CHAPTER I

INTRODUCTION

1.1 Introduction

The leading causes of the subsequent global increase in mortality are cardiovascular disease (CVDs) for individuals with myocardial infarction, hypertension, hyperlipidemia, diabetes mellitus, stenocardia, or coronary heart disease [1-3]. Every year, CVDs claim the lives of approximately 17.9 million people globally, causing them to be the primary reason of illness and death in both industrialized and developing nations [4-6]. As per the most recent data released by the World Health Organization (WHO), the top three causes of death in 2019 were Acute Myocardial Infarction (AMI), Brain Stroke (BS), and Chronic Obstructive Pulmonary Disease (COPD). AMI had the highest mortality rate, with 8.9 million occurrences [7]. The American Heart Association has released estimates about CVDs, stating that by 2030, 23.6 million deaths are expected worldwide [8].

A significant risk factor for cardiovascular diseases (CVDs) is thrombosis, a circulatory condition promoting intravascular coagulation within blood vessels. The principal protein constituent of the thrombus, fibrin, aggregates when the coagulation cascade components are too active. This condition may result in partial or complete blood flow restriction, ischemia, and tissue necrosis [9,10]. Therapeutic approaches, such as surgical intervention, anticoagulants, antiplatelet medicines, and fibrinolytic drugs, which dissolve acute thrombi in arteries, are frequently used to manage thrombosis [11].

The two mechanisms of thrombolysis that underpin the pharmacological approach of fibrinolytic agents are the indirect mechanisms of thrombi lysis, which rely on the biological conversion of plasminogen into plasmin through the action of plasminogen activators such as streptokinase, urokinase, and tissue plasminogen activating factor (t-PA); and the direct pathways of thrombus breakdown, which employ plasmin and nattokinase [12,13]. These thrombolytic medications can trigger allergic responses (urticaria), hemorrhagic consequences, and inflammatory response; as a result, their use may be limited or even avoided. Additionally, these agents often have expensive costs, limited half-lives, and poor selectivity for fibrin [14,15].

1.2 Progression of blood clotting or thrombus formation

The process of preventing loss of blood through clot formation is known as haemostasis. Thrombus formation is the term used to describe an obstructive clot that forms inside a blood vessel [16]. Primary haemostasis, or immediate platelet activation, and secondary

haemostasis, or additional coagulation cascade that produces fibrin strands, are the two forms of haemostasis [17]. Circulating platelets attach to damaged endothelium collagen using distinct surface glycoproteins when they come in contact with it. Platelet integrins are activated, causing platelets to adhere to the injury site [18]. Activated platelets transform their shape to stellate from spherical. They exclude several substrates, including thromboxane A_2, secretion, and platelet-activating Factor (PAF), which stimulate more platelets and improve their ability to bind fibrinogen [19].

In secondary haemostasis, many inactive enzyme precursors, or zymogens, are sequentially activated by proteolytic activity. Each activated zymogen triggers the activation of a subsequent zymogen, which ultimately causes blood to coagulate [19]. Three pathways make up the process of coagulation: the tissue factor pathway (also called the extrinsic pathway), the contact activation pathway (also known as the intrinsic pathway), and the final common pathway [20]. Prothrombin, a proteolytic enzyme, combines with active FX and FV to produce a prothrombinase complex, which converts prothrombin zymogen into active thrombin [21,22].

Throughout the tissue factor pathway, blood contains more FVII than other coagulation factors, and thrombin builds up rapidly. Thrombin's primary function is to change the inactivated fibrinogen zymogen into activated monomer, which combines with other components to form a haemostatic plug. Thrombin also has various functions, including activating platelets, FVIII, FV protein C (when thrombomodulin is present), and FXIII (which aids in the formation of a covalent link between fibrin polymer and zymogens) [19].

1.3 Thrombolysis and fibrinolysis

Thrombolysis normally refers to the dissolution of the thrombus, while fibrinolysis usually stands for the breakdown of fibrin within blood clots. The final product of the coagulation pathway is fibrin. The serine protease enzyme plasmin, which resembles trypsin, dissolves fibrin. Plasmin is the active component of plasma zymogen, also known as plasminogen. There are two main glycoforms of plasminogen in humans: type I plasminogen has two glycosylation molecules, while type II plasminogen only has one glycosylated molecule [23]. Circulating plasminogen acquires an open conformation upon binding to a blood clot or cell surface, which cleaves between Arg-561 and Val-562 to generate active plasmin. Numerous enzymes, including tissue plasminogen activator

(tPA), urokinase-type plasminogen activator (uPA), Kallilrein, and factor XII, are involved in this cleavage [24-26].

Plasmin converts fibrin into soluble breakdown products once it is fomed [27]. Two steps make up fibrinolysis: first, plasminogen activates on the surface of fibrin clot to generate plasmin; in the second stage, second, plasmin breaks fibrin, revealing more binding sites on the broken-down fibrin and hastening the dissolution of the clot [28]. While primary fibrinolysis is a natural physiological mechanism, secondary fibrinolysis can be induced by pharmaceutical agents like thrombolytics and fibrinolytics [29].

1.4 Molecular mechanism of fibrin formation

Fibrinogen, a 340 kDa trinodular protein secreted by hepatocytes and present in plasma at high concentrations (2-4 mg/mL), is the fundamental component of blood clots. It is made up of a dimer in which each subunit is made up of three polypeptide chains that are linked together as thread-like structures by 29 disulphide connections (αA, βB, and γY chains) [30,31]. It participates in various biological activities, including angiogenesis, atherosclerosis, thrombosis, inflammation, wound healing, haemostasis, etc [32].

The six polypeptide chains are arranged as follows: i) the N terminal of the E nodule; ii) the C terminal of the γ- and βB-chains from the D nodule facing outwards; iii) the globular C terminal of the αA-chains, which is located close to the E nodule (Figure 1 A) [31,32]. Active factors X and V convert prothrombin to thrombin, and fibrin is then generated from fibrinogen in a sequential process that leads to the formation of fibrin threads by the assembling of these fibrinogens [32-35].

Firstly, thrombin cleaves the N terminal peptides of the Aα- and Bβ-chains after adhering to the central E nodule (Figure 1 B). Secondly, fibrinopeptide A with an N terminal (16 residues) is released from Aα-chains when thrombin cleaves first and more quickly, revealing the binding site with Gly-Pro-Arg in the E region (A knob) (Figure 1 C). Thirdly, A knob possesses a γ-chain D region (a hole) that is complimentary to it, generating an interaction (A: a) that mediates the production of protofibrils, which are metastable peptide assemblies seen when a variety of peptides evolve into amyloid fibrils (Figure 1 D). Fourthly, after fibrinopeptide B with its N terminal (14 residues) is removed, a release occurs that reveals the Gly-His-Arg binding site in the E region (B knob) (Figure 1 C). Additionally, the B knob possesses a corresponding binding site of the β chain D

region (b hole), generating a (B: b) interaction that mediates the fibrinogen's lateral aggregation (Figure 1 D). This process produces protofibrils, and the blood clotting factor XIII subsequently helps convert them into fibrin fibres [36].

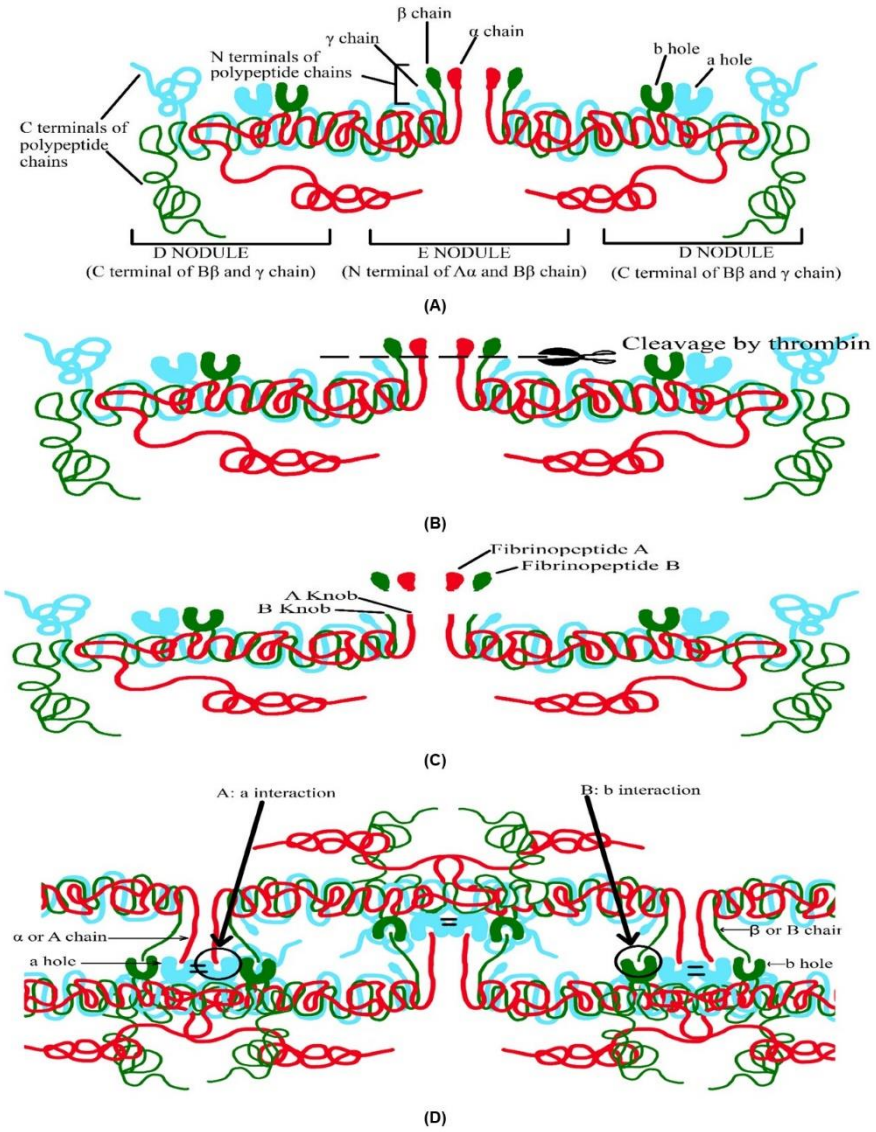

Figure 1.1: Clot formation mechanism. **(A)** The E nodules's N terminal, the γ- and βB-chain's terminals, and the αA-chain's C terminal are located close to the nodule; **(B)** Thrombin attaching itself to the central E nodule and cleaving the Aα- and Bβ-chains' N terminal peptides; **(C)** Thrombin-induced cleavage of Aα-chains; **(D)** Complementary binding sites of the D region's of γ- and β-chains mediated the production of protofibrils and lateral fibrinogen aggregation, respectively. This figure is adapted from [37].

