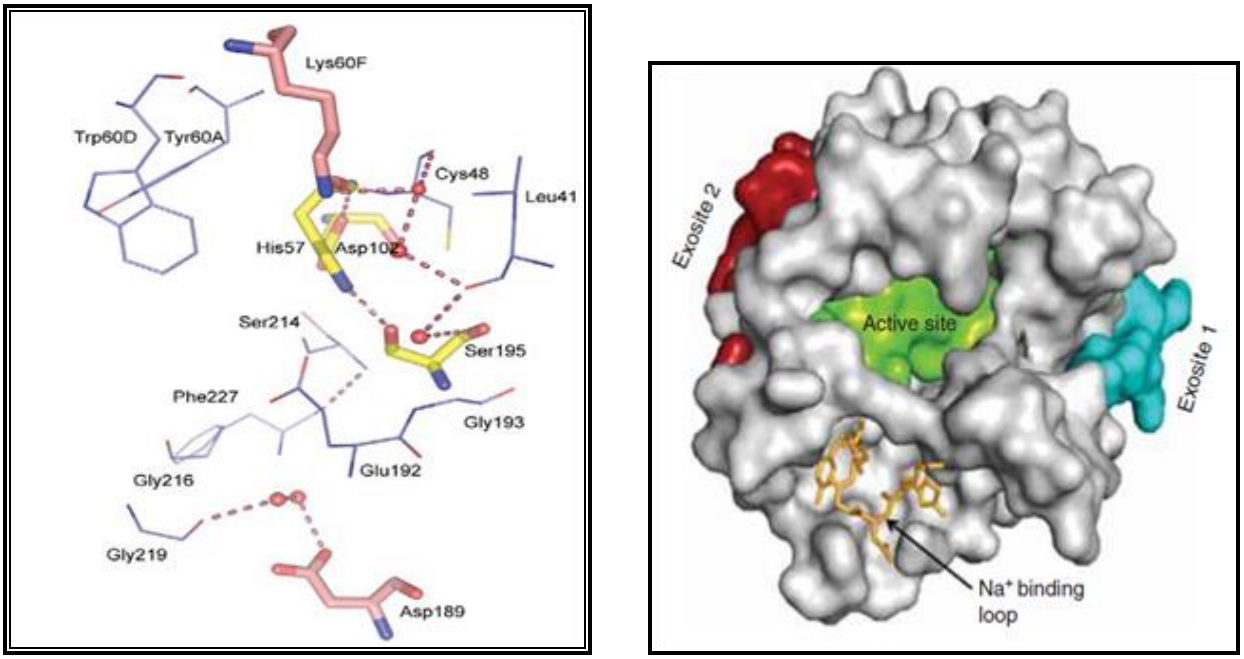


Figure 1.12: Human thrombin enzyme active sites (left) [66] and 3-D thrombin structure (right).[62]

The active proximal S2 pocket possesses alignment of thrombin specific YPPW insertion-loop (also called D-pocket) comprising of Tyr60A-Pro60B-Pro60C-Trp60D amino acid residues and is surrounded by Leu99 and His57 residues (**Figure 1.12**, left).[66] This pocket accommodating specific and small hydrophobic P2 residues *e.g.* valine and proline, acts as a distinguishing feature for the design of selective thrombin inhibitors. The larger distal S3 site is

normally flat and slightly acidic with Glu192 residue. The hydrophobic S4 pocket is lined by Leu99, Ile174 and Trp215 amino acids and forms edge to have van der Waals interactions with the aromatic groups.[47, 62, 63, 67]

Additionally thrombin also has two positively charged allosteric binding sites known as anion-binding exosite-1 and -2 located at opposite sides of the enzyme and they bind with various proteins (**Figure 1.12**, right).[62] Exosite-1 interacts with fibrinogen and platelet PAR-1 to orient the substrates into active site for thrombin activity. Also exosite-1 binds with thrombomodulin, heparin cofactor II and anionic C-terminal of hirudin and hirudin like peptides. Exosite-2 is responsible for binding with polysaccharides, heparin and dermatan sulphate. This type of binding results in antithrombin dependent thrombin inhibitory activity of heparin. Thrombin also displays ligand-dependent allosterism where both exosites cause conformational changes in the active sites that mediate enzyme specificity and catalytic activity.[62, 68-70]

