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RECOMBINANT PRODUCTION AND EVALUATION OF A MULTIFUNCTIONAL HAEMOSTATIC FUSION PROTEIN

by

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DECLARATION

I, the undersigned, hereby declare that the word contained within this thesis is my original and independent work and has not in its entirety or in part been submitted to any university for a degree.

All the sources I have made use of or quoted have been acknowledged by complete references.

W.B. van Zyl
October 1999

Dedicated to my husband, Ludi

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ABBREVIATIONS

α_2-PI – α_2-plasma inhibitor

^{111}In – 111-Indium

ABE – Anion binding exosite

ADP – Adenosine diphosphate

APC – Activated protein C

aPTT – Activated partial thromboplastin time

AT III – Antithrombin III

AV – Arteriovenous

cDNA – Complementary DNA

CHO – Chinese hamster ovary

EDTA – Ethylenedinitrilo tetraacetic acid

ELISA – Enzyme-linked immuno-sorbent assay

FDP – Fibrinogen degradation products

FPA – Fibrinopeptide A

FPLC – Fast protein liquid chromatography

Gp – Glycoprotein

HPI – Hookworm platelet inhibitor

HLA – Human leukocyte antigen

IPEC – Immortalized porcine endothelial cells

IPTG – Isopropyl-β-D-thiogalactopyranoside

kDa – Kilodalton

KGD – Lysine-Glycine-Aspartic acid

KGDS – Lysine-Glycine-Aspartic Acid-Serine

LAPP – Leech antiplatelet protein

Ni-NTA – Nickel-nitrilotriacetic acid

NTA – Nitrilotriacetic acid

PAI-1 – Plasminogen activator inhibitor-1

PAI-2 – Plasminogen activator inhibitor-2

PAI-3 – Plasminogen activator inhibitor-3

PCR – Polymerase chain reaction

PLATSAK – Platelet-Antithrombin-Staphylokinase

PPACK – D-Phenyl-L-Prolyl-L-Argenyl-chloromethylketone

Pr – Primer

psi – pressure per square inch

rAPC – Recombinant activated protein C

RGD – Arginine-Glycine-Aspartic acid

RGDNP – Arginine-Glycine-Aspartic acid-Asparagine-Proline

RGDS – Arginine-Glycine-Aspartic acid-Serine

RGDW – Arginine-Glycine-Aspartic acid-Tryptophan

rLAPP – Recombinant leech antiplatelet protein

rscu-PA – Recombinant single chain urokinase-type plasminogen activator

rTAP – Recombinant tick anticoagulant peptide

SAK – Staphylokinase

scu-PA – Single chain urokinase-type plasminogen activator

SDGE – Serine-Aspartic acid-Glycine-Glutamic acid

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TAFI – Thrombin activatable fibrinolysis inhibitor

TAP – Tick anticoagulant peptide

TAT – Thrombin-antithrombin III

TF – Tissue factor

TFPI – Tissue factor pathway inhibitor

t-PA – Tissue-type plasminogen activator

TRAP – Thrombin receptor activating peptide

TT – Thrombin time

TxA_2 – Thromboxane A_2

u-PA – Urokinase-type plasminogen activator

VWF – Von Willebrandt factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION:

Haemostasis is the processes involved in the prevention of blood loss during vascular injury, while thrombosis is a pathological outcome of vascular disease. Haemostasis involves complex interactions between damaged vessel wall surfaces, activated blood platelets and activated coagulation factors. These interactions ultimately lead to the production of thrombin, which is responsible for the conversion of soluble fibrinogen to insoluble fibrin. Thrombin also acitvates platelets and activates coagulation factors V and VIII. Cross-linking between fibrin strands results in the formation of a haemostatic plug at the site of vascular injury. Following wound repair, the haemostatic plug will be removed by a physiological process called fibrinolysis, which is also a delicately balanced cascade of interactions amongst several proteins.

Since thrombin plays a central role in the maintenance of vascular integrity, it has been the focus of research in the field of antithrombotic agents in the past few years. Hirudin, a potent inhibitor of thrombin, is produced by the medicinal leech and has been studied intensively (Markward, 1970; Rydel *et al*, 1990; Rydel *et al*, 1991). Fragments and derivatives of hirudin have also been studied thoroughly (Krstenansky and Mao, 1987; Mao *et al*, 1988; Naski *et al*, 1990; Schmitz *et al*, 1991). Much research has also been devoted to the prevention of platelet aggregation, in particular blockade of the receptor of fibrinogen on the activated platelet membrane, glycoprotein IIb/IIIa (Coller, 1985; Deckmyn *et al*, 1994; Foster *et al*, 1994). Several disintegrins from snake venoms were studied and their role in the inhibition of platelet activation has been elucidated (Dennis *et al*, 1990; Niewiarowski *et al*, 1990; Savage *et al*, 1990; Scarborough *et al*, 1993).

Exogenous activators of fibrinolysis, streptokinase and staphylokinase, have been studied for their potential as fibrinolytic agents (Collen *et al*, 1993; Collen and Lijnen, 1994; Collen, 1997).

Several combination proteins, which simultaneously target haemostasis at different levels, were recently developed. The activities of these novel proteins include combined antithrombin and antiplatelet activity (Knapp *et al*, 1992), fibrinolytic and antiplatelet activity (Smith *et al*, 1995) as well as fibrinolytic and antithrombin activity (Lijnen *et al*, 1995). Another interesting approach in terms of fibrin targeting of an antithrombin was followed by Bode *et al* (1994). They fused the Fab' of a monoclonal antibody, 59D8, to recombinant hirudin. The resultant antithrombin was substantially more effective than recombinant hirudin.

1.2 AIM AND SCOPE OF THE STUDY:

In this study, I have developed a novel chimeric protein that would target haemostasis at three levels. The protein, named PLATSAK, was designed to inhibit the action of thrombin, prevent platelet aggregation and activate fibrinolysis. It consists of staphylokinase (SAK), linked via a factor Xa cleavage site, to an antithrombotic and antiplatelet peptide (Fig. 1.1). The chimera was designed to act as a local drug delivery system. Theoretically, the high fibrin-specificity of SAK should transport PLATSAK to a fibrin-containing thrombus and thus to an environment that contains high concentrations of factor Xa. The peptide can then be released by factor Xa in the proximity of recently activated thrombin and activated platelets. Additional platelet aggregation and fibrin formation can subsequently be prevented by the antiplatelet and antithrombotic peptide. The peptide was designed to contain three inhibitory regions. Firstly, on its N-terminus it has the RGD-sequence for binding to the fibrinogen receptor (Gp IIb/IIIa) to prevent fibrinogen binding to platelets and so also platelet aggregation (Ruoslahti and Pierschbacher, 1987). That is followed by a part of fibrinopeptide A (residues 8-16) to block the active site of thrombin (Martin *et al*, 1992) and the C-terminus of hirudin (residues 54-65) to block the anion binding site of thrombin (Markwardt, 1970).

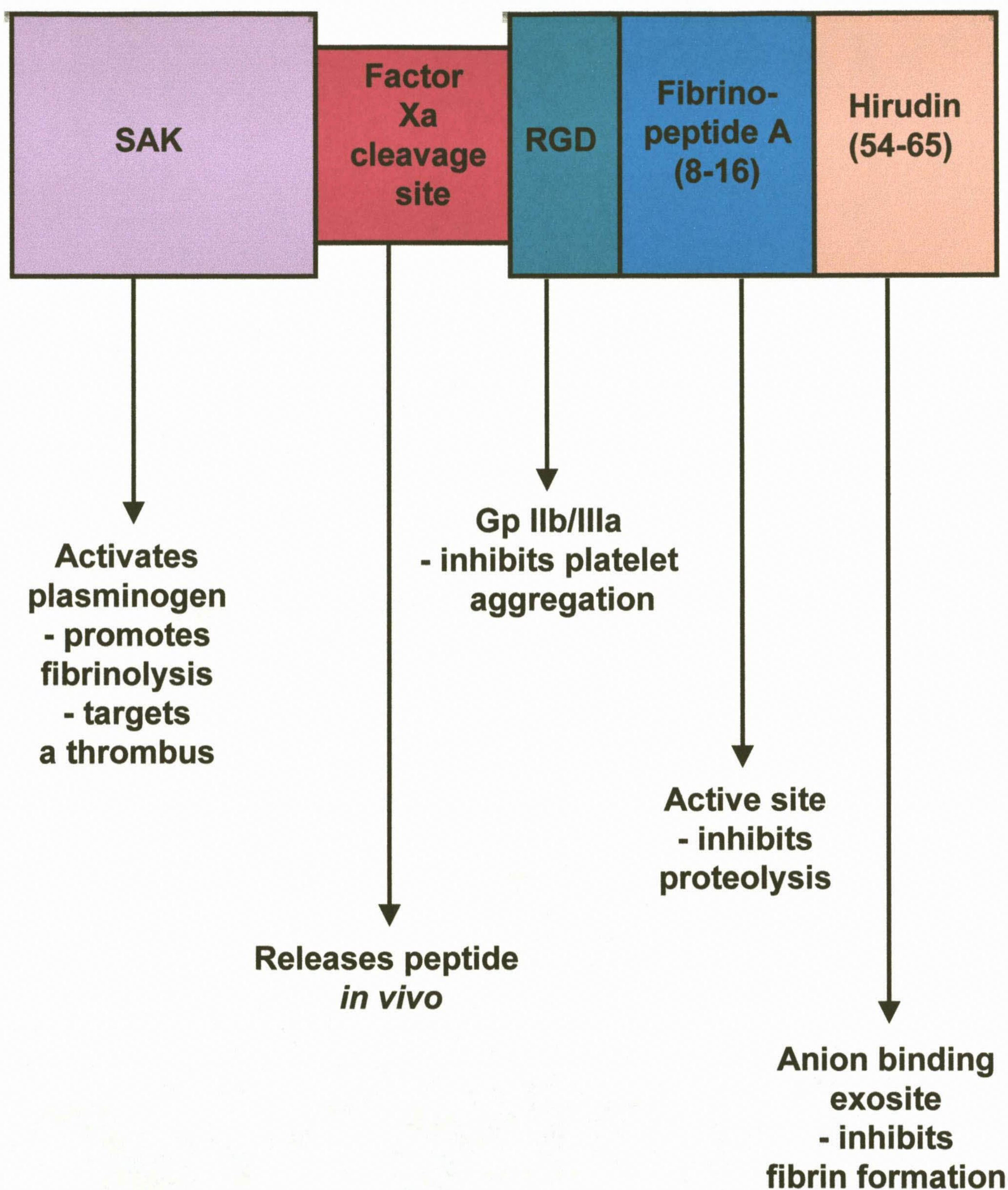

Fig. 1.1 Schematic representation of the composition of PLATSAK and the function of the individual components. The staphylokinase part is linked to the antiplatelet and antithrombotic peptide via the recognition sequence of factor Xa.

I have decided to use *Escherichia coli* cells as expression host for the recombinant production of PLATSAK. It is a well-known organism in terms of its genetic and biochemical characteristics and has been widely used as expression host for the production of novel agents. Furthermore, it is a fast growing organism and is easy and relatively inexpensive to cultivate.

The aim of the study was to successfully construct the hybrid gene, express it in *E.coli* cells and produce sufficient amounts of the protein to evaluate its biological activities *in vitro*. Furthermore, the project was aimed at the estimation of the potential of PLATSAK in an *in vivo* model.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION:

Remarkable strides have been made to unravel the molecular mechanisms that underlie thrombosis and haemostasis, especially with regard to the central role of thrombin and blood platelets. This knowledge and the high incidence of vascular related diseases led to a drastic increase in the development of novel and potent antithrombotic agents. These agents are targeted to either effectively prevent the formation of thrombin or inhibit its activity, or to inhibit platelet-platelet interactions. Furthermore, thrombolytic agents can be utilised to dissolve excessive fibrin clots and can also be used in conjunction with antithrombotic agents to prevent reocclusion of blood vessels following successful reperfusion.

This review will briefly focus on the molecular principles of thrombosis and haemostasis, especially with regard to the role of platelets, coagulation and fibrinolysis. The recent development and mode of action of several novel and potent antiplatelet, antithrombotic and fibrinolytic agents will also be discussed in detail.