A heterologous tetramer, Factor XIII comprises two catalytic A subunits (XIII A) made by bone marrow and two inhibitory/carrier B subunits (XIII B) formed by

hepatocytes. Inactive factor XIII is converted to active Factor XIII (active transglutaminase) by the concentrated activity of thrombin and calcium ions. Activation peptide XIII is first broken apart by thrombin, dissociating XIII B in the incidence of calcium ions. This cleaved XII A dimer is assumed to represent an active enzymatic structure of Factor XIII (XIIIa) [32]. The lateral structure and the individual protofibrils' elasticity and stability are increased when XIIIa creates an isopeptide bond among two adjacent αα- and αγ-chain monomers to produce fibrin fibres. To keep the blood clot from lysing, activated Factor XIII integrates α2-antiplasmin into fibrin α-chains [38].

1.5 Classification of thrombolytic agents

Medications known as thrombolytic medicines dissolve thrombus or blood clots that have developed inside blood vessels and reopen the channels (veins or arteries). Thrombolytic drugs can be used to treat a variety of CVDs, such as heart attacks, strokes, pulmonary emboli, deep vein thrombosis, and clots in deep leg veins. Thrombolytic drugs include urokinase, prourokinase, alteplase, an isolated, purified streptokinase activator complex (APSAC), and streptokinase. However, all thrombolytic medicines currently on the market are categorized as first, second, third, or fourth-generation agents [39,40]. Occasionally, fibrinolytic agents are divided into two groups: agents specific to fibrin and not[41].

However, the modes of action of various thrombolytic drugs vary. These mechanisms distinguish two categories of thrombolytic drugs. i) Plasminogen activators break down fibrin by converting plasminogen into active plasmin. These can come from bacterial sources, such as streptokinase, or they can occur natively in the blood, like tissue-type plasminogen activator (tPA) [42] and urokinase-type plasminogen activator [43]. ii) Plasmin-like proteins break down blood clot fibrin and disintegrate the thrombi.

Well-known plasmin-like proteins include snake venom fibrolase and earthworm venom lumbrokinase [44,45]. Alternatively, thrombolytic agents can be categorized based on the active site as either i) serine protease (NK, subtilisin DFE, and CK); ii) metalloprotease (jeot-gal enzyme, AMMP, and Bacillokinase II); or iii) a combination of serine and metalloprotease (R. chinensis 12 and Streptomyces sp. Y405) [46].

Table 1.1: List of different thrombolytic agents. This table is adapted from [47]

Sl. No.	Thrombolytic agents	Molecular weight	Source	Fibrin specificity	Dosage
1.	Streptokinase	47 kDa	β-hemolytic *Streptococci*	Non-specific	1.5 mU/hr
2.	Staphylokinase	16.5 kDa	*Staphylococcus aureus*	Specific	15 mg + 15 mg double bolus
3.	Urokinase	35-54 kDa	Human neonatal kidney cells	Non-specific	3 mU/hr
4.	Tissue plasminogen activator (t-PA)	70 kDa	Naturally in blood	Specific	Unknown
5.	Alteplase	70 kDa	Recombinant DNA technology from human melanoma cell line	Specific	15 mg bolus + 3 hr infusion upto 85 mg
6.	Reteplase	40 kDa	Single chain deletion variant of alteplase	Specific	Double bolus (10 U + 10 U, 30 min apart)
7.	Tenecteplase	70 kDa	Deletion and single point mutation of wild-type tPA	Specific	0.5 mg/kg single bolus
8.	Desmoteplase	52 kDa	From the saliva of the vampire bat *Desmodus rotundus*	Specific	0.125 mg/kg single bolus
9.	Nattokinase	27.7 kDa	Fermented food	Specific	100 mg/day

1.5.1 Direct-acting fibrinolytic enzymes

1.5.1.1 Nattokinase

First isolated by Sumi and colleagues, nattokinase is a serine protease produced from natto, a fermented food from Japan [48]. It is a single, 27.7 kDa polypeptide chain that is

275 aa long and functions at pH levels between 6-12 and temperatures as high as 60˚ C (Figure 2) [49]. Nattokinase strenghthens the body's inherent capability to break down blood clots and also has numerous benefits, including oral administration, effectiveness, affordability, long-lasting impacts, stability in the gastrointestinal tract, and the potential to enhance the body's production of plasmin and urokinase [50].

The innate capacity of nattokinase to enhance the body's processes for breaking down blood clots is accomplished in several ways: a) oral administration: this form of the fibrinolytic enzyme is thought to function directly since in vivo studies have shown decreased euglobulin clot lysis time (ECLT), prolonged partial thromboplastin time (PATT), and prevented platelet aggregation [51,52]; b) intraduodenal administration: data supports the movement of NK through the intestines and its hydrolysis of plasma fibrinogen[53]; c) efficacy: increases plasmin formation by increasing the synthesis of PLG activator and promotes fibrinolysis by breaking down plasminogen activator inhibitor-1 (PAI-1)[51,54]; d) affinity: increases affinity for cross-linked fibrin and reduces specificity for fibrinogen [55].

In human trials, two capsules of (2000 FU/capsule) were given orally to patients receiving dialysis, individuals with cardiovascular conditions, and healthy volunteers daily. Two months later, Factor VII and VIII were found to have decreased, which allowed fibrinogen to be seen in all three cases and the body weight, uric acid production, and heart rate remained stable with no adverse side effects. Four NK capsules (2000 FU/capsule) given orally to dogs caused a complete dissolution of the chemically generated blot clot (thrombi) in the major leg vein in 5 hours, re-establishing normal blood flow. Likewise, when NK was used to treat a rat's carotid artery thrombosis, 62% of the arterial blood flow was restored.

Additionally, in vitro and in vivo toxicology investigations have conclusively shown the safety of the enzyme for oral ingestion by humans [56]. As a result, nattokinase has been thoroughly investigated in China, Japan, and Korea as a promising enzyme for breaking up blood clots [57]. Moreover, the United States hosts clinical studies to prevent atherothrombosis [57]. In conclusion, compared to plasmin, using NK has many benefits, including its effectiveness in clot dissolution and the arterial blood flow restoration [58].

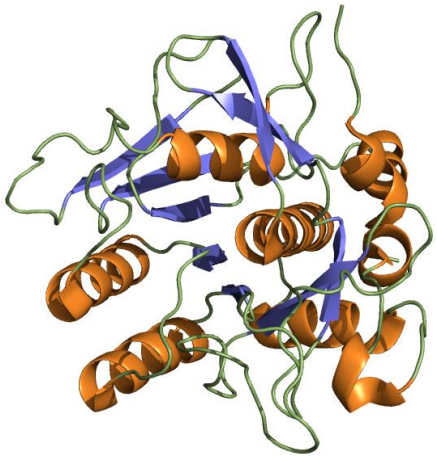

Figure 1.2: Structure of Nattokinase (Source: Wikipedia, https://en.wikipedia.org/wiki/Nattokinase)

1.5.2 Indirect-acting fibrinolytic enzymes

1.5.2.1 Streptokinase

The utmost widely used first-generation thrombolytic agent is streptokinase (SK). It is a 47 kDa protein with 414 amino acids (AA) residues that reaches its peak activity at a pH of about 7.5 (Figure 3) [59]. Streptokinase is attained from many β-hemolytic Streptococci strains belonging to Lancefield groups A, C, and G [60,61]. For the manufacture of streptokinase, *Streptococcus equisimilis* GCS strains H464 and group C have been primarily utilized [62]. Three structural domains, α, β, and γ, are present in SK and are catenated by two flexible coil sections at the A locations of 1-146, 147-290, and 291-414 [63,64]. Streptokinase half-life is about half an hour. Johnson and Tillett reported that intravenous infusion of Streptokinase effectively dissolved experimentally created intravascular clots in rabbits' ear veins caused by sodium morrhuate, indicating the thrombolytic potential of Streptokinase. This observation was made in 1952 [65].

Furthermore, the direct use of plasmin as a clot breaker was made possible by Kline's extensive purification of human plasminogen [66]. However, the significant caution associated with using plasmin directly or with Sk-mediated plasminogen activation may lead to indiscriminate systemic plasmin generation and significant reduction of circulating PLG and α-2-antiplasmin [67,68], which can seriously increase the risk of bleeding and significantly reduce blood clotting ability. Restricting the use of plasminogen activators as a therapeutic treatment instead of plasmin that is in circulation. The SK gene can be cloned from non-pathogenic microorganisms to produce fortified

recombinant SK (rSK), which reduces the chance of contracting potentially harmful Streptococci [69].

SK exerts control over the fibrinolysis cascade, resulting in the stoichiometric binding of plasminogen. The conformational change in plasminogen leads to the development of the enzymatically active streptokinase-plasminogen (SK-PLG) complex [70,71]. The highly selective SK-PLG complex protease can cleave additional circulating PLG molecules and transform them into active serine protease, plasmin, which can then break down the fibrin clot by binding to its particular lysine binding site [72,73].

SK domains consist of multiple functional areas, such as the α domain, where binding to PLG is regulated by the Asp41-His48 sequences between 1-59 aa residues [71,74]. It also includes single residues (V19F, V35E, and S44K) that are essential for substitution and the activation of the SK-plasmin complex [73,75]; (ii) the β domain, whose Lys 256, 257, and Val158-Arg219 region help recognize and process PLG and form the SK-plasmin complex [76]; and (iii) the γ domain, which is a coiled region (Leu314-Ala342) that is vital for stabilizing the SK-micro plasmin complex for PLG activation. The Food and Drug Administration (FDA) first authorized SK as a PLG activator for treating thrombosis.