b. **Thrombin inhibition and design of thrombin inhibitors**

Amongst the serine proteases of coagulation cascade, thrombin is the most extensively studied target for anticoagulation therapy and design of active-site inhibitors. Universal role of thrombin in maintaining hemostasis makes it a critical target for development of novel specific antithrombotic agents.[61] Enormous efforts have been made by researchers in the last few decades to develop low molecular-weight thrombin inhibitors having selectivity, high efficacy, oral bioavailability and improved safety profile in comparison to the traditional anticoagulants like warfarin but very few agents made it to the market.[65] Early thrombin inhibitors identified in 1950s were parenterally administered polypeptides like hirudin which was obtained from salivary glands of leech. Two recombinant forms of hirudin, viz. lepirudin and desirudin are now used today for treatment of HIT and prophylaxis of VTE in patients with hip-replacement surgery repectively.[71]

Early stage research on developing small direct thrombin inhibitors (DTIs) was targeted on *D*-Phe-Pro-Arg peptidomimetic derivatives which incorporated acidic moieties leading to discovery of argatroaban (**21**), first DTI approved in 2000, used parenterally for the treatment of thrombosis in HIT patients.[72] In pursuit of oral DTIs, many initial derivatives containing

guanidine or highly basic benzamidine moieties and acidic groups were developed that yielded zwitterionic compounds, but all of them exhibited poor oral bioavailability and pharmacokinetic properties hence later on non-peptide inhibitors were designed (**Figure 1.13**).[73] The issue of poor pharmacokinetics was resolved by designing of prodrugs. Ximelagatran (**22**), a double prodrug

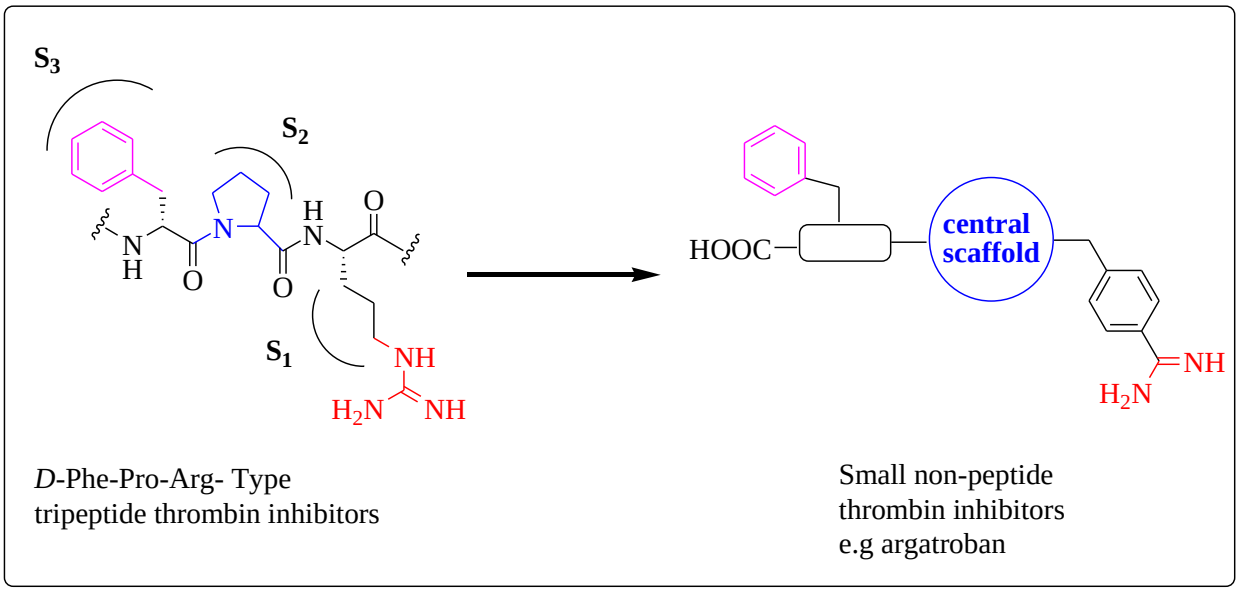
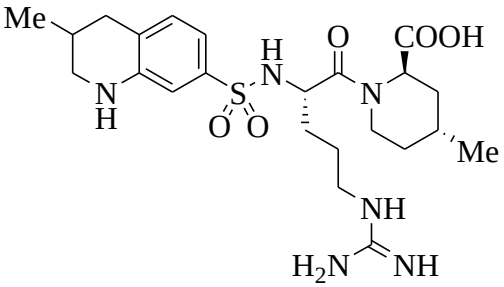
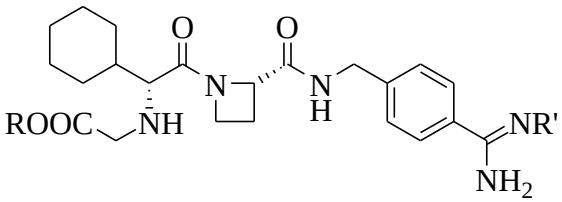