2.2 HAEMOSTASIS AND THROMBOSIS:

Haemostasis is a defence mechanism that prevents excessive blood loss by maintaining vascular integrity. Thrombosis on the other hand, is a pathological consequence of vascular disease. The haemostatic process involves complex and integrated interactions between damaged vessel wall surfaces, activated blood platelets and activated coagulation factors to form a localized haemostatic plug to prevent blood loss. Haemostasis involves complicated systems of activation and inhibition, which limit generalized extension of thrombosis beyond the area of

damage (Fig. 2.1). Under strictly controlled conditions the haemostatic plug is subsequently dissolved by fibrinolysis. When these contact mechanisms are overcome by excessive platelet activation, platelets and coagulation become major contributors to the development of thrombosis and can lead to morbidity and mortality due to myocardial infarction and stroke (Harker, 1990).

2.2.1 Platelet Function:

Blood platelets are produced by bone marrow megakaryocytes. They are about 3 μm in diameter and 1 μm thick and thus the smallest of all blood cells (Blockmans *et al*, 1995). Regardless of their small size they are biologically among the most active and play a substantial role in haemostasis, thrombosis and atherosclerosis (Wu, 1996). Resting platelets circulate the vascular system as discoid anuclear cells. Cellular constraints and factors like prostacyclin and nitric oxide prevent adhesion and aggregation of resting platelets and allow close contact to the endothelial cell lining without adhering to it (Wu, 1996). P-selectin, a cell adhesion molecule present in platelets and endothelial cells, is stored in the secretory granules and is rapidly expressed on the plasma membrane upon activation (Hsu-Lin *et al*, 1984). Both platelet and endothelial P-selectin mediate leukocyte adhesion (McEver *et al*, 1995) and are believed to be responsible to position circulating platelets close to the vessel wall.

Following vascular injury, platelets form the first line of defence to prevent excessive blood loss. This occurs through a series of well-defined reactions that culminate in the formation of a platelet plug at the area of injury. The reactions include activation of platelets, change in platelet shape, release of their granular content and ultimately platelet aggregation (Harker, 1990; Ruggeri, 1997). It is generally accepted that binding of platelets to collagen *in vivo* is the trigger that starts the haemostatic process. Intact endothelial cells, adjacent to the injury, produce inhibitors of both coagulation and platelets to control the size of the hemostatic plug and contain it to the area of injury (Blockmans *et al*, 1995).

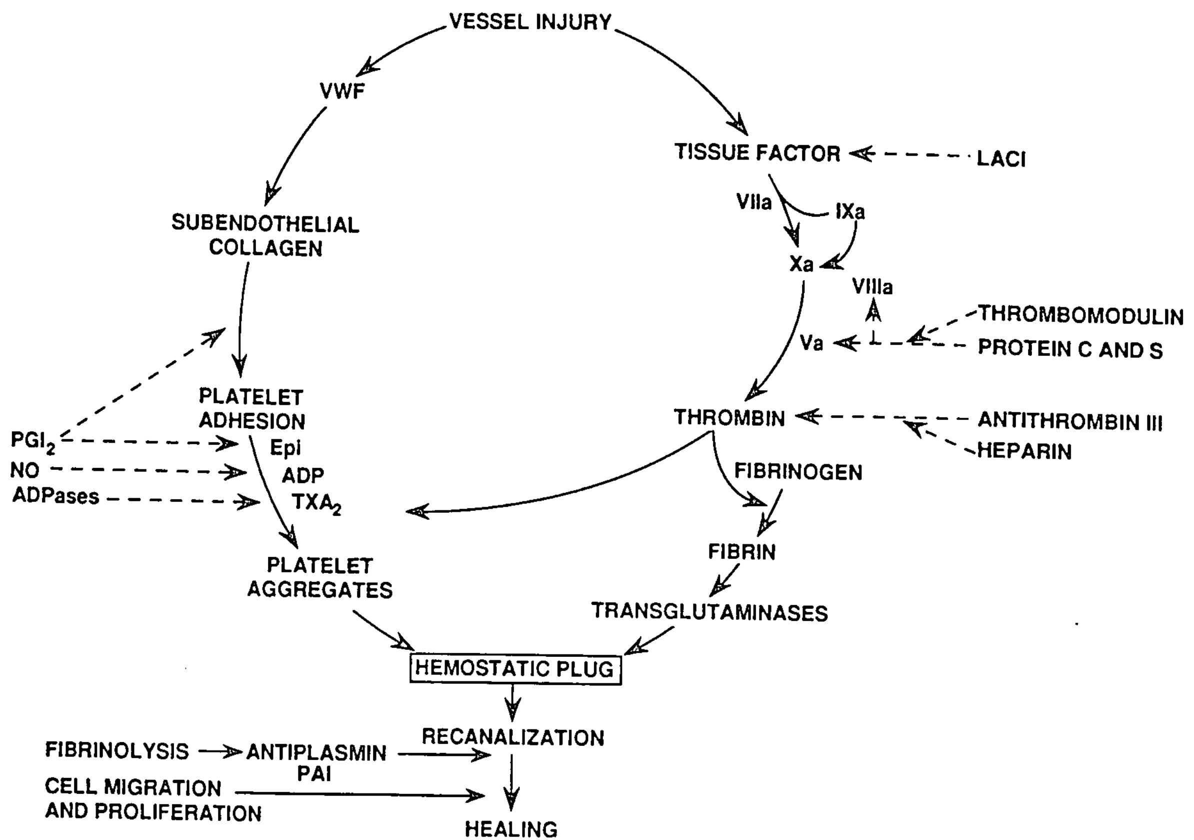

Fig. 2.1 An overview of haemostasis (Colman et al, 1994). Solid lines indicate activation and broken lines inhibition.

The platelet plasma membrane consists of a phospholipid bilayer containing the glycoproteins (Gp), which serve as receptors for adhesive proteins and seven transmembrane receptors for activating and inhibitory agents to bind to (Blockmans *et al*, 1995). The glycoproteins and their ligands play essential roles in platelet plug formation. Of particular interest are Gp Ia/IIa, Ib/V/IX and IIb/IIIa. Under low flow conditions, fibronectin binding to Gp Ia/IIa play the most important role in platelet adhesion (Weiss, 1995), while von Willebrand factor (VWF) is most important under high flow conditions (Ruggeri, 1997). Circulating VWF cannot bind to platelets, but is capable of binding to collagen. When bound to collagen, VWF undergoes certain conformational changes, which enable it to bind to Gp Ib/IX/V. Initially, the A1 domain of VWF rapidly binds to Gp Ibα on platelet membranes (Savage *et al*, 1996). This bond has a high dissociation rate, resulting in detachment of the tailing end of the platelet and a forward rotational movement due to the torque produced by the blood flow. It does, however, slow the progress of the platelets across the damaged area. There are two schools of thought on the mechanisms that anchor platelets at the site of injury. The first suggests that the rolling movement of the platelet continues until Gp IIb/IIIa becomes activated and binds to the RGDS sequence in the C1 domain of VWF (Savage *et al*, 1992; Ruggeri, 1997). The second proposes that the anchor is when Gp Ia/IIa binds to collagen in the subendotheluim (Vermylen *et al*, 1997).

Thrombin, adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2) are interdependent agonists that activate platelets (Harker 1994; Ware and Coller, 1995). Thrombin is the most potent and primary agonist of platelets and activation is achieved by binding to its receptor on platelets (Vu *et al*, 1991). ADP is released from activated platelets and is responsible for recruitment of surrounding platelets, while TxA_2 induces receptor activation (Harker, 1994). Activation results in a change in platelet shape to markedly increase the membrane surface area to facilitate platelet-subendothelium and platelet-platelet interactions. Activation also results in the release of the contents of platelet granules. The granules contain platelet specific proteins (platelet factor 4 and β-thromboglobulin), adhesion proteins (fibrinogen, von Willebrandt factor, fibronectin, vitronectin, and thrombospondin), coagulation factors (factors V and XIII and protein S), mitogenic

factors (platelet derived growth factor, endothelial cell growth factor and epidermal growth factor), fibrinolytic proteins (α_2-antiplasmin and plasminogen activator inhibitor-1) and plasma proteins (albumin and immunoglobulins).

Once activated, platelets can interact to form the hemostatic plug. The 40 000 – 50 000 Gp IIb/IIIa receptors and the adhesion proteins fibrinogen, VWF, fibronectin and vitronectin play essential roles in platelet aggregation. The Gp IIb/IIIa receptors on resting platelets bind fibrinogen weakly and are incapable of binding to VWF. Activation changes the Gp IIb/IIIa receptor into a high-affinity binding site for fibrinogen and VWF. Gp IIb/IIIa recognises two binding domains on fibrinogen: an RGD-sequence in each of the two A-alpha chains and a dodecapeptide in the gamma chain (Ruggeri, 1993; Ginsberg *et al*, 1995).

Fibrinogen molecules bind to Gp IIb/IIIa to form molecular bridges between two adjacent platelets to stabilise the thrombus. Activated Gp IIb/IIIa on the luminal side of the platelet plug may bind circulating fibrinogen or VWF, which in turn may interact with Gp IIb/IIIa of activated platelets in the neighbourhood. This process can be repeated several times to add a new layer of platelets to form the haemostatic plug (Ware and Coller, 1995). Platelet adhesion and aggregation and the relevant participants in thrombus formation are schematically shown in Fig. 2.2.

In concert with the events of platelet adhesion and aggregation, the coagulation cascade is activated and thrombin is responsible for the conversion of fibrinogen to fibrin (see Section 2.2.2). Activated platelets are responsible for providing a negatively charged surface for the coagulation factor complexes to bind to. The conversion of factor X to Xa and prothrombin to thrombin is facilitated to enhance thrombus formation on the activated platelet membrane (Tracy 1988; Mann *et al*, 1990).

Several factors control the growth of a hemostatic plug. Firstly, aggregating agents are removed from the site of plug formation by the flowing blood and its concentration is diluted in the area of thrombogenesis (Weiss, 1995). Secondly, thrombin stimulates adjacent intact endothelial cells to release platelet inhibitors