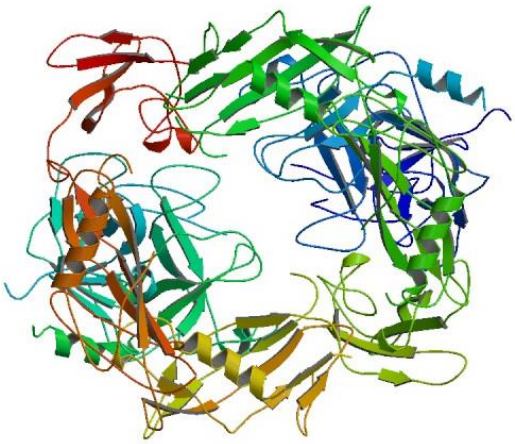

Figure 1.3: Structure of Streptokinase (Source: Drugbank online, https://go.drugbank.com/drugs/DB00086)

1.5.2.2 Stapkylokinase

The antithrombin activity of Staphylokinase, a third-generation plasminogen activator derived from Staphylococcus aureus GH38, is achieved by changing passive plasminogen

to active plasmin. Staphylokinase, a 136 aa monomer of 15.5 kDa, is made up of two domains of equal size that are shaped like flexible dumbbells (Figure 4) [77,78]. Like streptokinase, Staphylokinase also acts as a thrombolytic agent by changing plasminogen into plasmin. It can combine with plasmin or plasminogen to produce a 1:1 stoichiometric complex, activating other plasminogen molecules and ultimately dissolving the blood clot [79].

According to reports, methionine-26, or aa at position 26, has a structural role in PLG activation by Staphylokinase. Remarkably, substituting arginine or valine for this aa results in the loss of functional activity, while leucine or cysteine has little to no influence on functional activity [80].

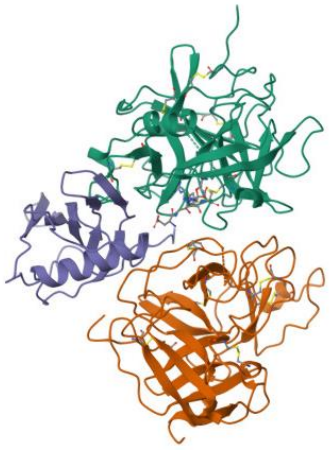

Figure 1.4: Structure of Staphylokinase (Source: RCSB PBD, https://www.rcsb.org/structure/1BUI)

1.5.2.3 Urokinase

In 1947, MacFarlane and Pilling [81] identified a new fibrinolytic enzyme in human urine; it was acknowledged as urokinase by Sobel, Mohler, Jones, Dowdy, and Guest a decade later (Figure 5) [82]. Renal epithelial cells, macrophages, endothelial cells, and specific tumour cells are among the sources of urokinase. Pro-urokinase, pro-uPA, or scu-PA is the term for urokinase in its active form, which is a single polypeptide glycosylated zymogen consisting of 411 amino acids [83]. Three different domains constitute this pro-urokinase: the growth factor domain (GFD), which covers amino acids 1 to 49; the kringle domain (KD), which covers amino acids 50 to 131 and is located at the N-terminus; and the serine protease domain (P), which is located at the C-terminus and covers amino acids 159 to 411. The linker segment consisting of amino acids 132 to 158 connects the N-

terminal and C-terminal domains [84]. Following secretion, the glycosylated zymogen (pro-uPA) is subjected to a two-step proteolytic process to convert it into its active form. The most effective proteolytic enzyme for this process is plasmin. Still, other proteolytic enzymes that work similarly include nerve growth factor-g, cathepsin B and L, trypsin, thermolysis, kallikrein, and mast cell tryptase [85-88].

Furthermore, proteases like thrombin and elastase can cleave pro-uPA at various sites [85]. The linker region connecting Lys 158 and Ile 159 is broken during the initial round of proteolysis, leaving two chains with a molecular weight of 54 kDa that are still connected by a disulphide bond. The complete division of the two uPA chains is then achieved by another phase of protein breakdown at the peptide link involving Lys 135 and Lys 136. As a result, an active low molecular weight version of uPA (33 kDa) comprising the serine protease domain forms in addition to an active amino-terminal fragment (ATF) consisting of the kringle domain and the growth factor domain (GFD). Lastly, pro-uPA and the two-chain uPA variants, or tcu-PA, which are composed of an inactive ATF with growth factor domain and kringle domain and an active serine protease domain, attach to their respective receptors, uPAR, with comparable affinity.

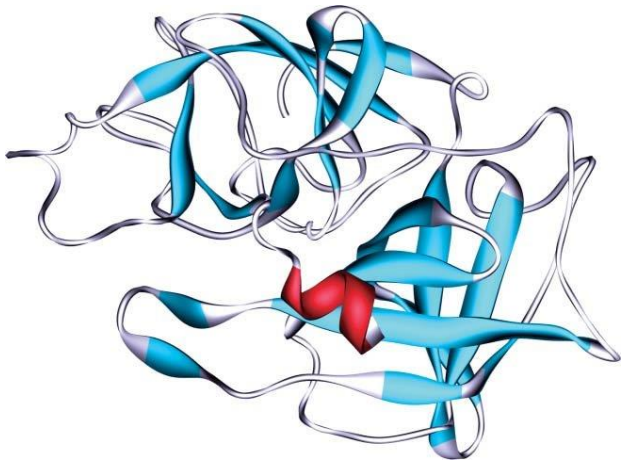

Figure 1.5: Structure of Urokinase (Source: RCSB PBD, https://www.rcsb.org/structure/1SQT)

1.5.2.4 Tissue plasminogen activator

A serine protease with thrombolytic activity specific to fibrin is called tissue plasminogen activator. It is composed of 527 amino acids and has a molecular weight of 70 kDa (Figure 6). There are five domains in it; i) a fibronectin type I domain at N-terminal with 47 amino

acid residues, ii) an epidermal growth factor domain, iii) kringle 1, iv) kringle 2, and v) a serine protease domain [78,89].

Without fibrin, plasminogen activator's action is negligible; however, fibrin significantly boosts the activator's activity. Moreover, PA-1 inhibits its activity in vivo, and the amino acids at positions 296-299 are essential for this inhibition [90]. Recombinant DNA technology has been used to create several recombinant tPA forms, such as Alteplase, Reteplase, Tenecteplase, and Desmoteplase, to get around these problems. Of them, only two-Tenecteplase and Reteplase-had clinical approval [91], yet they continue to have adverse effects related to allergic responses, fibrin specificity, and bleeding problems [90]. As a result, extensive research is underway to mitigate these detrimental effects and enhance the efficacy of these fibrinolytic enzymes. Desmoteplase, another r-tPA, is now in a Phase III clinical study. Lundbeck, a Danish pharmaceutical firm, has the global rights to this drug. Lanoteplase, saruplase, anistreplase, and pamiteplase are further recombinant tissue plasminogen activators (tPAs).

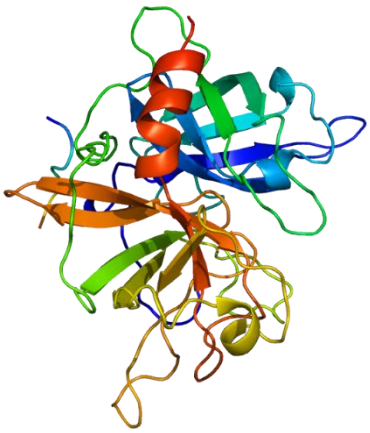

Figure 1.6: Structure of Tissue-plasminogen activator (Source: Wikipedia, https://en.wikipedia.org/wiki/Tissue-type_plasminogen_activator)

1.5.3 Anticoagulant

Anticoagulants are frequently used in the treatment and prevention of thromboembolic illness. Their use is indicated for the medical management and avoidance of venous thromboembolism (before thromboembolism, patients who are immomilized, patients following major surgery, etc.) as well as preventing embolic stroke (prosthetic heart valve patients, non-valvular atrial fibrillation patients) [92-95].

Anticoagulants function by modifying the procoagulation and anticoagulant physiological pathways [96], which is accomplished in two ways: one is by inhibiting the clot from forming, and the other is by decreasing the expansion of an already-existing clot [97]. The coagulation cascade is the basis for understanding the mechanism of the anticoagulant drugs now on the market. However, a new trend towards more targeted anticoagulant therapy has emerged due to advances in pharmacology and coagulation pathways [94,97]. Conventional anticoagulants, namely heparins and vitamin K antagonists (VKA), have drawbacks that have led to this trend. These drugs can have a limited therapeutic window and impulsive impact on clotting, necessitating periodic surveillance to guarantee safety and effectiveness [94,96-99].

Warfarin is still one of Australia's most widely used anticoagulants despite its erratic pharmacological profile. Because Warfarin may be taken orally, it continues to be the anticoagulant of choice when compared to parental options like Heparin. Warfarin is a beneficial medication for treating thromboembolic disease when used judiciously, but one drawback is that it necessitates frequent surveillance of a patient's international normalized ratio (INR). Patients may receive suboptimal anticoagulation as a result of noncompliance with warfarin medication and INR testing, which increases the risk of either uncontrolled bleeding or thromboembolic events.

Alternatives have been discovered to offer a useful, dependable oral anticoagulant due to the issues with Warfarin's limited efficacy for therapy and multiple drug-drug and food-drug interactions [93,97,98,100]. The Therapeutic Goods Administration (TGA) in Australia has approved using some drugs known as novel oral anticoagulants (NOAC's). Currently, the TGA has approved dabigatran (Pradaxa), rivaroxaban (Xarelto), and apixaban (Eliquis). These medications are being used more frequently to address issues related to warfarin use [93,101].

1.5.3.1 Heparin

In the 1930s, Heparin was first made available as an anticoagulant medication (Figure 7) [97]. Nevertheless, it needs careful observation and can only be administered as a parental agent, which restricts its mode of administration [97]. Heparin is given intravenously or subcutaneously to avoid venous thromboembolism (VTE), usually in the event of acute thromboembolic events or following major surgery that necessitates hospitalization [102]. Heparin prevents blood coagulation by attaching to and upregulating antithrombin by

inhibiting Factors IXa, Xa, and thrombin [99,102]. As a result, the thrombin-fibrinogen process is inhibited [102]. Heparins affect coagulation and prevent platelet activation caused by thrombin [102].

Heparins can be further separated into low-molecular-weight (LMW) heparin and unfractionated Heparin (UFH) [99,102]. UFH is linked to thrombocytopenia caused by heparinand has a brief half-life, frequently losing its anticoagulant effects six hours after discontinuing use [94,99,102]. On the other hand, compared to unfractionated Heparin, LMW heparin has a more prolonged period of action, improved bioavailability since it binds to fewer proteins, and fewer antiplatelet effects [99,102].