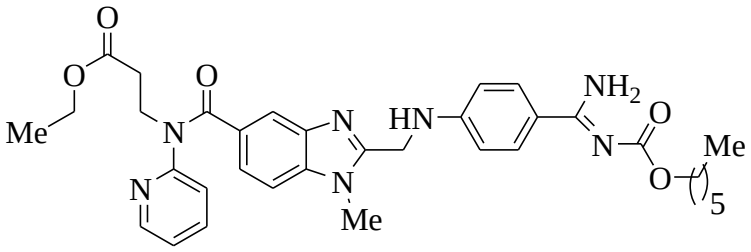
Figure 1.13: Development of early small molecular weight direct thrombin inhibitors.[73]



Argatroban (**21**)



Ximelagatran (**22**) R= Et, R'= OH
Melagatran (**23**) R= R'= H



Dabigatran etexilate (**24**)

of melagatran (**23**) was the first oral DTI introduced in market in 2004 but was withdrawn later due to liver toxicity.[47] Next oral DTI dabigatran etexilate (**24**), a double prodrug was launched in 2008 in Europe for prophylaxis of VTE and in 2010 in US for stroke prevention and presently, this is the only available oral DTI for anticoagulation.[74] Recently many novel allosteric thrombin inhibitors have been designed and developed which maintain some procoagulant action that results in lower bleeding tendencies by the compounds, but unfortunately all these agents were found to be orally inactive.[72, 75]

Thrombin inhibitors exhibit either direct or indirect mechanism of action. They block thrombin action by binding to one or two of its three domains viz. active site and exosites 1 and 2, as shown in **Figure 1.14**.[76] Exosite 1 serves as a dock for substrates like fibrin while exosite 2 acts as heparin binding domain. Heparin (UFH) and LMWHs bind to both antithrombin and exosite 2 simultaneously and form heparin-thrombin-antithrombin complex and thus inhibit free thrombin indirectly.[76] Direct thrombin inhibitors inhibit both soluble thrombin and fibrin-attached thrombin, without needing cofactor antithrombin. Bivalent DTIs e.g. hirudin and bivalirudin, interact with both of the active sites and exosite 1 of thrombin while univalent DTIs e.g. argatroban, dabigatran block the only active site for thrombin inhibition. Clinical and pharmacological properties of currently used DTIs are described in **Table 1.2**.[76,78] Examples of indirect thrombin inhibitors are warfarin, heparin, LMWH and fondaparinux while DTIs include hirudins, argatroban, dabigatran etexilate and ximelagatran. DTIs also exhibit some antiplatelet action owing to reduction in thrombin-induced platelet activation.[77]

Table 1.2: Pharmacological and clinical properties of direct thrombin inhibitors.[76, 78]