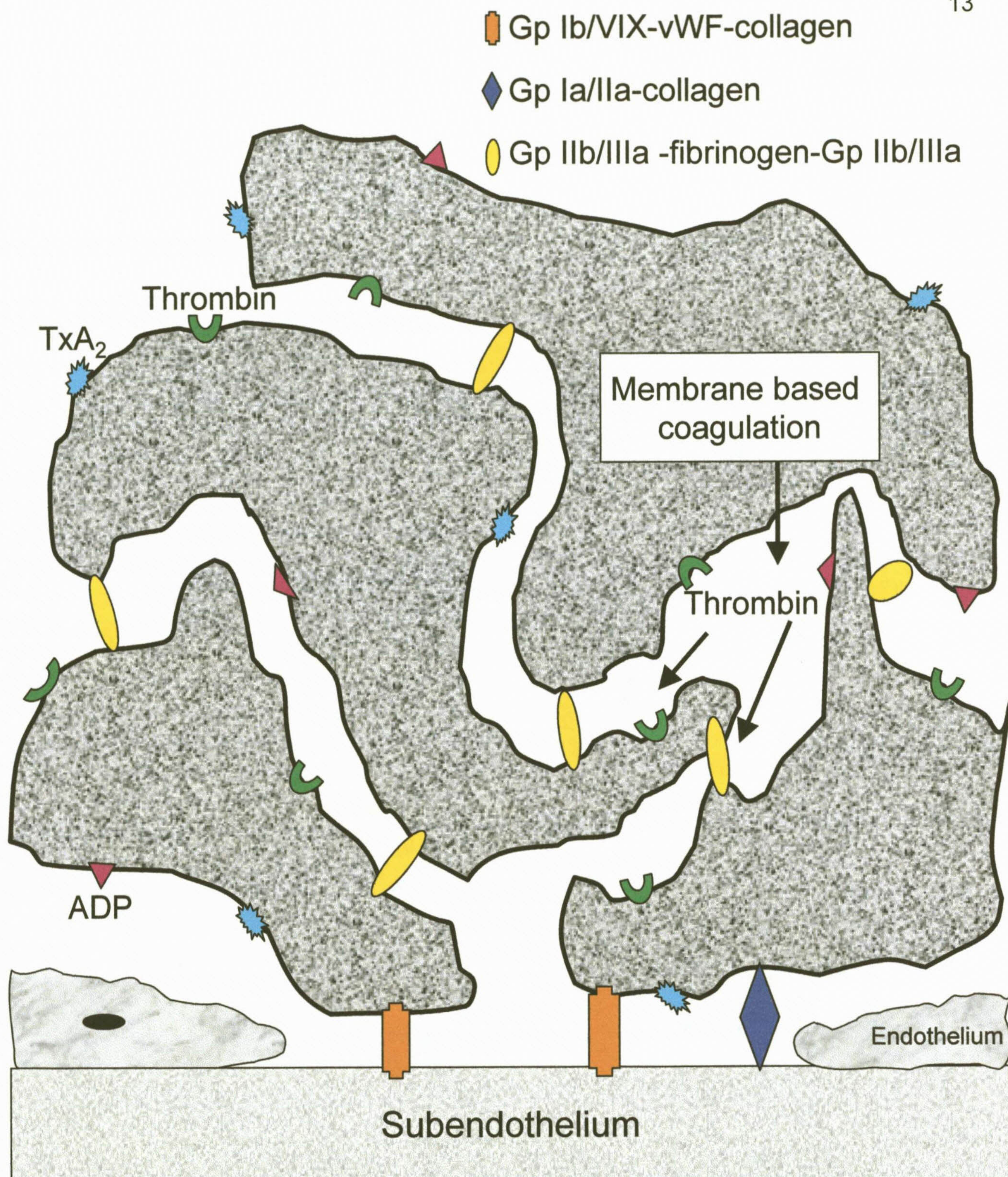

Fig. 2.2 A schematic representation of the important participants in thrombus formation. VWF binds to collagen and Gp Ib/V/IX to promote platelet adhesion to the exposed subendothelium. This process is helped by the binding of collagen to Gp Ia/IIa. Binding of fibrinogen, VWF, fibronectin or vitronectin to Gp IIb/IIIa on the membrane of activated platelets results in platelet aggregation. Thrombin, ADP and TxA_2 are important receptors for platelet agonists on the platelet membrane.

like prostacyclin and nitric oxide (Ware and Coller, 1995). Thirdly, thrombin binds to thrombomodulin on the endothelial cell membrane to activate protein C, which is regarded as the most important *in vivo* inhibitor of thrombin formation (Esmon, 1993).

2.2.2 Coagulation:

The factors of the coagulation cascade circulate as inert pro-enzymes. They are activated on negatively charged membrane surfaces, provided by activated platelets, by another activated coagulation factor in the presence of a co-enzyme and Ca-ions (Fig. 2.3). The coagulation factors are serine proteases and activation of a pro-enzyme lower down in the cascade is achieved by removing a peptide from the pro-enzyme at a serine residue in the active site (Kay, 1988). Thrombin is the final product of this series of reactions (Fig. 2.3). Prothrombin is cleaved through a series of steps to thrombin A and B and the biologically active α-thrombin.

Activation of thrombin leads to the exposure of the anion binding exosite (ABE), which enables thrombin to bind to negatively charged molecules like heparin (Rosenberg and Damus, 1973), fibrinogen (Fenton *et al*, 1988) and the C-terminus of hirudin (Grütter *et al*, 1990; Naski *et al*, 1990; Rydel *et al*, 1990). Residues 7-16 of thrombin are essential for the catalytic efficiency of the molecule and represents the anionic exosite domain (Berliner *et al,* 1985; Lord *et al,* 1990; De Cristofaro and Castagnola, 1991). The active site is responsible for its amidolytic activity and results in hydrolysis of small substrates like tripeptide *p*-nitroanilide and binding of peptides like fibrinopeptide A (Martin *et al*, 1992). Additionally, thrombin has an apolar binding site adjacent to its catalytic site in the fibrinopeptide groove, accounting for thrombin binding to compounds like proflavin (Sonder and Fenton, 1984).

Thrombin transforms soluble fibrinogen to insoluble fibrin by releasing fibrinopeptides A and B from fibrinogen (Blomback and Blomback, 1972). Fibrin

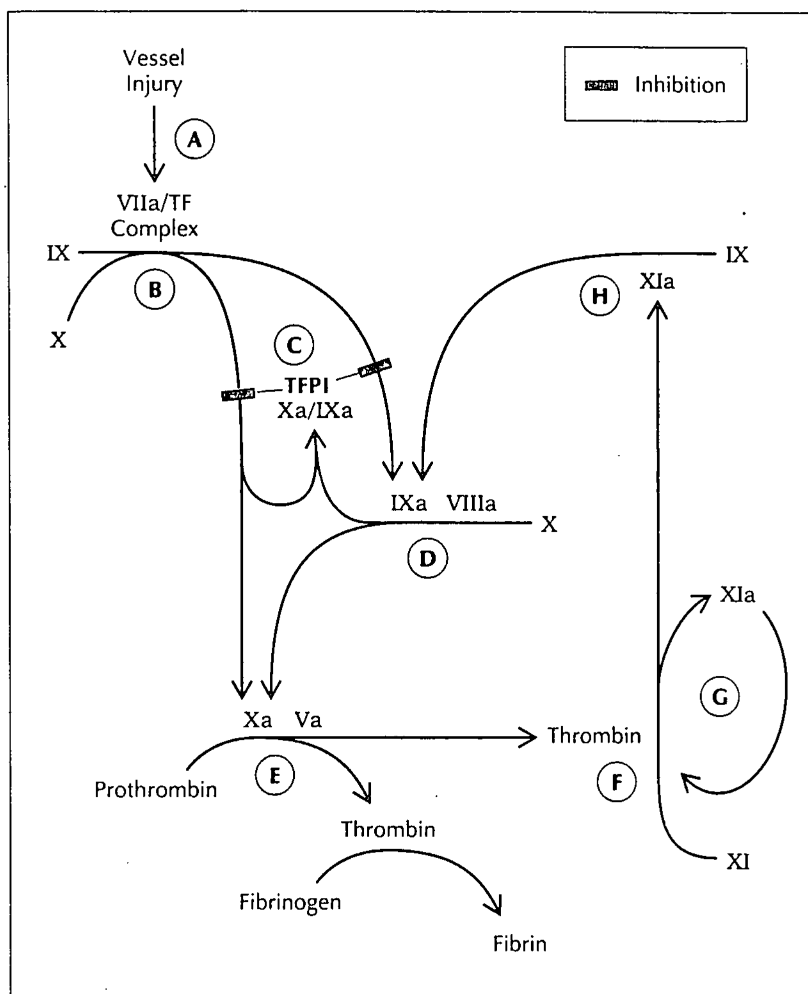

Fig. 2.3 Coagulation cascade hypothesised by Broze (1992). Exposure of tissue factor (TF) to FVIIa initiates coagulation (A). The VIIa/TF complex produces small amounts of factors IXa and Xa (B), until tissue factor pathway inhibitor (TFPI) inhibits this process (C). From this point only the action of factors IXa and VIIIa (D) can generate additional factor Xa (E) to maintain coagulation. Thrombin activation of factor XI (F) and XIa autoactivation (G) may produce additional factor IXa (H).

formation and subsequent lysis is illustrated in Fig. 2.4. Following release of fibrinopeptides A and B, fibrin dimerisation occurs. Factor XIIIa crosslinks a dimer with another monomer to enhance resistance of the thrombus towards plasmin degradation. Lengthening of the polymer occurs in a progressive, half-overlap, side-to-side interaction of monomers producing broad sheets of fibrin (Hermans and McDonagh, 1982). Lysis of the thrombus is discussed in Section 2.2.3.

Thrombin activates platelets by binding to its receptor on the platelet membrane (Coughlin, 1993) and is also responsible for activation of factor XIII, which cross-links fibrin threads. Furthermore, thrombin promotes its own production by activation of factors V and VIII. Simultaneously, it downregulates its own production by binding to thrombomodulin on the endothelium surface to activate the coagulation inhibitor, protein C (Esmon *et al*, 1982). Activated protein C, requiring protein S as a co-factor, inhibits thrombin production by inhibiting factors Va and VIIIa (Scully, 1992). Thrombin thus interacts with cells as well as with circulating proteins. All these interactions require the anion-binding site, while the catalytic domain of thrombin is responsible for its activational function (Arnaud *et al*, 1994).

Thrombin plays a central role in co-ordinating the molecular and cellular interactions essential for vascular lesion formation. Firstly, thrombin is the principal mediator of thrombus formation at *in vivo* sites of vascular injury. Secondly, thrombin is a potent growth factor that stimulates proliferation of vascular smooth muscle cells at vascular injury sites *in vivo*. Thirdly, thrombin controls the effects of other growth factors. Fourthly, thrombin regulates inflammatory processes in blood leukocytes and vascular vessel cells. On this basis, Harker *et al* (1995) suggested that inhibition of thrombin or its receptor function would ultimately lead to disruption of both thrombus formation and vascular lesion formation.

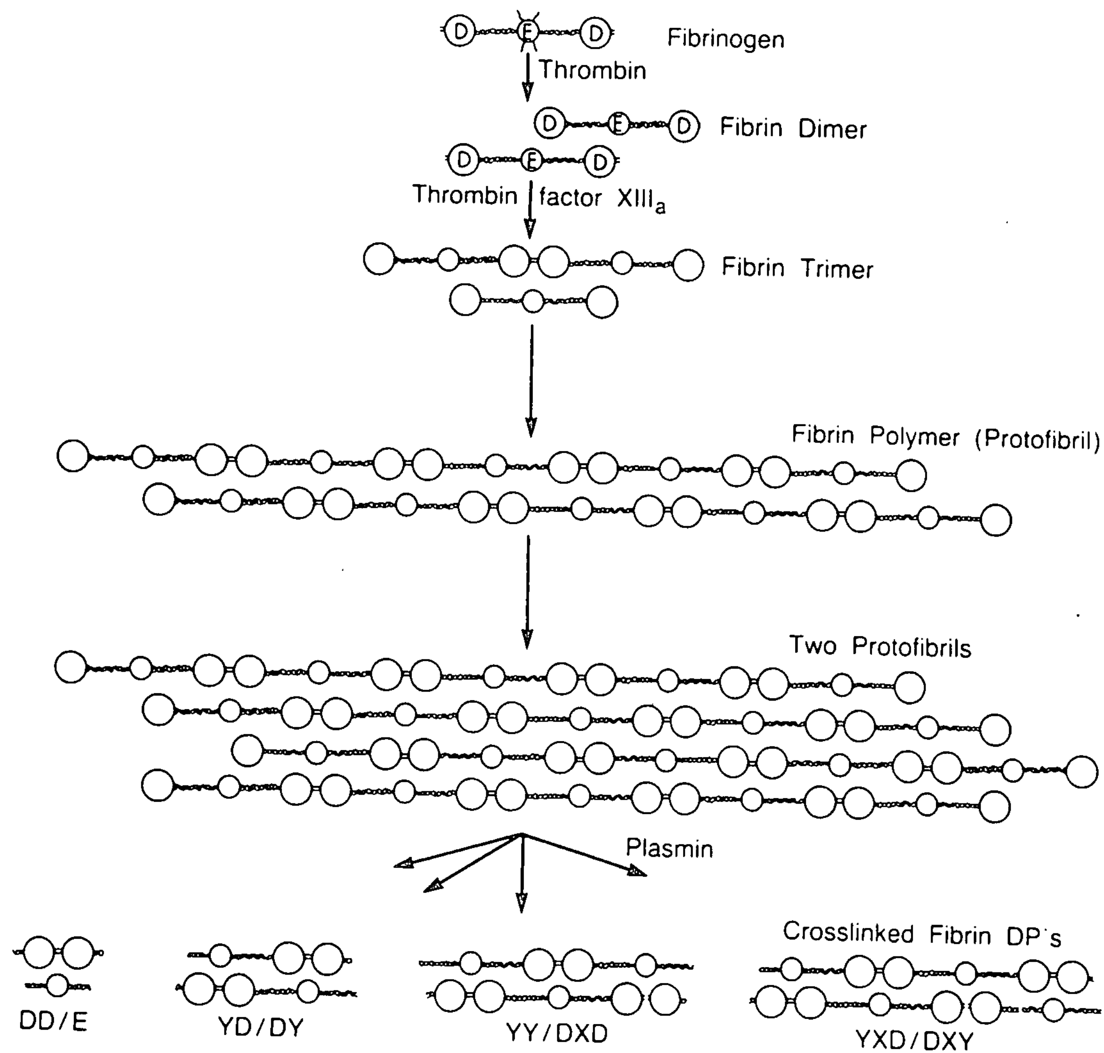

Fig. 2.4 Schematic representation of fibrin formation and subsequent lysis by plasmin (Colman et al, 1994).