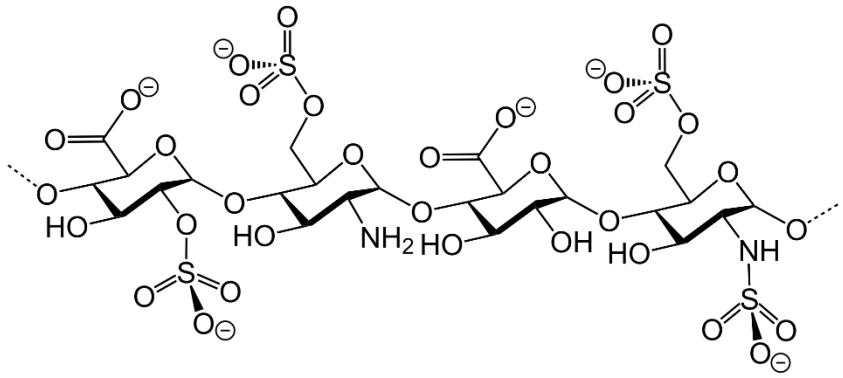

Figure 1.7: Structure of Heparin (Source: Wikipedia, https://en.wikipedia.org/wiki/Heparin)

1.5.3.2 Vitamin K antagonist

Vitamin K antagonists (VKA), such Warfarin (Figure 8) and its variations, which were created in 1940, were the only oral anticoagulants on the market until recently [97]. These oral anticoagulants resolved the issues related to Heparin's administration [97]. Warfarin is the most frequently used VKA, which the upper gastrointestinal system can absorb easily. After oral administration, the plasma concentration takes 60-90 minutes to peak [97,99,102]. Binding to vitamin K reductase opposes the production of coagulation components dependent on vitamin K [99,102]. Warfarin inhibits thrombin, Factor VII, IX, X, and proteins C and S, which are coagulation factors [99,102]. Though Heparin is rapidly absorbed, its effects take 8-12 hours to manifest and peak after 36 hours [102].

However, Heparin is still relevant when a quick anticoagulant action is needed [94]. The cytochrome p450 complex is primarily responsible for the liver's Warfarin metabolism, resulting in notable pharmacological variability among individuals

[94,99,102]. It is advised that INR be used to evaluate the effectiveness of patients on Warfarin due to the drug's limited therapeutic range and the high degree of patient variability in effect [96,99,102].

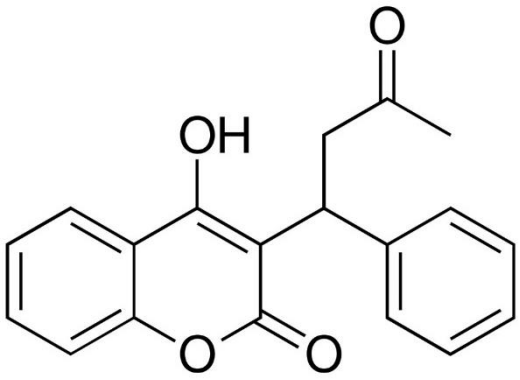

Figure 1.8: Structure of Warfarin (Source: Wikipedia, https://en.wikipedia.org/wiki/Warfarin)

1.5.3.3 Novel Oral Anticoagulants

Anticoagulant medications targeting thrombin and Factor Xa coagulation factors have been created to produce a more predictable result [93,94]. The NOAC class of medications is intended to address the drawbacks of traditional anticoagulants. These medications function by either directly suppressing thrombin or by blocking its synthesis [92-94]. Medication targeting Factor Xa can stop the production of thrombin. In contrast, thrombin inhibitors stop the fibrin meshwork from forming by slowing the transformation of fibrinogen to fibrin and thrombin-induced platelet aggregation [92].

The very first direct thrombin inhibitors demonstrated the effectiveness of direct thrombin inhibition, but their use was restricted to particular patient populations due to their unique pharmacokinetic and pharmacodynamic characteristics [94]. Inactivating both bound and soluble thrombin, direct thrombin inhibitors prevent thrombogenesis and thrombus formation [93,99]. In 2004, Ximelegatran, the first oral thrombin inhibitor, was introduced in Europe to assist patients recovering from orthopaedic surgery in avoiding VTE (Figure 9A) [93,94,99]. It was never certified for usage in the US or Australia, though, and was discontinued from the market in 2006 due to possible liver toxicity [93,94].

Another direct thrombin inhibitor, dabigatran, does not cause hepatotoxicity (Figure 9B) [93,99,101]. The TGA authorized dabigatran in November 2008 to treat VTE

in patients undergoing orthopaedic surgery. In April 2011, the drug was also licensed to prevent thromboembolism associated with atrial fibrillation. It takes 30 min for the onset of its action when taken via ingestion as a prodrug termed dabigatran etexilate [94,95,97,101]. To improve the bioavailability, it is produced in capsules containing tartaric acid, which facilitates absorption in the acidic milieu of the stomach and small intestine [101]. The medication is not dialyzable because about one-third is protein-bound [97,98]. The kidneys eliminate 80% of dabigatran, and hepatic metabolism does not influence plasma concentration. Instead, it is influenced by p-glycoprotein transporter activation or inhibition [94,97,101,103].

Direct Factor Xa inhibitors, unlike direct thrombin inhibitors, block the serine proteases mediating thrombin production. As a result, they may be more potent anticoagulants than those that block thrombin directly [93,94,99]. This is because the leading amplification site in the coagulation cascade is Factor Xa [94]. These medications are referred to as 'direct' because they inactivate both free Factor Xa and Factor Xa, which is linked to platelets inside the prothrombinase complex, altering the coagulation process without the need for antithrombin [92,97]. Rivaroxaban, one of the earliest Factor Xa inhibitors, demonstrated great promise in lowering thromboembolic events and established that Factor Xa can be specifically suppressed (Figure 9C) [94]. However, rivaroxaban raised the risk of significant bleeding when used in large dosages. As a result, apixaban, a novel Factor Xa inhibitor, became rivaroxaban [94].

In Australia, the TGA approved the use of apixaban in April 2013 (Figure 9D) and rivaroxaban in April 2012, respectively. These medications are prescribed to treat atrial fibrillation patients by preventing thrombosis. Additionally, rivaroxaban is recommended for the management of deep vein thrombosis (DVT) and the avoidance of pulmonary embolism or recurrent DVT.

The active version of rivaroxaban is taken orally and takes 1-4 hours for the onset of its action [94,95,97,98,101]. Unlike dabigatran, rivaroxaban is protein-bound to the extent that dialysis cannot be used to eliminate rivaroxaban rapidly [95,97,103]. Because rivaroxaban depends on the CYP344 system, it may interact with other medications using this pathway [97,98]. Furthermore, the p-glycoprotein system is also necessary for the clearance of rivaroxaban [97,98,101]. One-third of the medicine is eliminated unaltered

in urine, another third is broken down in the liver and removed through the faeces, and a third is broken down in the liver and excreted via kidneys [95,97,98,101,103].

Like rivaroxaban, apixaban is also taken via ingestion in its active version, and it takes around 3 hours to onset its action [97,98]. 87% is protein-bound, and the CYP3A4 system eliminates 75% [97,98]. When compared to the other NOACs, apixaban excretion is the least reliant; approximately 70% of processed apixaban is eliminated through faeces, and only 25% is eliminated via renal [94,95,97,103].

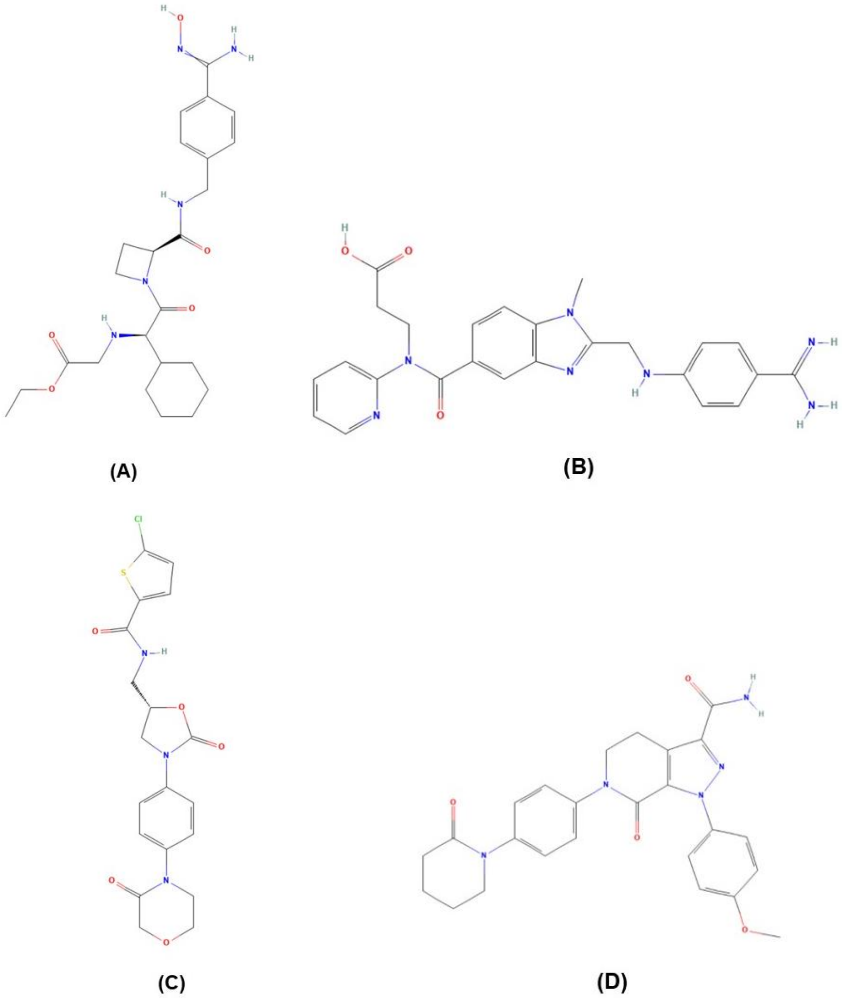

Figure 1.9: (A) Structure of Ximelegatran (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/656635); **(B)** Structure of Dabigatran (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Dabigatran#section=2D-Structure); **(C)** Structure of Rivaroxaban (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Rivaroxaban#section=2D-Structure); **(D)**

Structure of Apixaban (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Apixaban#section=2D-Structure)

1.5.4 Antiplatelet drugs

The mainstay of treatment for cardiovascular disorders, such as coronary artery disease, stroke, etc., which remain the leading cause of death and morbidity in the developed world, is antiplatelet medication [104]. Antiplatelet medications have the potential to both prevent and treat thrombotic disorders because platelets are essential to thrombosis [105]. Atherosclerotic plaque erosion or rupture is the primary source of thrombosis, which is the major event that culminates in acute coronary syndrome (ACS). The significant participants in this scenario are thrombin and platelets. Therefore, treating ACSs requires a thorough understanding of platelet activation, adhesion, and aggregation physiology. Numerous investigations have demonstrated that antiplatelet medication reduces mortality while averting recurrent ischaemia episodes [106].