Drugs	Lepirudin	Desirudin	Bivalirudin	Argatroban (21)	Dabigatran etexilate (24)
Thrombin affinity & K_i	Highest 0.23 pM	Highest 0.26 pM	Intermediate 1.9 nM	Lowest 5-38 nM	Intermediate 4.5 nM
Route of administration	Intravenous, subcutaneous	Intravenous, subcutaneous	Intravenous	Intravenous	Oral
Plasma half-life ($T_{1/2}$)	~ 80 min.	i.v. 60 min. s.c. 120 min.	~ 25 min.	~ 45 min.	12-14 hr.
Excretion	Renal	Renal	Renal	Hepatic	Renal
Monitoring	Yes	Yes	Yes	Yes	No
Approved indications	Prophylaxis and treatment of thrombosis in HIT patients	Postoperative prophylaxis of DVT after total hip replacement.	-Patients with unstable angina undergoing PCI. -Patients at high risk of HIT undergoing PCI.	-Prophylaxis and treatment of thrombosis in HIT patients. -Patients at high risk of HIT undergoing PCI.	-VTE prophylaxis after hip or knee replacement surgery. -Treatment and recurrence of DVT & PE. -Stroke prevention in nonvalvular patients.

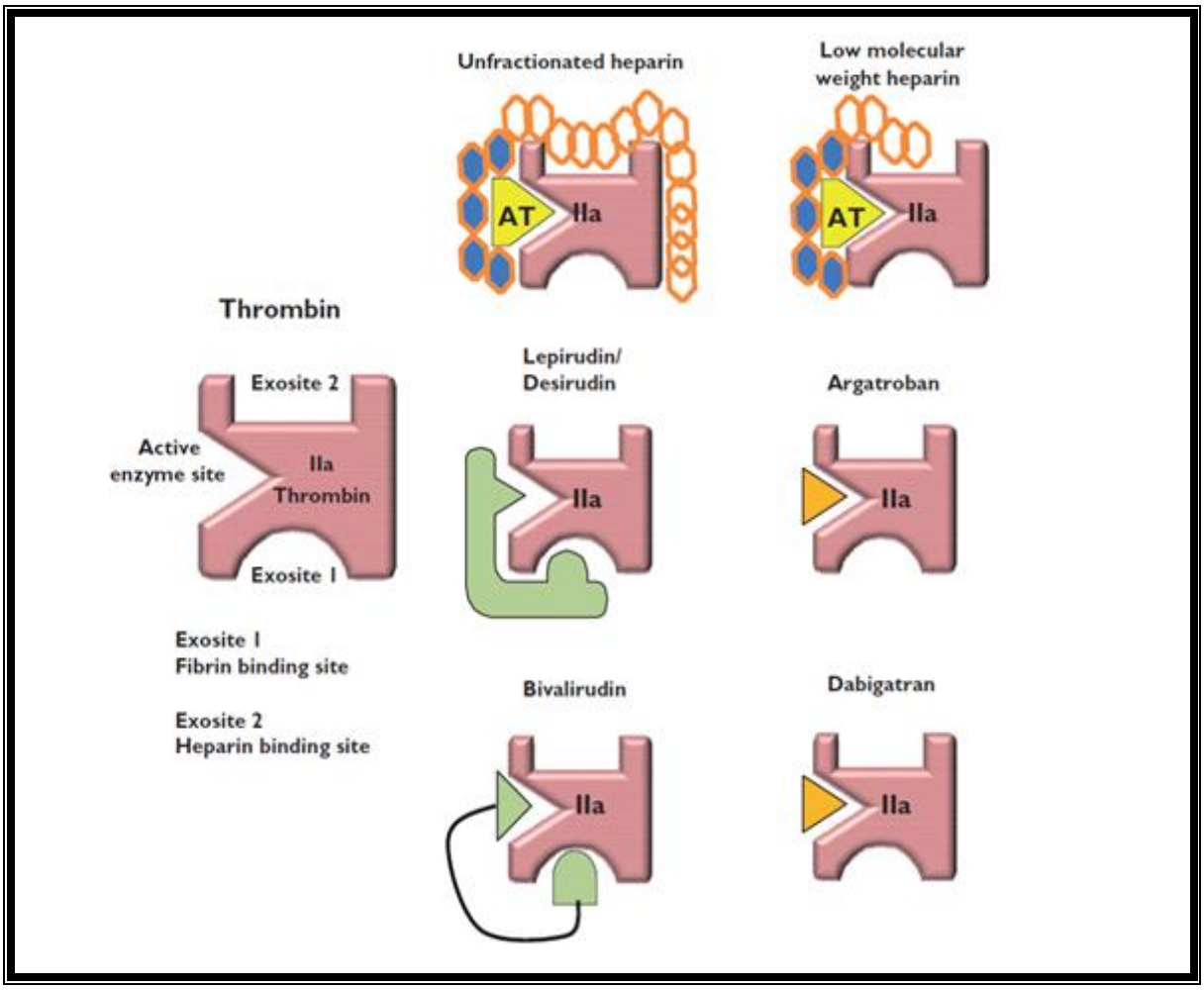


Figure 1.14: Mechanism of thrombin binding with different antithrombotic agents.[76]