2.2.3 Fibrinolysis:

Fibrinolysis is the final mechanism of haemostasis and involves remodelling and removal of a fibrin clot to maintain blood flow and promote healing. It consists of a cascade of zymogen-to-enzyme conversions, feedback potentiation and inhibition. The inactive circulating precursor fibrinolytic protein circulating the blood is plasminogen, which is an 88 kDa plasma glycoprotein. Platelets release plasminogen activator inhibitors during initial haemostasis to retard fibrin degradation. This allows sufficient fibrin formation to form the haemostatic plug (Plow and Collen, 1981). Precisely timed production of tissue-type plasminogen activator (t-PA) by endothelial cells (Lewin *et al*, 1984) as well as urokinase-type plasminogen activator (u-PA), which is found in plasma, leads to the activation of plasminogen to form plasmin (Lijnen and Collen, 1982). Activation is accomplished by proteolysis of the Arg^{561}-Val^{562} peptide bond. Plasmin has a positive feedback on its own production by cleaving an activation peptide from plasminogen, making it more susceptible to activation and enhancing its fibrin specificity. Like coagulation, fibrinolysis is also a delicately balanced cascade of interactions among several proteins. Fibrinolysis is responsible for clot lysis, endothelial cell regrowth and vessel recanalization (Colman *et al*, 1994).

Fibrin degradation is depicted in Fig. 2.4. Degradation by plasmin occurs by cleavage between the central E domain and one of the terminal D domains of a two-stranded protofibril. It results in the release of DD/E fragments, as well as intermediate products like YD/DY and YY/DXD fragments (Francis *et al*, 1980). The Y fragment consists of a central E domain attached to a single D domain, while an X fragment is composed of a central E domain connected to a D domain on both sides (Francis and Marder, 1995).

The major physiologic inhibitor of plasmin is α_2-antiplasmin, which circulates in plasma at a concentration of approximately 1 μM (Plow and Collen, 1981). Inhibition is achieved by formation of an irreversible bimolecular complex with the serine in the catalytic site of plasmin (Wiman and Collen, 1979). Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasminogen activators and is

produced by endothelial cells and hepatocytes. PAI-1 forms complexes with both t-PA and u-PA (Kruithof *et al*, 1986). The fibrinolytic role of PAI-2 and PAI-3 is still uncertain. PAI-2 is synthesised by the placenta and its plasma concentration is notably increased during the third trimester of pregnancy (Lesander and Åstedt, 1986). PAI-2 may be involved in inflammatory reactions and also in remodelling of connective tissue following arterial injury (Belin, 1993). PAI-3 acts as an inhibitor of u-PA in urine (De Munk *et al*, 1994).

The molecular interactions of physiological thrombolysis is locally enhanced, but systemically inhibited. Plasminogen and plasminogen activators are specifically bound to fibrin in the thrombus and fibrin facilitates the conversion of plasminogen to plasmin, by stimulating the release of t-PA from endothelial cells. PAI-1 and α_2-antiplasmin are not effective inhibitors of fibrin bound plasminogen activators and plasmin (Fig. 2.5). On the other hand, systemic fibrinolysis is prevented by the efficient inhibition of circulating plasminogen activator and plasmin by PAI-1 and α_2-antiplasmin, respectively (Francis and Marder, 1995).

Bajzar *et al* (1995) isolated a single chain polypeptide of 60 kDa, which they designated thrombin activatable fibrinolysis inhibitor (TAFI). TAFI appears to restrain fibrinolysis by removing lysine and arginine residues from the C-terminus of fibrin and so limits the cofactor activities of fibrin in plasminogen activation. The thrombin-thrombomodulin complex, which is responsible for activating the coagulation inhibitor protein C (Esmon *et al*, 1982), is also the activator for TAFI (Nesheim *et al*, 1997) as demonstrated in Fig. 2.6. Therefore, TAFI down-regulates both coagulation and fibrinolysis and forms the molecular link between the two processes (Bajzar *et al*, 1995).

Two exogenous activators of fibrinolysis are streptokinase and staphylokinase. Streptokinase is a bacterial protein produced by β-hemolytic streptococci (Davies *et al*, 1964), while staphylokinase is produced by *Staphylococcus aureus* (Collen *et al*, 1993). Both proteins have no enzymatic activity by itself, but form a 1:1

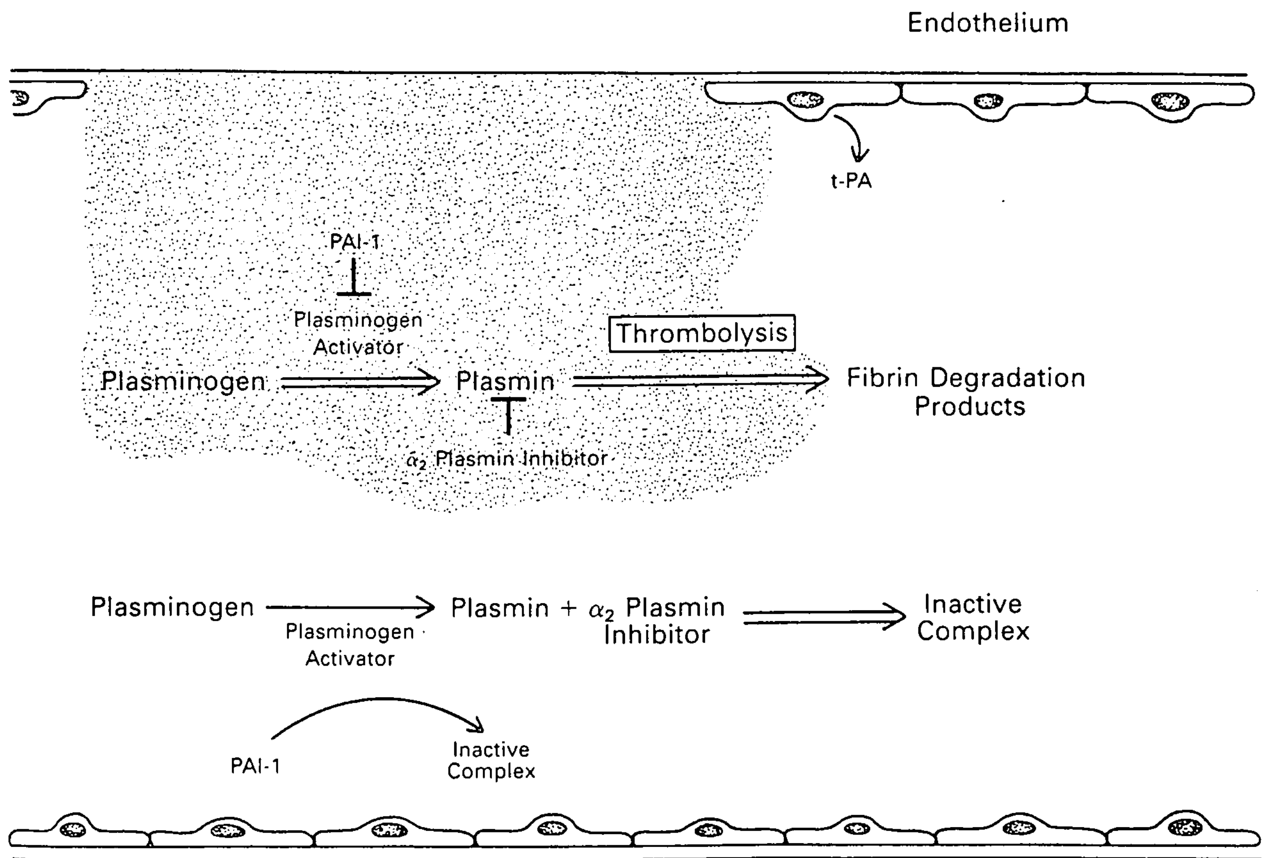

Fig. 2.5 The interactions of physiological thrombolysis in which the process is locally favoured but systemically inhibited. Fibrin bound plasminogen and plasmin is protected from inhibition by PAI-1 and α_2-plasmin inhibitor, respectively. In contrast, unbound plasminogen and plasmin are susceptible to inhibition (Francis and Marder, 1995).

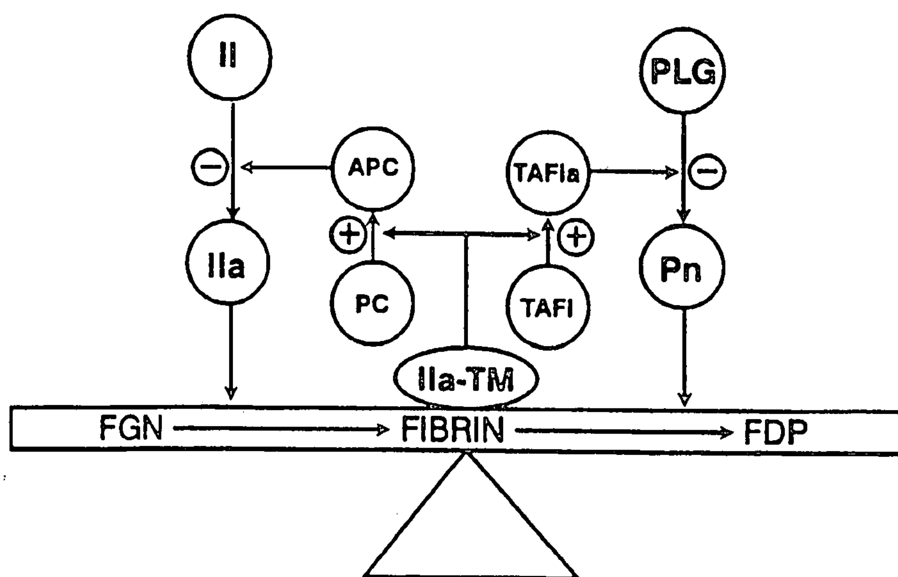

Fig. 2.6 A model showing the molecular link between coagulation and fibrinolysis. The thrombin-thrombomodulin complex activates both protein C and TAFI, which down-regulates the production of thrombin and plasmin, respectively (Nesheim *et al*, 1997).

complex with plasminogen. This complex is capable of converting plasminogen to plasmin (Collen *et al*, 1993). Streptokinase has a low affinity for fibrin (Wohl *et al*, 1978), while staphylokinse is highly fibrin-specific (Collen and Lijnen, 1994).

2.3 NOVEL ANTICOAGULANT AND ANTITHROMBOTIC AGENTS:

The principal *in vivo* inhibitor of thrombin is antithrombin III (AT III), which requires hepariniods to enhance its inactivation of thrombin. When heparin binds to AT III, it causes a conformational change to enhance its accessibility to bind to thrombin (Rosenburg and Damus, 1973). Heparin is widely used as an anticoagulant, but it has several disadvantages. Heparin does not inhibit thrombin-induced platelet activation and it can be inactivated by platelet factor 4, which is released from activated platelets in the vicinity of the thrombus (Harker, 1994). In addition, the heparin-antithrombin III complex cannot inhibit thrombin bound to a thrombus, most likely because it is too large to diffuse into the thrombus (Hogg and Jackson, 1989; Weitz *et al*, 1990). Furthermore, the efficiency of heparin therapy diversifies within and amongst patients and thrombocytopenia develops in 5-10% of patients (Bates, 1997). As a result, we have witnessed a marked increase in the development of new and more potent anticoagulant and antiplatelet agents in the past decade. In this regard, two major avenues have been followed. The first involved coagulation and initially thrombin was targeted. Subsequent developments include antagonists for factor Xa, factor VIIa and tissue factor. The other avenue targeted blood platelets, in particular the GpIIb/IIIa receptor on the platelet membrane. In the case where major pathological abnormalities are related to platelet deposition and not so much to fibrin formation, it may be more desirable to rely on antiplatelet agents than on antithrombins (Coller *et al*, 1991).