Upon exposure to platelet agonists, many transmembrane signalling pathways and molecules with enzymatic activity and/or functions participate in the complex process of platelet activation and aggregations [106]. Most antiplatelet medications under development target the enzymes or receptors found on the surface of platelets. Aspirin was the first antiplatelet medication ever developed; it inhibits the cyclo-oxygenase-1 enzyme. Even though other novel ways are being investigated to provide more potent drugs, Aspirin remains and will remain the cornerstone of antiplatelet therapy in the years to come.

Despite the advantages these antiplatelet medications offer, their broad application is restricted due to problems associated with their administration, such as their limited efficacy and narrow therapeutic window. Aspirin with Clopidogrel is currently thought to be the paradigm of care for preventing platelet activation and aggregation, which in turn prevents thrombosis and stroke. However, future initiatives for developing additional antiplatelet medications and treatment regimens are being investigated since the current antiplatelet therapies are laden with issues like resistance, optimal dosage, safety concerns, etc [104].

Antiplatelet medications restrict platelets in one way or another, which prevents them from aggregating and forming clots at the sites of vascular damage, where they serve as the initial haemostatic plug. Aspirin, ADP receptor inhibitors (ticlopidine), clopidogrel), and GPIIb/IIIa inhibitors (abciximab, tirofiban, eptifibatide) are the currently available antiplatelet medications currently in use [107].

1.5.4.1 Aspirin

Aspirin blocks the thromboxane-mediated pathway by inhibiting the cyclo-oxygenase-1 enzyme (Figure 10). Aspirin has a resistant effect on platelets that lasts for the platelet's lifespan, which is 7-10 days. Therefore, the daily aspirin administration will have a cumulative inhibitory effect on platelets. Aspirin has an antiplatelet impact even at low dosages, up to 160 mg daily, where it entirely and effectively inhibits the platelet's COX-1 enzyme. Higher doses come with a higher risk of bleeding, are more toxic, and are less effective. Numerous trials have demonstrated that it lessens the risk of mortality from cardiovascular causes, new myocardial infarction (MI), and repeated ischaemia by about 40% [107]. Although the most commonly utilized dose range is 75-350 mg, efficacy has also been shown at several other levels [108-110].

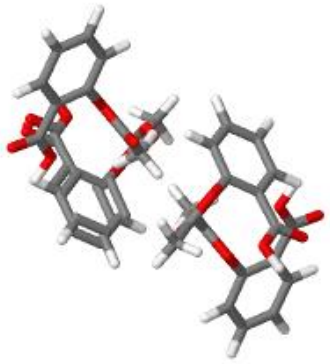

Figure 1.10: Crystal structure of Aspirin (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Aspirin#section=Crystal-Structures)

1.5.4.2 ADP receptor inhibitors: Ticlopidine and Clopidogrel

These medications inhibit platelets from aggregating when exposed to ADP. Currently on the market, ticlopidine and Clopidogrel are both irreversible thienopyridine P2Y12 receptor antagonists that have been confirmed to be effective in lowering the hazard of arterial thrombotic actions (Figure 11 A and B) [107]. Ticlopidine is a prodrug requiring the cytochrome P450 enzyme first to transform it into its active metabolite. Creating a

disulphide bond with the P2Y12 receptor is irreversible and blocks the receptor. The antiplatelet action peaks 8-11 days after the treatment begins. The release of the active metabolite from Clopidogrel depends on hepatic cytochrome P450. This metabolite binds permanently to the P2Y12 receptor through covalent modification, preventing the recovery of platelet function [107,111].

Regarding secondary stroke prevention, the medication is comparable to Aspirin, and when combined with Aspirin, it seems to be just as successful as ticlopidine and Aspirin [112]. It can be employed in established peripheral arterial disease, stroke, myocardial infarction, or acute coronary syndrome, according to FDA approval. Platelet aggregation and activation tests reveal a significant interindividual heterogeneity in clopidogrel response, with up to 25% of patients only partially reacting to the medication and 5-10% not responding [113].

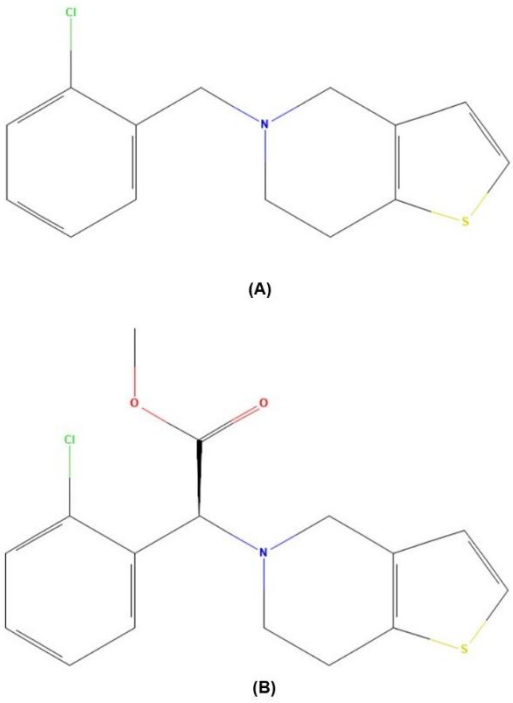

Figure 1.11: (A) Structure of Ticlopidine (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Ticlopidine#section=2D-Structure); **(B)** Structure of Clopidogrel (Source: Pubchem, https://pubchem.ncbi.nlm.nih.gov/compound/Zyllt#section=2D-Structure)

1.5.4.3 GPIIb/IIIa inhibitors

A dimeric receptor for fibrinogen and von Willebrand Factor (vWF), GpIIb/IIIa is a platelet surface integrin that anchors platelets to foreign surfaces and to one another, hence inducing platelets to aggregate [105,114]. As a result, abciximab, tirofiban, and eptifibatide, which are inhibitors of this receptor, are effective antiplatelet medications (Figure 12 A-C). These medications are all parental formulations. Numerous pharmacodynamic investigations on these medications showed an antiaggregatory impact more significant than 80%. Abciximab was the first GPIIb/IIIa receptor antagonist utilised in a therapeutic context. The Fab segment of a humanized monoclonal antibody that targets the GPIIb/IIIa receptor is known as abciximab.

Additionally, it attaches itself to the vitronectin receptor found on smooth muscle, vascular endothelium, and platelets. Even after the infusion is stopped, the effects last 18-24 hours. Nevertheless, there are some issues with using abciximab, including its high cost, immunogenicity, and irreversibility of the impact [107,115]. As a result, micromolecule GPIIb/IIIa receptor antagonists such as tirofiban and eptifibatide have been established. Eptifibatide is a cyclic peptide inhibitor that inhibits the GPIIb/IIIa receptor's fibrinogen binding site. Because it doesn't also bind to the vitronectin receptor, the advantage observed with it is slightly lesser than that of abciximab. Its duration of action is transient; that is, platelet activity resumes 6-12 hours after the infusion is stopped [104,107]. As a non-peptide with a short half-life, tirofiban functions similarly to eptifibatide and is effective in treating non-Q-wave myocardial infarction associated with unstable angina.

Furthermore, it doesn't block vitronectin receptors. Because tirofiban and eptifibatide have low molecular weights, they cannot trigger an immunological response [107,116]. Extensive clinical studies have confirmed their safety and clinical properties in acute coronary syndrome. They are employed as supplemental treatment for percutaneous coronary intervention (PCI). These medications are reportedly linked to bleeding risks, though [116].

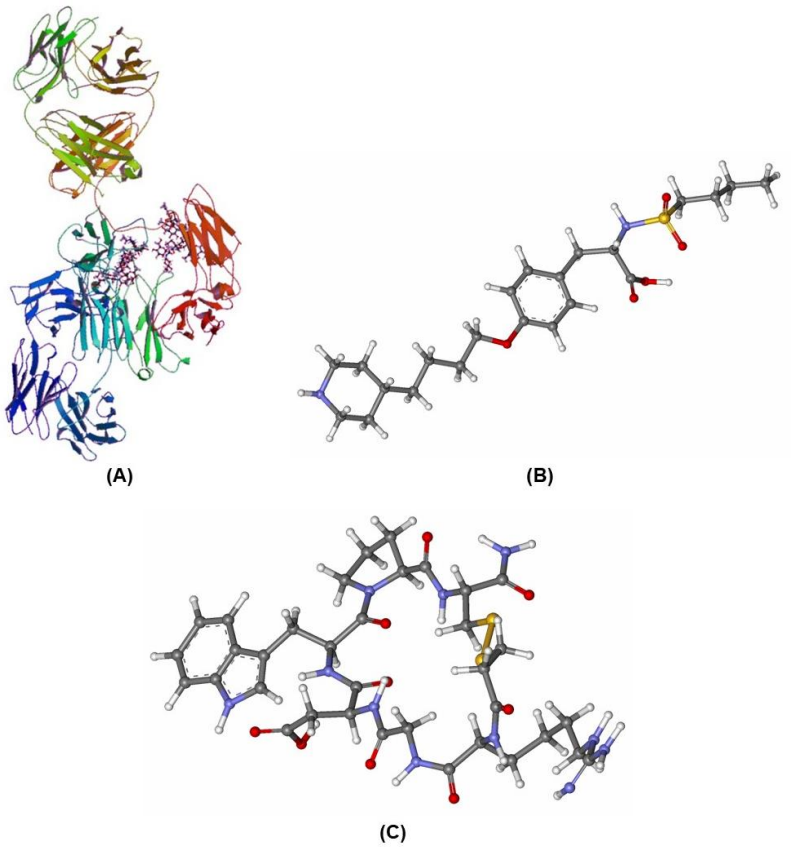

Figure 1.12: (A) Structure of Abciximab (Source: Wikipedia, https://en.wikipedia.org/wiki/Abciximab); **(B)** Structure of Tirofiban (Source: Wikipedia, https://en.wikipedia.org/wiki/Tirofiban); **(C)** Structure of Eptifibatide (Source: Wikipedia, https://en.wikipedia.org/wiki/Eptifibatide)

1.6 Limitations of thrombolytic drugs

A haemostasis imbalance can cause uncontrolled bleeding or the development of a thrombus, which is an unsuitable blood clot that sticks to the intact walls of blood vessels. The fibrin buildup in the blood vessels may impede the flow of blood, leading to myocardial infarction along with other severe cardiovascular conditions. The tissue may perish or suffer significant harm if blockages are not removed promptly. Massive injuries would prevent the ventricles from conducting normal electrical signals, which could lead to irregular heartbeats, cardiac arrests, or even death.