1.3.3 Fibrinolytic or Thrombolytic agents

The fibrinolytic process is vital in limiting the propagation of thrombi formation by dissolution and removal of unwanted clots thereby restoring normal blood flow. Though anticoagulants are effective in prevention and propagation of thrombus formation they are not active against pre-formed clots. Fibrinolytic or thrombolytic agents activate the fibrinolytic mechanism in the body that lead to lysis of thrombus clot. They act by conversion of inactive zymogen plasminogen to active protease plasmin which catalyses hydrolysis of insoluble fibrin matrix to soluble fibrin degradation products.[78] Presently approved fibrinolytic drugs are streptokinase, urokinase, anistreplase and recombinant tissue-plasminogen activator (rt-PA, also

called alteplase) and derivatives of rt-PA i.e. reteplase and tenecteplase. All these agents are high molecular weight compounds obtained from either bacterial sources or recombinant technologies. They are indicated in conditions like acute MI, ischemic stroke, DVT and PE. They are administered systemically or through catheters into thrombus site. Because of nonspecificity of plasmin which degrades fibrin as well as fibrinogen and also other clotting factors ultimately lead to serious bleeding. The major drawbacks of thrombolytic agents are risks of bleeding, short half-life and necessity of parenteral administration.[16,30,79]

1.4 Advantages and drawbacks of current antithrombotic drugs therapy

Antithrombotic drugs are used in the treatment of thrombotic diseases since the early last century with the introduction of pioneer drugs e.g. heparin and vitamin-K antagonists like warfarin. Though effective, over a period of time these agents showed many adverse effects and limitations during the treatment. Bleeding is the most common adverse effect for all antithrombotic drugs.[80] Some drug-specific side effects are mentioned in **Table 1.3**. Additionally they also possess many limitations. Warfarin shows delayed on-and off-set of action, patient variability, long half-life, very narrow therapeutic index and it needs constant monitoring. Heparin (UFH) needs parenteral administration and daily monitoring due to unpredictable pharmacokinetic properties. The disadvantages and limitations of these early anticoagulants have prompted the need for development of safe and effective anticoagulants.[55]

DOACs furnish many advantages over VKAs and heparin analogs. They are comparatively safe drugs exhibiting lower bleeding tendencies. They are conveniently used without any monitoring, show quick onset of action and predictable PK-PD properties, minor food and drug interactions and broad therapeutic window. Inspite of relative safety of DOACs with respect to bleeding complications, these side effects still persist and patients under therapy may need urgent intervention or surgical emergency hence require reversal agents urgently.[39,81,82] Currently two antidotes are approved for reversal of uncontrolled bleeding effects of new oral anticoagulants viz. idarucizumab (Praxbind), a monoclonal antibody fragment for dabigatran etexilate in 2015 and andexanet alfa (Andexaa), a recombinant FXa decoy for direct FXa inhibitors rivaroxaban and apixaban in 2018, while ciraparantag as a common antidote for all anticoagulants is under development.[83] DOACs are contraindicated in patients with mechanical

heart valves due to reports of valve thrombosis and renal and liver failure because these drugs are excreted by these routes. Higher costs involved in their acquisition is responsible for their poor accessibility and patient compliance.[20]

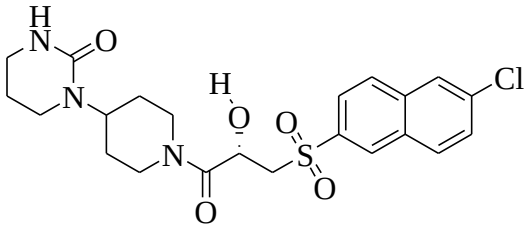
Table 1.3: Current antithrombotic drugs with their side effects.[30, 84, 85]