As early as the 1950's antithrombotic agents were identified and isolated from bloodsucking animals like ticks, assassin bugs, leeches and horse flies (Markwardt, 1994). It was, however, only during the past ten to fifteen years that such anticoagulant agents could be fully characterised using recombinant DNA

technology. This literature review will focus on some of the antiplatelet and antithrombotic agents found in nature, as well as on synthetic agents developed from these native haemostatic agents.

2.3.1 Inhibition of platelet aggregation:

Some antiplatelet agents prevent the action of a single aggregatory agonist, without affecting the activity of other agonists. For example, aspirin prevents formation of TxA_2, but does not effect platelet aggregation stimulated by other agonists such as ADP or thrombin (Oates *et al*, 1988). Fibrinogen binding to Gp IIb/IIIa is a mutual event during platelet aggregation and is independent of the initiating stimulus. Inhibition of fibrinogen binding to Gp IIb/IIIa is thus a more effective pharmacological approach than prevention of the activity of individual agonists (Foster *et al*, 1994).

The murine monoclonal antibody, 7E3, was the first platelet Gp IIb/IIIa antagonist to be developed (Coller, 1985). It completely inhibited *in vitro* platelet aggregation and prevented thrombosis in animal models of arterial thrombosis and thrombolysis (Hanson *et al*, 1988; Gold *et al*, 1988). The high immunogenicity of the antibody led to the development of a derivative product, a chimaeric monoclonal 7E3 Fab, via recombinant technology. This hybrid molecule consists of mouse-derived variable regions of the original molecule linked to the constant region of human immunoglobulin IgG (Coller *et al*, 1989).

The primary mechanism of c7E3 Fab is to block the Gp IIb/IIIa receptor on activated platelet membranes to prevent adhesive ligands such as fibrinogen and VWF to bind to it. This prevents platelet aggregation and ultimately leads to impaired thrombus formation. Christopoulos *et al* (1993) observed with flow cytometry that c7E3 Fab can move from one platelet to another and can thus be redistributed to new platelets entering the circulation. Additionally, Gp IIb/IIIa receptors have been shown to be involved in platelet adhesion and platelet spreading (Weiss *et al*, 1986) and inhibition can result in a decrease in the release of granular contents due to a lower level of platelet activation. This may in turn result in a decrease in the local concentration of the inhibitors of fibrinolysis,

plasminogen activator inhibitor-1 (PAI-1) and α_2-plasma inhibitor (α_2-PI), which are both released from platelets (Coller, 1996). Another profibrinolytic effect of c7E3 Fab may evolve from its prevention of factor XIIIa to bind to platelets (Cox and Devine, 1994). In addition, factor XIIIa cross-linking of fibrin enhances its resistance to fibrinolysis and factor XIIIa also mediates cross-linking of α_2-PI to fibrin. Furthermore, reducing the number of platelets in a thrombus, due to inhibition of Gp IIb/IIIa, could decrease the local availability of platelet factor XIII. Factor XIIIa cross-linking reactions would thus be reduced even further (Coller, 1997). Therefore, c7E3 Fab has the potential to serve as both an antithrombotic and a profibrinolytic drug.

Another murine monoclonal antibody, MA-16N7C2, inhibits Gp IIb/IIIa function and is the first antibody described with an echistatin-like RGD-containing sequence in the CDR_3-region of its heavy chain (Deckmyn *et al*, 1994). The antibody also recognises the Gp IIb/IIIa complex on resting platelets, but platelet aggregation accelerates binding and increases its affinity for the complex. The antiplatelet activity of MA-16N7C2 was confirmed in a baboon model of platelet-dependent arterial thrombosis. The effects were dose-dependent and long lasting, suggesting that MA-16N7C2 is a potent and long-acting Gp IIb/IIIa inhibitor (Kotzé *et al*, 1995).

In order to produce more powerful fibrinogen receptor antagonists, Foster *et al* (1994) studied the inhibition of platelet aggregation by non-peptidic compounds. They were constructed by replacing the Arg-Gly of RGD by alkyl chains of varying lenghts. The most potent *in vitro* compound was GR91669 {8-[aminoiminomethyl)thio]-L-aspartyl-L-phenylalanine}. In *in vitro* studies GR91669 inhibited marmoset platelet aggregation in whole blood, similarly to inhibition of platelet aggregation in human whole blood. Marmosets were thus chosen as the animal model for *ex vivo* studies. These studies showed that GR91669 inhibited platelet aggregation significantly, but reversibly over a period of a few hours. The short half-life of GR91669 and the lack of activity when taken orally of other peptidic fibrinogen receptor agonists, accentuate the potential of non-peptidic compounds (Foster *et al*, 1994).

The physical characteristics of novel antiplatelet agents found in nature are summarised in Table 2.1. Disintegrins isolated from snake venoms represent a new class of low molecular weight RGD-containing peptides (Niewiarowski *et al*, 1990). A novel platelet aggregation inhibitor, contortrostatin, was isolated from southern copperhead snake venom and it has an apparent molecular weight of 9 kDa (Trikha *et al*, 1990). Dennis *et al* (1990a) described the purification, complete amino acid sequence and biological activity of several snake venom proteins, which are potent Gp IIb/IIIa antagonists and inhibitors of platelet aggregation. These proteins are kistrin from *Agkistrodon rhodostoma*, bitan from *Bitis arietans*, three isoforms of trigramin from *Trimeresusus gramineus* and an isoform of echistatin from *Echis carinatus*. All four peptides consist of between 47 and 83 residues and were able to inhibit platelet aggregation significantly (Dennis *et al*, 1990a).

Kistrin contains six intramolecular disulfide bonds and it binds reversibly to Gp IIb/IIIa in nanomolar concentrations (Dennis *et al*, 1990a). Yasuda and Gold (1991) used kistrin in conjunction with a recombinant tissue-type plasminogen activator in a canine model of coronary artery thrombosis. Kistrin increased both the rate and extent of thrombolysis and prevented reocclusion. Shebuski *et al* (1990) successfully used echistatin in an animal model of thrombosis. Musial *et al* (1990) compared the action of RGDS and four disintegrins from viper venoms (echistatin, flavoridin, albolabrin and bitistatin). Their results confirmed that disintegrins are potential candidates for antiplatelet agents (Musial *et al*, 1990).

Savage *et al* (1990) studied the platelet-binding characteristics of two snake venom-derived proteins, applaggin and echistatin, from *Agkistrodon piscivorus piscivorus* and *Echis carinatus*, respectively. Applaggin is a disulfide-linked homodimer and its RGD-motif is situated at residues 50-52, while echistatin is a single chain, with its RGD-motif at residues 24-26. Both proteins were able to inhibit platelet secretion and aggregation of platelets stimulated by ADP, collagen and human thrombin. A monoclonal antibody LJ-CP3, which inhibits binding of

Table 2.1: Summary of antiplatelet proteins.

Name	Species	Size	Reference	Expression host	Reference
Accutin	*Agkistrodon acutus*	47 residues 7 Cys 5.2 kDa	Yeh *et al* (1998a)	-	-
Albolabrin	*Trimeresurus albolabris*	73 residues	Musial *et al* (1990)	-	-
Applaggin	*Agkistrodon piscivorus piscivorus*	72 residues 17.7 kDa	Savage *et al* (1990)	-	-
Barbourin	*Sistrurus m. barbouri*	73 residues 12 Cys	Scarborough *et al* (1991)	-	-
Basilicin	*Crotalus basilicus*	72 residues	Scarborough *et al* (1993a)	-	-
Bitan	*Bitis arietans*	83 residues	Dennis *et al* (1990a)	-	-
Bitistatin	*Bitis arietans*	84 residues 9 kDa	Musial *et al* (1990)	-	-
Calin	*Hirudo medicinales*	65 kDa	Munro *et al* (1991)	-	-
Cerastin	*Crotalus cerastes cerastes*	73 residues	Scarborough *et al* (1993a)	-	-
Cereberin	*Crotalus viridis cereberus*	72 residues	Scarborough *et al* (1993a)	-	-
Concortrostatin	Southern copperhead snake	70-80 residues 9 kDa	Trika *et al* (1990)	-	-
Cotiarin	*Bothrops cotiara*	72 residues	Scarborough *et al* (1993a)	-	-

Table 2.1 Continued

Name	Species	Size	Reference	Expression host	Reference
Crotatroxin	*Crotalus atrox*	72 residues	Scarborough *et al* (1993a)	-	-
Decorsin	*Macrobdella decora*	39 residues 4.4 kDa	Krezel *et al* (1994)	-	-
Durissin	*Crotalus durissus durissus*	72 residues	Scarborough *et al* (1993a)	-	-
Echistatin	*Echis carinatus*	48 residues 5.4 kDa	Dennis *et al* (1990a)	-	-
Flavoridin	*Trimeresurus flavoviridis*	70 residues	Musial *et al* (1990)	-	-
Flavostatin	*Trimeresurus flavoviridis*	68 residues 12 Cys	Maruyama *et al* (1997)	-	-
Hookworm platelet inhibitor	*Ancylostoma caninum*	15 kDa	Chadderdon and Cappello (1999)		
Jararacin	*Bothrops jararaca*	73 residues	Scarborough *et al* (1993a)	-	-
Kistrin	*Agkistrodon rhodostoma*	68 residues 12 Cys	Dennis *et al* (1990a)	-	-
Lachesin	*Lachesis mutus*	73 residues	Scarborough *et al* (1993a)	-	-
Leech antiplatelet protein	*Haementeria officinalis*	16 kDa	Connolly *et al* (1992)	Yeast	Keller *et al* (1992)
Lutosin	*Crotalus viridis lutosus*	73 residues	Scarborough *et al* (1993a)	-	-
Molossin	*Crotalus molossus molossus*	73 residues	Scarborough *et al* (1993a)	-	-

Table 2.1 Continued

Name	Species	Size	Reference	Expression host	Reference
Moubatin	*Ornithodoros moubata*	17 kDa	Waxman and Connolly (1993)	-	-
Pallidipin	*Triatoma pallidipensis*	19 kDa	Noeske-Jungblut *et al* (1994)	Baby hamster kidney cells	Noeske-Jungblut *et al* (1994)
Trigramin	*Trimeresusus gramineus*	72 residues 7.5 kDa	Dennis *et al* (1990a)	-	-
Viridin	*Crotalus viridis viridis*	71 residues	Scarborough *et al* (1993a)	-	-

RGD-containing proteins to Gp IIb/IIIa, also prevented applaggin binding to platelets. These findings confirm that the binding of the two venom proteins are mediated by an RGD-motif (Savage *et al*, 1990).

Scarborough *et al* (1993a) characterized the disintegrin specificities of eleven different disintegrins isolated from the venoms of the American pit viper genera *Bothrops*, *Crotalus* and *Lachesis*. All disintegrins consisted of 71-73 amino acids, containing twelve highly conserved cysteine residues. These disintegrins all share a high homology of primary amino acid sequence with other peptides in this family of 71-73 residue integrins, such as trigramin (Huang *et al*, 1987; Huang *et al*, 1989), albolabrin (Musial *et al*, 1990), kistrin (Dennis *et al*, 1990a), applaggin (Chao *et al*, 1989) and flavoridin (Musial *et al*, 1990).