Commercial thrombolysis medicines such as tissue plasminogen activator, streptokinase, and urokinase have been explored as a potential substitute for surgical procedures aimed at dissolving blood clots or avoiding heart blockages. These cardiovascular medications do, however, have certain drawbacks, including the

possibility of gastrointestinal bleeding and allergic reactions. Additionally, these medications are costly, making them unaffordable for the average person, particularly in underdeveloped countries.

Thrombolytic therapy's significant side effect is bleeding from fibrinogenolysis or fibrinolysis at the site of vascular damage. It is essential to monitor hypo-fibrinogenemia with laboratory testing as it may occur. The incidence of second- and third-generation agents occurs similarly for all agents at effective thrombolytic dosages. Halting treatment, providing whole blood, platelets, or fresh frozen plasma, and administering protamine (if Heparin is present) may be necessary in cases of life-threatening cerebral bleeding.

Bleeding is the primary side effect of Warfarin. The side effects of long-term coumarin-type anticoagulant therapy are comparatively low. Bleeding can be visible (on the skin or mucous membrane) or invisible (in the gastrointestinal tract, Uterine, renal, hepatic, pulmonary or cerebral). Less common adverse effects include dermatitis, urticaria, alopecia, purple toes, diarrhoea, and small intestine necrosis.

Haemorrhage is also the main adverse effect following heparin therapy. Adrenal glands, the gastrointestinal system, and the urinary tract can also bleed. There can be wound ecchymosis, hemarthrosis, severe haemorrhagic pancreatitis, and subdural haematoma. Although rare, life-threatening bleeding occurs occasionally. 3-30% of patients may experience acute and delayed onset thrombocytopenia caused by Heparin. The delayed reaction involves the development of heparin-dependent antiplatelet antibodies and the elimination of platelets from circulation.

In contrast, the rapid-type reaction is temporary and may not necessitate platelet destruction. Heparin-induced thrombocytopenia may accompany white clot syndrome, characterized by irreversible platelet aggregation. Hypersensitivity responses (rash, pruritus, urticaria), alopecia, osteoporosis, fever, hypoaldosteronism, and ostealgia are among the supplementary adverse effects of heparin treatment. Bleeding is the most frequent adverse effect or risk of using antiplatelet medications because they all interfere with normal blood coagulation. Bleeding risks vary from extremely small (nose bleeds) to large, potentially fatal (bleeding into the brain). Fortunately, there is little to no risk of bleeding, no threat to life, and no need for a blood transfusion. Patients on antiplatelet treatment for acute myocardial infarctions and cerebrovascular accidents most frequently experience bleeding in the locations where vascular catheters, such as intravenous

catheters, have been placed. This include the region in groin, which is usually the location of access during coronary angioplasty. Gastrointestinal bleeding is infrequent. Following the administration of antiplatelet medicine, any bleeding from these places necessitates further investigation, as such hemorrhaging sometimes stems from previously undetected conditions, including malignancies or peptic ulcers. Uncommon adverse effects associated with antiplatelet agents encompass rashes, hypersensitivity to Aspirin, and reduced platelet count (thrombocytopenia) when administered with glycoprotein IIb/IIIa inhibitors.

1.7 Aims and objectives of the study

The aim of this study, as delineated in the following parts, has taken into consideration the scopes above. Because fibrinolytic protease(s) may be used to treat cardiovascular-related illnesses, they have become more important in medicine. Although several fibrinolytic enzymes have been previously described, there is limited information on non-toxic target-specific medications and their potential for thrombolysis in vivo. The current state of industrial biotechnology is a gold mine for finding more substantial and commercially viable products due to the astounding abilities of microbial enzymes and their diverse specificity and efficiency. Therapeutic enzymes are the best illustration of this kind of effort. In particular, the *Bacillus* genus is widely known for synthesizing a wide range of highly significant industrial enzymes, such as fibrinolytic enzymes.

The biochemical characterizations of most enzymes have substantially enhanced their applicability in developing thrombolytic drugs. It is crucial to emphasize that food-derived microorganisms are favoured in the pharmaceutical sector due to their multiple benefits in producing fibrinolytic enzymes. Considering Northeast India is a global mega-biodiversity hotspot, exploring new fibrinolytic enzymes from this region is promising. The preliminary step in investigating the microbial diversity of these regions involves isolating bacteria from diverse fermented food samples commonly utilized for both consumption and non-toxic applications. The present work sought to employ in-silico techniques to analyze fibrinolytic enzymes from the *Bacillus* genus and to discover potent fibrinolytic enzyme-producing bacteria from the starter cultures of Northeast India. Further, the gene sequence encoding for fibrinolytic enzyme derived from *Bacillus subtilis* was codon-optimized according to *Escherichia coli* codon preference, and this gene was synthetically cloned into the pET26b(+) vector followed by expression in *E.*

coli under the T7 promoter, optimization of refolding conditions, purification using affinity chromatography, pharmacological characteriation and *in vivo* toxicity assessment.

The following objectives formed the basis of this investigation:

1. Computational (*in silico*) analysis to study bacterial fibrinolytic proteases' structure-function and evolutionary relationship.
2. Isolation of fibrinolytic protease produces bacterial strains from fermented foods in northeastern India.
3. Cloning, expression, purification and characterization of recombinant fibrinolytic protease enzyme.

Bibliography

[1] Koene, R. J., Prizment, A. E., Blaes, A., and Konety, S. H. Shared risk factors in cardiovascular disease and cancer. *Circulation*, 133(11): 1104-1114, 2016.

[2] Hu, Y., Yu, D., Wang, Z., Hou, J., Tyagi, R., Liang, Y., and Hu, Y. Purification and characterization of a novel, highly potent fibrinolytic enzyme from Bacillus subtilis DC27 screened from Douchi, a traditional Chinese fermented soybean food. *Scientific reports*, 9(1): 9235, 2019.

[3] Mackman, N., Bergmeier, W., Stouffer, G. A., and Weitz, J. I. Therapeutic strategies for thrombosis: new targets and approaches. *Nature reviews Drug discovery*, 19(5): 333-352, 2020.

[4] Zhou, S.-s., Jin, J.-p., Wang, J.-q., Zhang, Z.-g., Freedman, J. H., Zheng, Y., and Cai, L. miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges. *Acta Pharmacologica Sinica*, 39(7): 1073-1084, 2018.

[5] Hogas, S., Bilha, S. C., Branisteanu, D., Hogas, M., Gaipov, A., Kanbay, M., and Covic, A. Potential novel biomarkers of cardiovascular dysfunction and disease: cardiotrophin-1, adipokines and galectin-3. *Archives of medical science*, 13(4): 897-913, 2017.

[6] Sharma, C., Salem, G. E. M., Sharma, N., Gautam, P., and Singh, R. Thrombolytic potential of novel thiol-dependent fibrinolytic protease from Bacillus cereus RSA1. *Biomolecules*, 10(1): 3, 2019.

[7] Organization, G. World health statistics 2022: monitoring health for the SDGs, sustainable development goals. *Geneva: World Health Organization*, 2022.

[8] Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., Chiuve, S. E., Cushman, M., Delling, F. N., and Deo, R. Heart disease and stroke statistics—2018 update: a report from the American Heart Association. *Circulation*, 137(12): e67-e492, 2018.

[9] Kim, C., Ri, K., and Choe, S. A novel fibrinolytic enzymes from the Korean traditional fermented food—Jotgal: Purification and characterization. *Journal of Food Biochemistry*, 44(7): e13255, 2020.

[10] Ten Cate, H., Hackeng, T. M., and de Frutos, P. G. Coagulation factor and protease pathways in thrombosis and cardiovascular disease. *Thrombosis haemostasis*, 117(07): 1265-1271, 2017.

[11] Kotb, E. Purification and partial characterization of a chymotrypsin-like serine fibrinolytic enzyme from Bacillus amyloliquefaciens FCF-11 using corn husk as a novel substrate. *World journal of microbiology biotechnology*, 30: 2071-2080, 2014.

[12] Asgari, M., Javaran, M. J., Moieni, A., Masoumiasl, A., and Abdolinasab, M. Production of human tissue plasminogen activator (tPA) in Cucumis sativus. *Preparative Biochemistry Biotechnology*, 44(2): 182-192, 2014.

[13] Ali, A. M. M. and Bavisetty, S. C. B. Purification, physicochemical properties, and statistical optimization of fibrinolytic enzymes especially from fermented foods: a comprehensive review. *International journal of biological macromolecules*, 163: 1498-1517, 2020.

[14] Bonnard, T., Law, L., Tennant, Z., and Hagemeyer, C. Development and validation of a high throughput whole blood thrombolysis plate assay. *Scientific reports*, 7(1): 2346, 2017.

[15] Liu, X., Kopparapu, N.-k., Li, Y., Deng, Y., and Zheng, X. Biochemical characterization of a novel fibrinolytic enzyme from Cordyceps militaris. *International journal of biological macromolecules*, 94: 793-801, 2017.

[16] Key, N. S., Makris, M., and Lillicrap, D. *Practical hemostasis and thrombosis*. John Wiley & Sons, 2017.

[17] Furie, B. and Furie, B. C. Thrombus formation in vivo. *The Journal of clinical investigation*, 115(12): 3355-3362, 2005.

[18] Rivera, J., Lozano, M. L., Navarro-Núñez, L., and Vicente, V. Platelet receptors and signaling in the dynamics of thrombus formation. *haematologica*, 94(5): 700, 2009.

[19] Pallister, C. and Watson, M. *Haematology*. Scion Publishing Ltd, 2010.

[20] Hoffbrand, A. (Blackwell Science, Oxford, 2002).

[21] Coughlin, S. R. Thrombin signalling and protease-activated receptors. *Nature*, 407(6801): 258-264, 2000.

[22] Cardigan, R. A., Mcgloin, H., Mackie, I. J., Machin, S. J., and Singer, M. Activation of the tissue factor pathway occurs during continuous venovenous hemofiltration. *Kidney international*, 55(4): 1568-1574, 1999.