S. No.	Drug	Adverse effects
1	Warfarin	Skin necrosis, fetal abnormalities, alopecia, potential CYP-based drug-drug interactions.
2	Heparin	Antigenic hypersensitivity, hair loss, thrombocytopenia, osteoporosis after prolonged use.
3	Lepirudin	Hematuria, hematoma, pneumonia, multi organ failure
4	Apixaban	Rare hepatotoxicity
5	Dabigatran	Gastrointestinal bleeding, dyspepsia, rebound thrombosis
6	Danaparoid	Coughing up blood, paralysis, tarry stool, discoloured urine, breathlessness, vomiting, dizziness
7	Aspirin	Gastric bleeding, ulcers, hepatotoxicity, skin reaction
8	Ticlodipine	Thrombocytopenia, cholestatic changes, hepatotoxicity, rashes, colitis, and arthritis
9	Clopidogrel	Neutropenia, dyspepsia, dizziness, aplastic anemia
10	Abiciximab	Cardiac side effects, heart burn, change in vision, abdominal pain
11	Dipyridamole	Rashes, dizziness, hypotension, itching, redness of skin, diarrhoea, bruising.

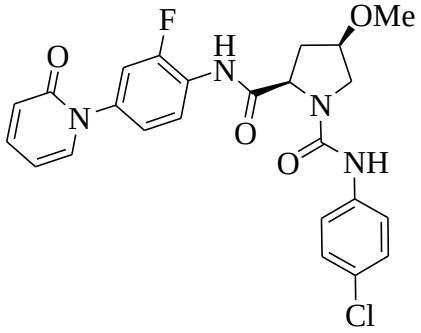
1.5 Future Scope and perspectives

Thrombosis remains the major culprit behind high mortality resulting from diseases like venous thromboembolism, MI and strokes. Hence development of effective and safe antithrombotic drugs is need of the time. For prophylaxis and initial treatment of thrombosis, promptly acting parenteral agents e.g. LMWHs and sometimes fondaparinux and bivalirudin are

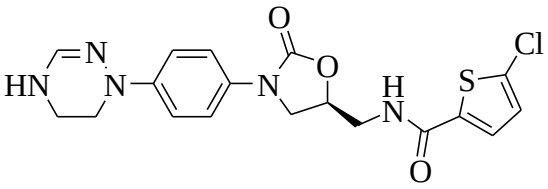
available while vitamin K antagonists and DOACs are recommended for long-term anticoagulation therapy. The annual global sales of antithrombotic agents for the year 2017 is shown in **Figure 1.15** and it is expected to grow further with the introduction of new oral anticoagulants due to the advantages they would have over the traditional agents and also due to introduction of generic versions of rivaroxaban and dabigatran in a few years.[86] The ease of use and better safety profile of DOACs has prompted more investigations for new indications like ACS, CAD and peripheral artery disease.[87]



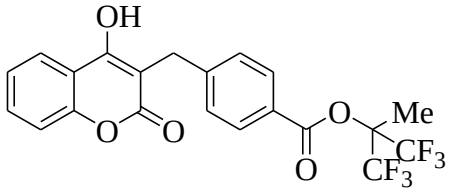
Letaxaban (**25**)



Eribaxaban (**26**)



Nokoxaban (**27**)



Tecarfarin (**28**)

Though offering advantages over VKAs, bleeding events and some other limitations do not make DOACs as the ideal anticoagulant, hence the search for new drugs still continues. Without any doubt, introduction of factor Xa and thrombin inhibitors have significantly improved both efficacy and safety of anticoagulant drug therapy and also provided impetus to the academic and industrial drug discovery research. Some novel FXa inhibitors e.g. betrixaban, letaxaban (**25**), eribaxaban (**26**), nokxaban (**27**) and VKA like tecarfarin (**28**) are under development. The current status of these agents in phase trials are mentioned in **Table 1.4.**[88] Factors XII and XI as new targets for anticoagulation are under studies as safer alternatives, since upstream inhibition of coagulation is safer than downward level and deficiency of these

factors have negligible effect on hemostasis. Though sharing similar mechanism of action DOACs still exhibit variable efficacy and safety leading to approval of a few compounds while discontinuation of other agents. Selection of proper anticoagulants need knowledge about their oral bioavailability, metabolism and routes of excretion, benefits and risks involved and these factors majorly depend on pharmacological and pharmacokinetic properties of the drugs and also on patient age and disease state, dose adjustment and frequency of administration.[88] All of these pose economical and organizational challenges in assessment of antithrombotic agents making the job burdensome for professionals. The development of new oral anticoagulants with favorable benefit-risk profiles including new indications and availability of their antidotes has opened a new era in antithrombotic drugs therapy and certainly they would be helpful to achieve the optimistic goal of maximum efficacy plus safety without disturbance of hemostasis.