The eleven disintegrins studied by Scarborough *et al* (1993a) could be organized in two distinct groups in terms of structure and function. The first group consisted of cerastin, lutosin, crotatroxin and durissin and they had only four amino acid differences in their primary amino acid sequence. All members had identical C-terminal sequences from residues 50-73 and all had an RGDW-sequence at residues 51-54. This group was more potent in preventing fibrinogen binding to Gp IIb/IIIa than inhibiting the binding of vitronectin to $\alpha_v\beta_3$. The second group included molossin, viridin, cereberin, basilicin, lachesin, jararacin and cotiarin. In contrast to the first group, the disintegrin activity of the second group resided in the RGDNP-sequence at residues 51-55 and it was more effective in inhibition of vitronectin binding to $\alpha_v\beta_3$ than fibrinogen binding to Gp II/IIIa. These results indicated that the amino acid sequence immediately downstream from the RGD-sequence play a crucial role in determining integrin specificity and affinity (Scarborough *et al*, 1993a).

Yeh *et al* (1998a) isolated a new disintegrin, accutin, from *Agkistrodon acutus* venom, which contains an RGD-sequence and seven Cys residues at positions highly homologous to other disintegrins. Although accutin did not affect the change in platelet shape caused by thrombin, ADP, collagen or U46619 activation, it did inhibit platelet aggregation stimulated by these agonists. Furthermore, it

prevented binding of the monoclonal antibody 7E3 to activated platelets. Accutin thus belongs to the short chain disintegrin family, acting specifically on the binding epitope of IIb/IIIa, overlapping with that of 7E3 and blocks the receptor for fibrinogen binding (Yeh *et al*, 1998a). In addition, accutin also inhibited binding of 7E3 to integrin $\alpha_v\beta_3$, its receptor on human umbilical vein endothelial cells. Yeh *et al* (1998b) studied the effect of accutin on the binding of other anti-integrin monoclonal antibodies to several other receptors, such as $\alpha_{II}\beta_1$, $\alpha_{III}\beta_1$ and $\alpha_v\beta_1$ on human umbilical vein endothelial cells, but no inhibition was detected. Interestingly, accutin had an unexpected *in vivo* anti-angiogenic effect on ten day old chick embryo cells and it induced apoptotic DNA fragmentation in human umbilical vein endothelial cells. These characteristics give accutin great potential in the field of antimetastatic agents (Yeh *et al*, 1998).

Scarborough *et al* (1991) isolated a novel disintegrin, barbourin, from the venom of *Sistrurus m. barbouri*. In contrast to all other disintegrins, barbourin inhibited Gp II/IIIa binding via a KGD-sequence instead of the RGD-sequence. Scarborough *et al* (1993b) incorporated the KGD-motif on cyclic peptides and optimized the peptides in terms of cyclic ring size, hydrophobic binding site interactions and lysyl side chain function. An optimal display of KGD on cyclic peptides resulted in a high affinity and selectivity for Gp IIb/IIIa, which were virtually identical to that of barbourin. This study demonstrated that the specificity and potency of disintegrins could successfully be mimicked by small cyclic peptides (Scarborough *et al*, 1993b). This synthetic cyclic KGD-heptapeptide, with high affinity and specificity for the Gp IIb/IIIa integrin, was named integrelin. Binding of integrelin inhibits platelet aggregation and prevents thrombosis. It is a rapid-acting and highly potent agent with a short half-life (Charo *et al*, 1992). Tcheng *et al* (1995) performed the first clinical trial of integrelin during a routine, elective, low- and high-risk coronary intervention study. Profound and sustained inhibition of platelet function was achieved (Tcheng *et al*, 1995).

Flavostatin is a novel 68 amino acid disintegrin found in the venom of *Trimeresurus flavoviridis* (Maruyama *et al*, 1997). It contains an RGD-sequence

and twelve conserved Cys residues. Flavostatin effectively inhibited ADP, collagen and thrombin receptor activating peptide (TRAP)-induced platelet aggregation (Maruyama *et al*, 1997).

Decorsin originates from the leech *Macrobdella decora*. It has a single RGD-sequence, which is situated at the apex of an extended loop (Krezel *et al*, 1994). This structural positioning of the RGD-sequence is commonly found amongst disintegrins like kistrin (Adler *et al*, 1991), echistatin and flavoridin (Dennis *et al*, 1993). Decorsin is far more effective in preventing platelet aggregation than the pentapeptide GRGDV. Although it contains an RGD-sequence, decorsin shows only approximately 16% amino acid sequence similarity with other snake venom Gp IIb/IIIa antagonists. Decorsin was the first Gp IIb/IIIa antagonist and inhibitor of platelet aggregation isolated from leeches (Seymour *et al*, 1990).

Connolly *et al* (1992) described a protein that inhibited collagen-induced platelet aggregation. The protein was isolated from *Haementeria officinalis* and the purified protein was designated leech antiplatelet protein (LAPP). According to Keller *et al* (1992), LAPP prevented platelet adhesion to collagen, but had no effect on aggregation when platelets were stimulated by ADP, thrombin, arachidonic acid, U46619 or A23187 (Connolly *et al*, 1992). The gene encoding LAPP was cloned and expressed in yeast (Keller *et al*, 1992), enabling *in vivo* studies in an animal model of thrombosis (Schaffer *et al*, 1993). rLAPP was able to completely inhibit collagen-mediated platelet aggregation, but had no significant effect on the rate and extent of platelet deposition on a collagen surface. In contrast, a peptidyl fibrinogen receptor antagonist, L-366763 (acetylated-Cys-Asn-Pro-Arg-Gly-Asp-Cys-NH_2), completely prevented platelet deposition at the same dosage that inhibited *ex vivo* aggregation. These results demonstrated that inhibition of collagen-induced platelet aggregation by rLAPP alone was not sufficient to prevent platelet-dependent thrombosis in the animal model studied and that other mechanisms are crucial for the development of thrombosis (Schaffer *et al*, 1993).

The medicinal leech, *Hirudo medicinales*, became famous for the production of hirudin, the most potent known thrombin inhibitor in nature (Markwardt, 1970). Interestingly, Munro *et al* (1991) isolated an inhibitor of collagen-mediated platelet adhesion and aggregation from the same leech and the inhibitor was named calin. It inhibits aggregation by rapidly (1-10 min) binding to collagen. However, no cleavage of collagen occurs as in the case of collagenases. Calin's rapid interaction with collagen may explain the prolonged bleeding phenomenon seen after leech bites (Munro *et al*, 1991).

Waxman and Connolly (1993) purified an antiplatelet protein from yet another blood-sucking organism, the soft tick, *Ornithodoros moubata*. The protein was called moubatin and interfered in haemostasis by preventing collagen-stimulated platelet aggregation. The blood-sucking bug, *Triatoma pallidipensis*, also produces an inhibitor of collagen-induced platelet aggregation, called pallidipin (Noeske-Jungblut *et al*, 1994). Interestingly, it had no effect on platelet adhesion to collagen, but inhibited the release of ADP from platelets. No inhibition of aggregation in response to ADP, thrombin, TxA2, mimetic U44619 or phorbol ester was observed. Its gene was cloned from a cDNA library and the recombinant product was produced in baby hamster kidney cells. Recombinant pallidipin had antiplatelet effects identical to those of the native inhibitor (Noeske-Jungblut *et al*, 1994).

Recently, Chadderdon and Cappello (1999) isolated an inhibitor of platelet aggregation and adhesion from adult *Ancylostoma caninum* hookworms. The protein of approximately 15 kDa was named hookworm platelet inhibitor (HPI). HPI blocked platelet aggregation in response to epinephrine, thrombin and ADP. Furthermore, it also inhibited the binding of resting platelets to immobilized fibrinogen and collagen, suggesting interactions with Gp IIb/IIIa and Gp Ia/IIa. Monoclonal antibodies were used to confirm blockade of cell surface integrins Gp IIb/IIIa and Gp Ia/IIa (Chadderdon and Cappello, 1999).

It is clear that there are a vast number of products that can inhibit platelet-dependent thrombogenesis by preventing the binding of inhibitors to Gp IIb/IIIa. Integrelin and c7E3 Fab have been tested in clinical studies where their efficiency

was proven. Results document that Gp IIb/IIIa blockade reduces the incidence of clinically significant ischemic events in the entire spectrum of patients undergoing coronary intervention. It is proposed that in the cases where major pathological abnormalities are related to platelet deposition and not so much to fibrin formation, it may be more desirable to rely on antiplatelet agents than on antithrombins (Coller *et al*, 1991).

2.3.2 Direct inhibition of thrombin:

The development of direct and indirect thrombin inhibitors has led to a new dimension in the management of thrombotic and vascular disorders and had immense implications on drug development research (Fareed *et al*, 1999). De Simone *et al* (1998) schematically compared the binding of fibrinogen to thrombin and the binding of the different classes of direct thrombin inhibitors to thrombin (Fig. 2.7). Table 2.2 summarises the physical characteristics of the direct antithrombins. More detailed descriptions of the design and mode of action of the different direct thrombin inhibitors will follow in the text.

Hirudin is produced by the salivary glands of the leech *Hirudo medicinales* and is the most potent and specific thrombin inhibitor found in nature (Markwardt, 1970). The molecule is a single carbohydrate-free polypeptide and is stabilised by three intramolecular disulphide bridges. It contains a sulphated tyrosine on position 63. Unlike heparin, hirudin does not require any cofactors for its anticoagulant activity. Hirudin is composed of a cysteine-rich amino terminus and an acidic carboxy-terminus. Inhibition of thrombin is accomplished by the formation of a tight 1:1 stoichiometric complex. The 48-amino acid globular N-terminus, which is stabilized by three disulphide bridges, binds within the active site of thrombin (Rydel *et al*, 1990; Rydel *et al*, 1991). The C-terminus, which is a highly acidic region and also contains a sulphated tyrosine at position 63, binds through ionic and hydrophobic interactions to the ABE of thrombin (Rydel *et al*, 1990). Hirudin binds to thrombin in a non-substrate mode (Fig. 2.7F), with the N-terminal tail of hirudin parallel to thrombin segment Ser^{214}-Glu^{217} (Stone and Hofsteenge, 1986; Grütter *et al*, 1990; Rydel *et al*, 1990, Markwardt, 1994). This results in inhibition of both the proteolytic and cellular activities of the enzyme (Fenton, 1989).

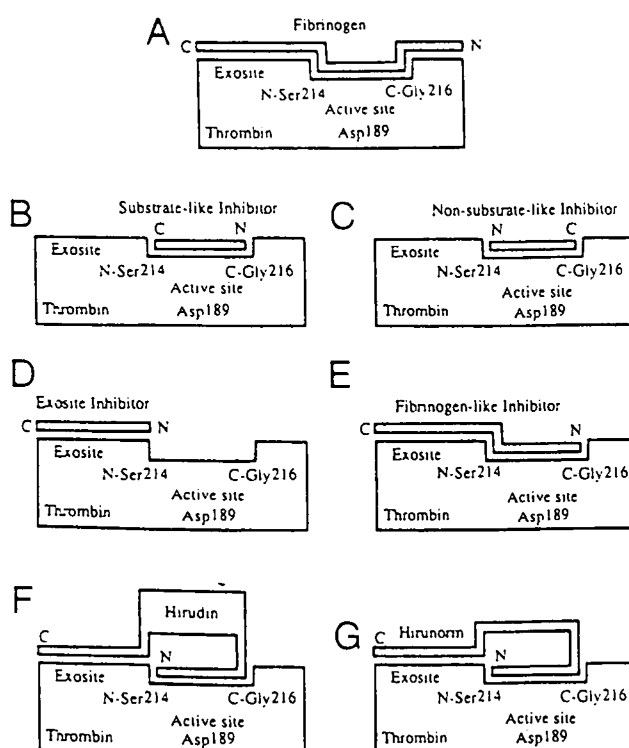

Fig. 2.7 Schematic summary of the different interaction mechanisms between thrombin and its inhibitors (De Simone *et al*, 1998). **A**: Interaction of fibrinogen with ABE and the active site of thrombin. **B**: Peptide-based inhibitors like PPACK interact with the active site in a substrate mode by aligning its backbone (like fibrinogen) in an antiparallel manner to thrombin segment Ser^{214}-Glu^{217}. **C**: Some active site-directed peptidic inhibitors also interact in a nonsubstrate mode and align their backbone in parallel fashion to thrombin segment Ser^{214}-Glu^{217}, eg. BMS-183507 (Iwanowich *et al*, 1994). **D**: ABE-directed inhibitors interact merely with the fibrinogen recognition sequence of thrombin. They include hirugen and hirugen-related peptides, $hirudin^{54-65}$ and $hirullin^{50-62}$. **E**: Multisite-directed inhibitors bind to both the active site and the ABE in an antiparallel manner and are hirulogs, hirulog derivatives and hirutonins. **F**: Hirudin interacts with the ABE and with the active site in a parallel fashion. **G**: Hirunorms bind in a similar manner to thrombin than hirudin.