[23] Hajjar, K. The molecular basis of fibrinolysis. *Hematology of infancy childhood*, 2: 1557-1573, 1998.

[24] Miyata, T., Iwanaga, S., Sakata, Y., and Aoki, N. Plasminogen Tochigi: inactive plasmin resulting from replacement of alanine-600 by threonine in the active site. *Proceedings of the National Academy of Sciences*, 79(20): 6132-6136, 1982.

[25] Forsgren, M., Råden, B., Israelsson, M., Larsson, K., and Hedén, L.-O. Molecular cloning and characterization of a full-length cDNA clone for human plasminogen. *FEBS letters*, 213(2): 254-260, 1987.

[26] Law, R. H., Caradoc-Davies, T., Cowieson, N., Horvath, A. J., Quek, A. J., Encarnacao, J. A., Steer, D., Cowan, A., Zhang, Q., and Lu, B. G. The X-ray crystal structure of full-length human plasminogen. *Cell reports*, 1(3): 185-190, 2012.

[27] Cesarman-Maus, G. and Hajjar, K. A. Molecular mechanisms of fibrinolysis. *British journal of haematology*, 129(3): 307-321, 2005.

[28] Medved, L. and Nieuwenhuizen, W. Molecular mechanisms of initiation of fibrinolysis by fibrin. *Thrombosis haemostasis*, 89(03): 409-419, 2003.

[29] Dugdale, D., Cesarman-Maus, G., and Hajjar, K. Primary or secondary fibrinolysis. *Retrieved. MedlinePlus*, 2011.

[30] Chapin, J. C. and Hajjar, K. A. Fibrinolysis and the control of blood coagulation. *Blood Reviews*, 29(1): 17-24, 2015.

[31] La Corte, A. L. C., Philippou, H., and Ariëns, R. A. Role of fibrin structure in thrombosis and vascular disease. *Advances in protein chemistry structural biology*, 83: 75-127, 2011.

[32] Lord, S. T. Fibrinogen and fibrin: scaffold proteins in hemostasis. *Current opinion in hematology*, 14(3): 236-241, 2007.

[33] Schuliga, M., Grainge, C., Westall, G., and Knight, D. The fibrogenic actions of the coagulant and plasminogen activation systems in pulmonary fibrosis. *The international journal of biochemistry cell biology*, 97: 108-117, 2018.

[34] Wolberg, A. S. Thrombin generation and fibrin clot structure. *Blood Reviews*, 21(3): 131-142, 2007.

[35] Cooper, A. V., Standeven, K. F., and Ariëns, R. A. Fibrinogen gamma-chain splice variant γ′ alters fibrin formation and structure. *Blood*, 102(2): 535-540, 2003.

[36] Bagoly, Z., Koncz, Z., Hársfalvi, J., and Muszbek, L. Factor XIII, clot structure, thrombosis. *Thrombosis Research*, 129(3): 382-387, 2012.

[37] Singh, R., Gautam, P., Sharma, C., and Osmolovskiy, A. Fibrin and fibrinolytic enzyme cascade in thrombosis: unravelling the role. *Life*, 13(11): 2196, 2023.

[38] Ariëns, R. Fibrin (ogen) and thrombotic disease. *Journal of thrombosis haemostasis*, 11: 294-305, 2013.

[39] Zhang, J., Ma, G., Lv, Z., Zhou, Y., Wen, C., Wu, Y., and Xu, R. Targeted thrombolysis strategies for neuroprotective effect. *Neural Regeneration Research*, 9(13): 1316-1322, 2014.

[40] Kirmani, J. F., Alkawi, A., Panezai, S., and Gizzi, M. Advances in thrombolytics for treatment of acute ischemic stroke. *Neurology*, 79(13_supplement_1): S119-S125, 2012.

[41] Kumar, A., Pulicherla, K., Ram, K. S., and Rao, K. Evolutionary trend of thrombolytics. *International Journal of Bio-science Bio-technology*, 2(4): 51-68, 2010.

[42] Collen, D. and Lijnen, H. R. Tissue-type plasminogen activator: a historical perspective and personal account. *Journal of thrombosis haemostasis*, 2(4): 541-546, 2004.

[43] Duffy, M. J. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies. *Clinical chemistry*, 48(8): 1194-1197, 2002.

[44] Chen, H. á., Guan, A. á., and Markland Jr, F. Immunological properties of the fibrinolytic enzyme (fibrolase) from southern copperhead (Agkistrodon contortrix contortrix) venom and its purification by immunoaffinity chromatography. *Toxicon*, 29(6): 683-694, 1991.

[45] Mihara, H., Sumi, H., Yoneta, T., MizuMOTO, H., Ikeda, R., Seiki, M., and Maruyama, M. A novel fibrinolytic enzyme extracted from the earthworm, Lumbricus rubellus. *The Japanese journal of physiology*, 41(3): 461-472, 1991.

[46] Liu, J., Xing, J., Chang, T., Ma, Z., and Liu, H. Optimization of nutritional conditions for nattokinase production by Bacillus natto NLSSE using statistical experimental methods. *Process Biochemistry*, 40(8): 2757-2762, 2005.

[47] Akhtar, T., Hoq, M. M., and Mazid, M. A. Bacterial proteases as thrombolytics and fibrinolytics. *Dhaka University Journal of Pharmaceutical Sciences*, 16(2): 255-269, 2017.

[48] Mukherjee, A. K., Rai, S. K., Thakur, R., Chattopadhyay, P., and Kar, S. K. Bafibrinase: A non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from Bacillus sp. strain AS-S20-I exhibits in vivo anticoagulant activity and thrombolytic potency. *Biochimie*, 94(6): 1300-1308, 2012.

[49] Maeda, H., Mizutani, O., Yamagata, Y., Ichishima, E., and Nakajima, T. Alkaline-resistance model of subtilisin ALP I, a novel alkaline subtilisin. *The Journal of Biochemistry*, 129(5): 675-682, 2001.

[50] Kotb, E. and Kotb, E. *Fibrinolytic bacterial enzymes with thrombolytic activity*. Springer, 2012.

[51] Sumi, H., Hamada, H., Nakanishi, K., and Hiratani, H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinases. *Acta haematologica*, 84(3): 139-143, 1990.

[52] Park, K.-J., Kang, J. I., Kim, T.-S., and Yeo, I.-H. The antithrombotic and fibrinolytic effect of natto in hypercholesterolemia rats. *Preventive nutrition food science*, 17(1): 78, 2012.

[53] Fujita, M., Hong, K., Ito, Y., Misawa, S., Takeuchi, N., Kariya, K., and Nishimuro, S. Transport of nattokinase across the rat intestinal tract. *Biological Pharmaceutical Bulletin*, 18(9): 1194-1196, 1995.

[54] Urano, T., Ihara, H., Umemura, K., Suzuki, Y., Oike, M., Akita, S., Tsukamoto, Y., Suzuki, I., and Takada, A. The profibrinolytic enzyme subtilisin NAT purified frombacillus subtilis cleaves and inactivates plasminogen activator inhibitor type 1. *Journal of Biological Chemistry*, 276(27): 24690-24696, 2001.

[55] Fujita, M., Ito, Y., Hong, K., and Nishimuro, S. Characterization of nattokinase-degraded products from human fibrinogen or cross-linked fibrin. *Fibrinolysis*, 9(3): 157-164, 1995.

[56] Lampe, B. J. and English, J. C. Toxicological assessment of nattokinase derived from Bacillus subtilis var. natto. *Food Chemical Toxicology*, 88: 87-99, 2016.

[57] Weng, Y., Yao, J., Sparks, S., and Wang, K. Y. Nattokinase: an oral antithrombotic agent for the prevention of cardiovascular disease. *International journal of molecular sciences*, 18(3): 523, 2017.

[58] Fujita, M., Hong, K., Ito, Y., Fujii, R., Kariya, K., and Nishimuro, S. Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. *Biological Pharmaceutical Bulletin*, 18(10): 1387-1391, 1995.

[59] Banerjee, A., Chisti, Y., and Banerjee, U. Streptokinase—a clinically useful thrombolytic agent. *Biotechnology advances*, 22(4): 287-307, 2004.

[60] Babu, V. and Subathra Devi, C. In vitro thrombolytic activity of purified streptokinase extracted from Streptococcus equinus VIT_VB2 isolated from bovine milk. *Journal of thrombosis thrombolysis*, 39: 71-78, 2015.

[61] Yadav, S., Datt, M., Singh, B., and Sahni, G. Role of the 88–97 loop in plasminogen activation by streptokinase probed through site-specific mutagenesis. *Biochimica et Biophysica Acta -Proteins Proteomics*, 1784(9): 1310-1318, 2008.

[62] Vadla, P. K., Tummuru, M., and Kumar, D. Purification and characterization of recombinant Streptokinase expressed in E. coli from Streptococcus equisimilis with N-terminal methionine. *Jurnal Teknologi Laboratorium*, 8(1): 8-17, 2019.

[63] Anderson, J. L. Development and evaluation of anisoylated plasminogen streptokinase activator complex (APSAC) as a second generation thrombolytic agent. *Journal of the American College of Cardiology*, 10(5): 22B-27B, 1987.

[64] Nguyen, T. H. T. and Quyen, D. T. High-level expression, purification and properties of a fully active even glycosylated staphylokinase variant SakfC from Staphylococcus aureus QT08 in Pichia pastoris. *Afr. J. Microbiol. Res*, 6: 2129-2136, 2012.

[65] Johnson, A. J. and Tillett, W. S. The lysis in rabbits of intravascular blood clots by the streptococcal fibrinolytic system (streptokinase). *The Journal of Experimental Medicine*, 95(5): 449-464, 1952.

[66] Kline, D. L. The purification and crystallization of plasminogen (profibrinolysin). *Journal of Biological Chemistry*, 204(2): 949-955, 1953.

[67] Alkjaersig, N., Fletcher, A. P., and Sherry, S. The mechanism of clot dissolution by plasmin. *The Journal of clinical investigation*, 38(7): 1086-1095, 1959.

[68] Fletcher, A. P., Sherry, S., Alkjaersig, N., Smyrniotis, F. E., and Jick, S. The maintenance of a sustained thrombolytic state in man. II. Clinical observations on patients with myocardial infarction and other thromboembolic disorders. *The Journal of clinical investigation*, 38(7): 1111-1119, 1959.

[69] Malke, H. and Ferretti, J. J. Streptokinase: cloning, expression, and excretion by Escherichia coli. *Proceedings of the National Academy of Sciences*, 81(11): 3557-3561, 1984.