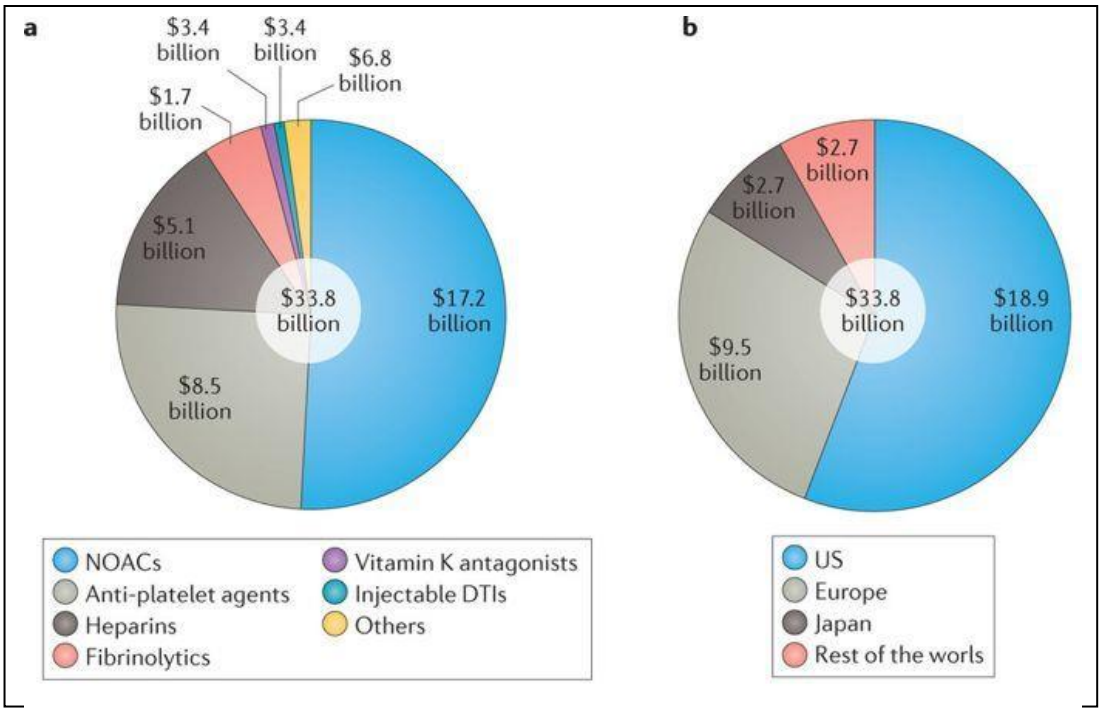


Fig. 1.15: Worldwide sales of antithrombotic drugs in 2017.[86]

Table 1.4: Current oral anticoagulants under development.[88]

Drug	Phase	Dose	Onset of action	Half-life	Clinical indications under study
Betrixaban	Approved by US-FDA; EMA and TGA approval pending.	Oral, 40 or 80 mg	3-4 hr	19-27 hr	Prevention of VTE in hospitalized patients approved by US-FDA; prevention of VTE in patients with AF.
Eribaxaban	Phase II completed	Oral, 2.5-150 mg	3-4 hr	10 hr	Prevention of VT
Letaxaban	Phase II completed	Oral, 10-160 mg	NA	9-12 hr	Prevention of VTE after orthopaedic surgery; treatment of ACS.
Nokxaban	Phase I completed	Oral, 20-40 mg	< 1 hr	3-9 hr	Still not defined

FDA: U. S. Food and Drug Administration, EMA: European Medical Agency, TGA: Australian Therapeutic Goods Administration.

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