Table 2.2: Summary of direct antithrombins.

Name	Species	Size	Reference	Expression host	Reference
Bifrudin	*Hirudinaria manillensis*	7 kDa	Electricwala *et al* (1991)	-	-
Bothrojaracin	*Bothrops jararaca*	27 kDa	Zingali *et al* (1993)	COS cells	Arocas *et al* (1997)
Haemadin	*Haemadipsa sylvestris*	57 residues 5 kDa	Stube *et al* (1993)	*Escherichia coli*	Stube *et al* (1993)
Hirudin	*Hirudo medicinales*	65 residues 6 Cys 7 kDa	Markwardt (1970)	*Escherichia coli*	Dodt *et al* (1986)
Hirullin P18	*Hirudinaria manillensis*	61 residues	Krstenansky *et al* (1990)	-	-
Rhodnin	*Rhodnius prolixus*	103 residues 11 kDa	Friedrich *et al* (1993)	*Escherichia coli*	Friedrich *et al* (1993)

Two unique characteristics distinguish hirudin from conventional protease inhibitors. Firstly, most serine protease inhibitors contain a reactive site for interaction with the active site of the target enzyme. In contrast, none of the three lysine residues of hirudin is involved in such an interaction (Braun *et al*, 1988). Secondly, hirudin contains a compact N-terminus, but a disordered C-terminus, in contrast to most other serine protease inhibitors, which are compact molecules (Folkers *et al*, 1989)

Unlike native hirudin, recombinant hirudin lacks a sulphate group on Tyr^{63} (Dodt *et al*, 1984; Dodt *et al*, 1986). As a result, the desulphonated compound has a tenfold-reduced affinity for α-thrombin (Stone and Hofsteenge, 1986). The affinity of r-hirudin for thrombin could, however, be restored to equivalent levels to that of wild type hirudin by introducing phosphotyrosine into position 63 (Hofsteenge *et al*, 1990). A negatively charged Tyr^{63} thus plays a substantial role in determining the affinity of hirudin for thrombin.

Over the last decade, several groups investigated the activity of different hirudin fragments. Schmitz *et al* (1991) found that the N-terminal fragment of hirudin (Hir^{1-47}) inhibited all enzymatic functions of thrombin. Krstenansky and Mao (1987) studied a chemically synthesized unsulphated N^{α}-acetyl-hirudin$^{45-65}$ (Hir^{45-65}). This fragment was able to inhibit blood coagulation and the release of fibrinopeptide A by thrombin, but was unable to inhibit the amidolytic activity of thrombin. This indicates that the C-terminus of hirudin occupies the ABE, which was later confirmed by Dodt *et al* (1990). Schmitz *et al* (1991) showed that Hir^{45-65} inhibits the interaction between thrombin and thrombomodulin. This results in inhibition of the activation of protein C, activation of platelets and endothelial cells, and interactions between thrombin and factors V and VIII. The binding of hirudin to thrombin does not inhibit binding of antithrombin III and thrombin, since antithrombin III does not recognize the anion-binding site (Dennis *et al*, 1990b). Mao *et al* (1988) determined that the shortest C-terminal fragment with inhibitory activity was Hir^{56-65}, while maximum activity was obtained with Hir^{54-65}. The binding of thrombin exosite inhibitors, like Hir^{54-65}, to thrombin is presented in Fig. 7D (Banner and Hadvary, 1991; Stubbs *et al*, 1992; Priestle *et al*, 1993).

Phe^{56} appeared to be crucial for maintaining activity, since replacement with Glu or Leu lead to complete loss of inhibitory activity of hirudin and its fragments. Even when Phe^{56} was replaced with D-Phe to determine conformational requirements, inhibition was also completely lost. Circular dishroism spectra showed that binding of hirudin C-terminal peptides to thrombin lead to significant conformational changes and that loss of thrombin activity might be due to a lack of conformational change in the case of D-Phe (Mao *et al*, 1988).

The leech *Hirudinaria manillensis* belongs to the same family as *Hirudo medicinales* and it produces an antithrombin similar to hirudin, called bifrudin (Electricwala *et al*, 1991). The antithrombotic effect of bufrudin in human plasma had a potency similar to hirudin variant I (Dodt *et al*, 1984) at equivalent dosage. It did, however, not cross-react with monoclonal antibodies directed against recombinant hirudin variant 1. Amino acid sequence analysis showed four differences up to residue 25 between the two antithrombins. These results indicate that bufridin is a potent thrombin inhibitor with biological activity similar to hirudin, but it differs in terms of structural and immunological properties (Electricwala *et al*, 1991).

Naski *et al* (1990) studied the effect of a synthetic *N*-acetylated C-terminus of hirudin, called hirugen. They found that hirugen [Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3)Leu] competitively inhibited the action of α-thrombin towards fibrinogen, but with minimal inhibition of the active site. These results again illustrated the interaction between the C-terminus of hirudin and the anion-binding site of thrombin. The binding of hirugen as exosite inhibitor is shown in Fig. 2.7D (Krstenansky and Mao, 1987; Krstenansky *et al*, 1987; Jakubowski and Maraganore, 1990; Skrzypczak-Junken *et al*, 1991). *In vitro* studies with hirugen revealed that it competitively inhibited fibrinogen cleavage and platelet activation by thrombin (Jakubowski *et al*, 1990; Cadroy *et al*, 1991). Hirugen prevented *ex vivo* platelet deposition in low-shear flow chambers connected to arteriovenous shunts in baboons. However, it failed to affect *ex vivo* platelet deposition on collagen type I-coated tubing (Cadroy *et al*, 1991).

Another hirudin variant produced by *Hirudinaria manillensis* was designated hirullin P18. Like hirudin, it has a highly acidic C-terminus. Phe^{51} is a crucial residue and corresponds to the important Phe^{56} of hirudin. The $hirullin^{50-62}$ peptide (SDFEEFSLDDIEQ) binds thrombin with similar affinity as unsulfated $hirudin^{54-65}$ (Krstenansky et al, 1990). Crystallographic studies showed that the association of residues 51-55 of hirullin with thrombin is similar to that of hirudin and hirugen. The remaining residues also interact with and bind to thrombin, but binding is achieved through a conformational adjustment of the peptide with respect to the conformation of hirudin and hirugen. It causes the side group of Ile^{60} of hirullin to point in the opposite direction of Tyr^{63} of hirudin and hirugen, but allows the residues to interact with the 3_{10} turn of the hydrophobic binding pocket of thrombin. The hydrophobic interaction is thus accomplished through a conformational readjustment (Qiu *et al*, 1993).

Maraganore *et al* (1990) designed a range of antithrombotic peptides called hirulogs. They consist of an active-site specific sequence with an Arg-Pro scissile bond, a polymeric linker of glycyl residues (6 to 18 Å in length) and a sequence for binding to the ABE of thrombin. In contrast to synthetic hirudin C-terminal peptides, hirulogs inhibited both the fibrinogen clotting activity of thrombin and its proteolytic activity. Fig. 2.7E shows the binding of hirulogs to both the active site and the ABE of thrombin in an antiparallel manner (Maraganore *et al*, 1990; Skrzypczak-Jankun *et al*, 1991; Qiu *et al*, 1992).

Hirulog-1 [(D-Phe)-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu] was capable of inhibiting thrombin catalyzed hydrolysis of *p*-nitroanilide at nanomolar concentrations (DiMaio *et al*, 1990; Maraganore *et al*, 1990). The optimal length of the oligoglycyl spacer forming a molecular bridge between the active-site and the ABE sequence appeared to be at least three to four glycyl residues (Maraganore *et al*, 1990). Witting *et al* (1992b) studied the effect of hirulog-B2 on thrombin inhibition. Hirulog-B2 has D-cyclohexylalanine substituted in the first position and is highly specific for binding to the active site and to the ABE of thrombin and could have strong pharmaceutical potential. The efficiency of hirulogs as antithrombotic drugs was clearly demonstrated in animal models of both venous and arterial thrombosis (Maraganore and Adelman, 1996). The slow

cleavage of the Pro-Arg bond by thrombin is however a major limitation. The direct thrombin inhibition by hirulogs is therefore only temporary and in time, hirulogs are converted into less potent hirugen-like peptides (Witting *et al*, 1992a).

A new class of thrombin inhibitors, called hirunorms, was designed to mimic the binding mode of hirudin, without the undesirable substrate-like features of hirulogs. Hirunorm V is a synthetic polypeptide of 26 residues and was constructed to interact with the active site of thrombin through its N-terminus. Its C-terminal domain should bind the ABE of thrombin (Lombardi *et al*, 1996). The two binding regions are divided by a spacer segment of the appropriate length to allow interaction with both the active site and ABE. The interaction between hirunorm and thrombin is shown in Fig. 2.7G (De Simone *et al*, 1998). The molecular structure of hirunorms makes them resistant to proteolysis by thrombin. Hirunorm V showed high antithrombotic activity in different rat models and compared well with hirudin in inhibiting arterial thrombosis (Lombardi *et al*, 1996). The primary structure of hirunorm V is presented and compared to the amino acid sequence of hirudin variant 2 (Harvey *et al*, 1986) in Table 2.3.

Additionally, the activities of other proteases such as plasmin, tPA and trypsin are not affected by hirunorms (Cirillo *et al,* 1996). The hirunorms are also insensitive to plasma proteases and are readily inactivated by rat liver or kidney extracts (Cirillo *et al*, 1996). X-ray crystal structure analysis of hirunorm V showed that its C-terminus interacts with the ABE, similarly to the interaction between hirudin and thrombin (De Simone *et al*, 1998). These crystallographic studies confirm the accuracy of the hirunorm design and provide convincing proof of its interaction as a synthetic thrombin inhibitor with high potency.

In the inhibition of thrombin by hirudin-derivatives, the focus is on the segments inhibiting the active site and ABE. The role of the linker (residues 49-54) was limited to provision of an appropriate spacer between the two inhibitory segments. None of the side chains of the linker contributed significantly to binding and could be replaced without considerably affecting the activity of hirudin (Yue *et al*, 1992).

Table 2.3: Primary structure of hirunorm V and its comparison with hirudin variant 2 (De Simone *et al*, 1998).

Hirudin	**Hirunorm V**
Ile^{1}	Chg^{1}
Thr^{2}	Val^{2}
Tyr^{3}	2-Nal^{3}
Thr^{4}	Thr^{4}
Asp^{5}	Asp^{5}
|	D-Ala^{6}
Globular domain	Gly^{7}
|	β-Ala^{8}
Pro^{48}	Pro^{9}
Glu^{49}	Glu^{10}
Ser^{50}	Ser^{11}
His^{51}	His^{12}
Asn^{52}	h-Phe^{13}
Asn^{53}	Gly^{14}
Gly^{54}	Gly^{15}
Asp^{55}	Asp^{16}
Phe^{56}	Tyr^{17}
Glu^{57}	Glu^{18}
Glu^{58}	Glu^{19}
Ile^{59}	Ile^{20}
Pro^{60}	Pro^{21}
Glu^{61}	Aib^{22}
Glu ^{62}	Aib^{23}
Tyr^{63}	Tyr^{24}
Leu^{64}	Cha^{25}
Gln^{65}	D-Glu^{26}

In complex with α-thrombin, the linker was mainly exposed to the solvent outside of a deep groove between the active site and the ABE. The linker had exceptional conformational flexibility, but no interaction with thrombin (Yue *et al*, 1992). N^{α}-Acetyl[D-Phe^{45},Arg^{47}]hirudin$^{45\text{-}65}$ (P53) is a bivalent thrombin inhibitor with a K_i-value of 5.6 nM (DiMaio et al, 1990). It consists of an active site inhibitor segment, [N^{α}-Acetyl-(dF)PRP], an ABE inhibitor segment, hirudin$^{55\text{-}65}$ (DFEEIPEEYLQ-OH), and a connecting linker, hirudin$^{49\text{-}54}$ (QSHNDG). Szewczuk *et al* (1993) used P53 as a model to study the structure-function relationship of the connecting linker. They used ω-amino acids, which modified the length of the linker as well as the number and location of the peptide bonds. The length of the linker was calculated in terms of the number of atoms participating in the formation of the backbone. No side chains were included in the synthesis of the linker. A minimum of eleven atoms was required to bridge the two inhibitory segments, but the potency of this linker was weak (K_i-value of 26 nM). An inhibitor with a 13-atom linker showed the highest potency with a K_i-value of 0.51 nM. Molecular dynamic simulation of inhibitors with a 13-atom linker suggested that the linker interacted with thrombin through hydrogen bonds. These inhibitors may be reclassified as trivalent rather than bivalent inhibitors (Szewczuk *et al*, 1993). A schematic representation of its interaction with thrombin is shown in Fig.7E (Szewczuk *et al*, 1993).