[70] Nolan, M., Bouldin, S. D., and Bock, P. E. Full time course kinetics of the streptokinase-plasminogen activation pathway. *Journal of Biological Chemistry*, 288(41): 29482-29493, 2013.

[71] Verhamme, I. M. and Bock, P. E. Rapid binding of plasminogen to streptokinase in a catalytic complex reveals a three-step mechanism. *Journal of Biological Chemistry*, 289(40): 28006-28018, 2014.

[72] Castellino, F. J. Recent advances in the chemistry of the fibrinolytic system. *Chemical Reviews*, 81(5): 431-446, 1981.

[73] Kazemi, F., Arab, S. S., Mohajel, N., Keramati, M., Niknam, N., Aslani, M. M., and Roohvand, F. Computational simulations assessment of mutations impact on streptokinase (SK) from a group G streptococci with enhanced activity–insights into the functional roles of structural dynamics flexibility of SK and stabilization of SK–μplasmin catalytic complex. *Journal of Biomolecular Structure Dynamics*, 37(8): 1944-1955, 2019.

[74] Sazonova, I. Y., Robinson, B. R., Gladysheva, I. P., Castellino, F. J., and Reed, G. L. α Domain deletion converts streptokinase into a fibrin-dependent plasminogen activator through mechanisms akin to staphylokinase and tissue plasminogen activator. *Journal of Biological Chemistry*, 279(24): 24994-25001, 2004.

[75] Aneja, R., Datt, M., Yadav, S., and Sahni, G. Multiple exosites distributed across the three domains of streptokinase co-operate to generate high catalytic rates in the streptokinase–plasmin activator complex. *Biochemistry*, 52(49): 8957-8968, 2013.

[76] Tharp, A. C., Laha, M., Panizzi, P., Thompson, M. W., Fuentes-Prior, P., and Bock, P. E. Plasminogen substrate recognition by the streptokinase-plasminogen catalytic complex is facilitated by Arg253, Lys256, and Lys257 in the streptokinase β-domain and kringle 5 of the substrate. *Journal of Biological Chemistry*, 284(29): 19511-19521, 2009.

[77] Bokarewa, M. I., Jin, T., and Tarkowski, A. Staphylococcus aureus: staphylokinase. *The international journal of biochemistry cell biology*, 38(4): 504-509, 2006.

[78] Nedaeinia, R., Faraji, H., Javanmard, S. H., Ferns, G. A., Ghayour-Mobarhan, M., Goli, M., Mashkani, B., Nedaeinia, M., Haghighi, M. H. H., and Ranjbar, M. Bacterial staphylokinase as a promising third-generation drug in the treatment for vascular occlusion. *Molecular Biology Reports*, 47: 819-841, 2020.

[79] Adivitiya and Khasa, Y. P. The evolution of recombinant thrombolytics: Current status and future directions. *Bioengineered*, 8(4): 331-358, 2017.

[80] Schlott, B., Hartmann, M., Gührs, K.-H., Birch-Hirschfeld, E., Gase, A., Vettermann, S., Collen, D., and Lijnen, H. R. Functional properties of recombinant staphylokinase variants obtained by site-specific mutagenesis of methionine-26. *Biochimica et Biophysica Acta -Protein Structure Molecular Enzymology*, 1204(2): 235-242, 1994.

[81] Macfarlane, R. G. and Pilling, J. Fibrinolytic activity of normal urine. *Nature*, 159(4049): 779-779, 1947.

[82] Sobel, G., Mohler, S., Jones, N., Dowdy, A., and Guest, M. in *American Journal of Physiology*. 768-769 (AMER PHYSIOLOGICAL SOC 9650 ROCKVILLE PIKE, BETHESDA, MD 20814).

[83] Rijken, D. and Lijnen, H. New insights into the molecular mechanisms of the fibrinolytic system. *Journal of thrombosis haemostasis*, 7(1): 4-13, 2009.

[84] Egelund, R., Petersen, T. E., and Andreasen, P. A. A serpin-induced extensive proteolytic susceptibility of urokinase-type plasminogen activator implicates distortion of the proteinase substrate-binding pocket and oxyanion hole in the serpin inhibitory mechanism. *European Journal of Biochemistry*, 268(3): 673-685, 2001.

[85] Schmitt, M., Goretzki, L., Jänicke, F., Calvete, J., Eulitz, M., Kobayashi, H., Chucholowski, N., and Graeff, H. Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer. *Biomedica biochimica acta*, 50(4-6): 731-741, 1991.

[86] Stack, M. S. and Johnson, D. A. Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). *Journal of Biological Chemistry*, 269(13): 9416-9419, 1994.

[87] Sadasivan, R., Cherian, R., and Wyatt, A. Urokinase-type plasminogen activator expression in human prostate carcinomas. *The American journal of the medical sciences*, 312(1): 8-11, 1996.

[88] Goretzki, L., Schmitt, M., Mann, K., Calvete, J., Chucholowski, N., Kramer, M., Günzler, W. A., Jänicke, F., and Graeff, H. Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L. *FEBS letters*, 297(1-2): 112-118, 1992.

[89] Lee, H.-J. and Im, H.-N. Soluble expression and purification of human tissue-type plasminogen activator protease domain. *Bulletin of the Korean Chemical Society*, 31(9): 2607-2612, 2010.

[90] Ram, K. S., Peravali, J., Kumar, A., Rao, K., and Pulicherla, K. Staphylokinase: A Boon in Medical Sciences—Review. *Mintage J. Pharmaceut. Med. Sci*, 2(2): 28-34, 2013.

[91] Wander, G. S. and Chhabra, S. T. Critical analysis of various drugs used for thrombolytic therapy in acute myocardial infarction. *Med Updat*, 23: 109-116, 2013.

[92] Bates, S. M. and Weitz, J. I. New anticoagulants: beyond heparin, low-molecular-weight heparin and warfarin. *British journal of pharmacology*, 144(8): 1017-1028, 2005.

[93] Brieger, D. and Curnow, J. Anticoagulation: a GP primer on the new oral anticoagulants. *Australian family physician*, 43(5): 254-259, 2014.

[94] Lassen, M. R. and Laux, V. Emergence of new oral antithrombotics: a critical appraisal of their clinical potential. *Vascular Health Risk Management*, 4(6): 1373-1386, 2008.

[95] Gibbs, N. M., Weightman, W. M., and Watts, S. A. New antithrombotic agents in the ambulatory setting. *Current Opinion in Anesthesiology*, 27(6): 589-596, 2014.

[96] Favaloro, E. J. and Lippi, G. The new oral anticoagulants and the future of haemostasis laboratory testing. *Biochemia Medica*, 22(3): 329-341, 2012.

[97] Goel, R. and Srivathsan, K. Newer oral anticoagulant agents: a new era in medicine. *Current cardiology reviews*, 8(2): 158-165, 2012.

[98] Toschi, V. and Lettino, M. Inhibitors of propagation of coagulation: factors V and X. *British journal of clinical pharmacology*, 72(4): 563-580, 2011.

[99] Suryanarayan, D. and Schulman, S. Potential antidotes for reversal of old and new oral anticoagulants. *Thrombosis Research*, 133: S158-S166, 2014.

[100] Majeed, A. and Schulman, S. Bleeding and antidotes in new oral anticoagulants. *Best Practice Research Clinical Haematology*, 26(2): 191-202, 2013.

[101] Brighton, T. New oral anticoagulant drugs-mechanisms of action. *Australian prescriber*, 33(2), 2010.

[102] Scully, C. and Wolff, A. Oral surgery in patients on anticoagulant therapy. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, Endodontology*, 94(1): 57-64, 2002.

[103] Adewale Fawole, M. D. Practical management of bleeding due to the anticoagulants dabigatran, rivaroxaban, and apixaban. *Cleveland Clinic Journal of Medicine*, 80(7): 443, 2013.

[104] Yeung, J. and Holinstat, M. Newer agents in antiplatelet therapy: a review. *Journal of blood medicine*: 33-42, 2012.

[105] Xiang, Y.-Z., Xia, Y., Gao, X.-M., Shang, H.-C., Kang, L.-Y., and Zhang, B.-L. Platelet activation, and antiplatelet targets and agents: current and novel strategies. *Drugs*, 68: 1647-1664, 2008.

[106] Penela, D., Diaz-Ricart, M., and Heras, M. New antiplatelet drugs in perspective. 2011.

[107] Majerus, P. W. Anticoagulant, thrombolytic, and antiplatelet drugs. *Goodman Gilman's the pharmacological basis of therapeutics*: 1519-1538, 2001.

[108] Berger, J. S., Brown, D. L., and Becker, R. C. Low-dose aspirin in patients with stable cardiovascular disease: a meta-analysis. *The American journal of medicine,* 121(1): 43-49, 2008.

[109] Campbell, C. L., Smyth, S., Montalescot, G., and Steinhubl, S. R. Aspirin dose for the prevention of cardiovascular disease: a systematic review. *Jama,* 297(18): 2018-2024, 2007.

[110] Burger, W., Chemnitius, J. M., Kneissl, G., and Rücker, G. Low-dose aspirin for secondary cardiovascular prevention–cardiovascular risks after its perioperative withdrawal versus bleeding risks with its continuation–review and meta-analysis. *Journal of internal medicine,* 257(5): 399-414, 2005.

[111] Schrör, K. The basic pharmacology of ticlopidine and clopidogrel. *Platelets,* 4(5): 252-261, 1993.

[112] Bellavance, A. Efficacy of ticlopidine and aspirin for prevention of reversible cerebrovascular ischemic events. The Ticlopidine Aspirin Stroke Study. *Stroke,* 24(10): 1452-1457, 1993.

[113] Husted, S. New developments in oral antiplatelet therapy. *European heart journal supplements,* 9(suppl_D): D20-D27, 2007.

[114] Andrews, R. K. and Berndt, M. C. Platelet physiology and thrombosis. *Thrombosis Research,* 114(5-6): 447-453, 2004.

[115] Lippi, G., Montagnana, M., Danese, E., Favaloro, E. J., and Franchini, M. Glycoprotein IIb/IIIa inhibitors: an update on the mechanism of action and use of functional testing methods to assess antiplatelet efficacy. *Biomarkers in medicine,* 5(1): 63-70, 2011.

[116] Ji, X. and Hou, M. Novel agents for anti-platelet therapy. *Journal of hematology oncology,* 4: 1-7, 2011.