Hirutonins belong to a family of bifunctional inhibitors containing a noncleavable moiety mimicking the scissile bond (Zdanov *et al*, 1993). Hirutonin-2 is an analog of (D)Phe-Pro-Arg-Gly-hirudin$^{49\text{-}65}$. The crystal structure of the thrombin-hirutonin-2 complex shows that the C- and N-terminal segments are well ordered, while the linker is partially disordered. Hirutonin-6 has the same N-terminus as hirutonin-2, connected to a shortened ABE binding segment by a short non-peptidyl linker. The linker is situated near die bottom of the groove connecting the active site and the ABE, forming a short antiparallel β-sheet-like organisation with Leu^{40}-Leu^{41} and interacting with Glu^{39}-Leu^{40}-Leu^{41} via van der Waals forces. The N-terminal fragment of the two hirutonins binds thrombin similarly to other inhibitors with this motif, while binding of the C-terminal fragments to the ABE are similar to that of hirudin and hirulogs. Interaction between thrombin and hirutonins are shown in Fig 2.7E (Zdanov *et al*, 1993).

A slow tight-binding thrombin inhibitor, haemadin, was isolated from the Indian leech, *Haemadipsa sylvestris* (Strube *et al*, 1993). Haemadin is highly thrombin specific, with no inhibition of other proteases like trypsin, chymotrypsin, factor Xa and plasmin. The N-terminus of haemadin shows no sequence similarity with hirudin, but six of the twelve C-terminal residues are identical to those of hirudin. In contrast to hirudin, haemadin does lack a tyrosine residue in its C-terminus. The haemadin gene was cloned and expressed in *E. coli*. Recombinant haemadin displayed a similar inhibition constant and specific activity than native haemadin, suggesting that posttranslational modifications are not essential for its activity (Strube et al, 1993).

Bothrojaracin is a potent thrombin inhibitor that was isolated from the venom of *Bothrops jararaca*. This unique thrombin inhibitor does not bind to the active site, but interacts with both anion-binding exosites 1 and 2 (Zingali *et al*, 1993). Bothrojaracin has two disulfide-linked polypeptides, chains A and B with molecular masses of 15 kDa and 13 kDa, respectively. In contrast to other ligands, which recognise exosite 1, bothrojaracin lacks an acidic sequence similar to the C-terminal tail of hirudin. Arocas *et al* (1997) used COS cells to express functional bothrojaracin, which was able to bind to and inhibit the function of thrombin.

A highly specific thrombin inhibitor was isolated from the assassin bug, *Rhodnius prolixus,* and was named rhodniin (Friedrich *et al*, 1993). It displays an internal sequence homology of residues 6-48 with residues 57-101, indicating a two-domain structure. Rhodniin forms a 1:1 complex with thrombin. Domain 1 binds to the active site of thrombin, with His^{10} pointing into the specificity pocket. The cDNA of rhodnin could be cloned and expression in *E. coli* yielded a potent recombinant thrombin inhibitor (Friedrich *et al*, 1993).

D-Phenyl-L-Prolyl-L-Arginyl-chloromethylketone (PPACK) is a specific inhibitor of the catalytic site of thrombin. The effect is brought about by irreversible alkylation of the active site histidine (Kettner and Shaw, 1979). Bode *et al* (1989) did crystallographic studies to investigate the formation of the stoichiometric complex between human α-thrombin and PPACK. They found that the exceptional

specificity of PPACK could be explained by a hydrophobic cage in thrombin, formed by Ile^{174}, Trp^{215}, Leu^{99}, His^{57}, $Tyr^{60}A$ and $Trp^{60}D$. PPACK interacts with the active site of thrombin in a substrate mode by aligning its backbone in an antiparallel manner to thrombin segment Ser^{214}-Glu^{217} (Bode *et al*, 1989) as illustrated in Fig. 2.7B. This interaction is similar to that of fibrinogen, which is also in a substrate mode (Fig. 2.7A).

Lumsden *et al* (1993) investigated the effect of PPACK on thrombosis following endarterectomy in baboons. Continuous intravenous infusion of PPACK (100 nmol/kg/min) permanently interrupted thrombosis, by irreversibly inactivating the thrombin that was bound to the thrombus. Infusion was started after surgical haemostasis was established to avoid abnormal surgical bleeding. These safe and lasting characteristics make PPACK an attractive candidate for use in mechanical interventional vascular procedures applied in the management of symptomatical arterial disease in humans (Lumsden et al, 1993).

2.3.3 Indirect inhibition of thrombin:

Thrombin generation is not affected by direct antithrombins and some thrombin molecules may escape inhibition. This can, however, be prevented by inhibition of factor Xa and thus disruption of the thrombin feedback loop. The physical characteristics of the indirect thrombin inhibitors are summarised in Table 2.4.

Waxman *et al* (1990) isolated a factor Xa inhibitor form the soft tick, *Ornithodoros moubata*. The inhibitor has a single chain and was designated tick anticoagulant peptide (TAP). Interaction between TAP and factor Xa involves initial low-affinity binding to an exosite followed by high-affinity binding to the catalytic site of factor Xa (Vlasuk *et al*, 1991). TAP was superior to heparin in the prevention of venous thrombi in a rabbit model (Vlasuk *et al*, 1991) and also in preventing heparin-resistant platelet thrombi in a primate model of arteriovenous shunts grafted with collagen and endarterectomised carotids (Schaffer *et al*, 1991; Kotzé *et al*, 1997). Biologically active recombinant TAP (rTAP) was produced in the yeast *Saccharomyces cerevisiae*. Native TAP and rTAP have the same amino acid

Table 2.4: Summary of indirect antithrombins.

Name	Species	Size	Reference	Expression host	Reference
Annexin V	*Homo sapiens* placenta	319 residues 36.5 kDa	Funakoshi *et al* (1987a)	*Escherichia coli*	Thiagarajan and Benedict (1997)
Antistasin	*Haementeria officinalis*	119 residues 15 kDa	Nutt *et al* (1988)	Insect baculovirus	Nutt *et al* (1991)
Protein C	*Homo sapiens*	62 kDa	Esmon *et al* (1982)	AV12 cell line (Syrian hamster tumor cells)	Ehrlich *et al* (1989)
Tick anticoagulant peptide	*Ornithodoros moubata*	60 residues 6.8 k Da	Waxman *et al* (1990)	Yeast	Neeper et al (1990)

composition, primary structure, electrophoretic mobility and inhibition of factor Xa (Neeper et al, 1990). Due to its small molecular size, TAP is a weak immunogen, which makes it an attractive option for the prevention and treatment of thrombosis (Verstraete and Zoldhelyi, 1995).

The Mexican leech *Haementeria officinalis* produces a tight-binding and highly specific inhibitor of factor Xa, which was purified to homogeneity and called antistasin (Dunwiddie et al, 1993). Antistasin has a high cysteine content (Nutt *et al*, 1988). Recombinant antistasin was produced in an insect cell baculovirus host vector system (Nutt *et al*, 1991). Antistasin has been studied thoroughly in various thrombosis models and found to be superior to heparin in preventing platelet-rich thrombi in dacron-grafted arteriovenous femoral grafts (Schaffer et al, 1992). Clinical development of antistasin is, however, highly unlikely, because of its strong immunogenicity, (Verstraete and Zoldhelyi, 1995). A similar factor Xa inhibitor was isolated from *Haementeria ghilianii* (Condra *et al*, 1989). A factor Xa inhibitor was also found in the hookworm, *Ancylostoma duodenalis* (Capello *et al*, 1993).

Annexins are a family of calcium-dependent anionic-phospholipid-binding proteins (Barton *et al*, 1991). Annexin V was originally isolated from the placenta and characterised as placental anticoagulant protein I (Funakoshi *et al*, 1987a; Funakoshi *et al*, 1987b). It binds to anionic phospholipid on the platelet membrane and prevents the binding of factors Xa and Va to platelets (Thiagarajan and Tait, 1990; Thiagarajan and Tait, 1991). Annexin V was used effectively as inhibitor of thrombosis in a venous thrombosis model (Romoisch *et al*, 1991; Van Ryn McKenna *et al*, 1993; Van Heerde *et al*, 1994). Thiagarajan and Benedict (1997) successfully used recombinant annexin V as an inhibitor of arterial thrombosis in a rabbit carotid artery injury model. Intravenous infusion of annexin V significantly inhibited arterial thrombosis, without impairing the haemostatic response (Thiagarajan and Benedict, 1997).

Protein C is a human coagulation factor inhibitor and is involved in the down-regulation of thrombin production. Thrombin, bound to thrombomodulin on the endothelium surface, activates protein C (Esmon *et al*, 1982). Activated protein C

(APC) requires protein S as a co-factor and inhibits thrombin production by inhibiting coagulation factors Va and VIIIa (Scully, 1992). Gruber *et al* (1989) investigated the *in vivo* antithrombotic properties of APC in a baboon model of thrombus formation on prosthetic vascular grafts. Infusion of human APC led to inhibition of clotting as measured by aPTT and to reduction of vascular graft platelet deposition as determined by the real-time scintillation camera imaging of ^{111}In-labeled platelet deposition. Template bleeding times showed that haemostatic plug formation remained normal. Gruber *et al* (1990) proved in a primate model of arterial thrombosis that recombinant activated protein C (rAPC), like human plasma-derived APC, inhibited thrombus formation without impairing primary haemostasis. Hanson *et al* (1993) presented a novel and effective antithrombotic strategy by the infusion of thrombin to activate endogenous protein C in a baboon model. Gruber *et al* (1993) observed that APC levels are elevated during thrombolytic therapy and that this may help to prevent reocclusion during or after thrombolysis (Gruber *et al*, 1993). In 1994 Gruber *et al* observed that APC indeed reduced the diameter and relative number of fibrin fibers in plasma clots. Protein C is thus also involved in enhancing the efficacy of thrombolysis (Gruber *et al*, 1994).

In conclusion, there are many direct and indirect thrombin inhibitors that inhibit thrombin with different potency. One must always keep in mind that complete inhibition of thrombin can lead to the development of a bleeding tendency, which may in itself be as dangerous to the patient as thrombosis. It was therefore suggested that a mild to medium strength antithrombotic may be the best choice (Fenton, 1998).

2.3.4 Activation of fibrinolysis:

Fibrinolysis is an essential process to dissolve fibrin clots and to prevent excessive fibrin formation that can disrupt normal blood flow. Several profibrinolytic proteins have been studied intensively to assess their potential as therapeutic agents. The physical characteristics of these profibrinolytic proteins are summarized in Table 2.5.

Table 2.5: Summary of fibrinolytic proteins.

Name	Species	Size	Reference	Expression host	Reference
Bat plasminogen activator (Bat-PA)	*Desmodus rotundus*	49 kDa	Gardell *et al* (1989)	*Escherichia coli*	Gardell *et al* (1989)
Hemetin	*Haementaria ghilianii*	120 kDa	Malinconico *et al* (1984)	-	-
Plasminogen activator	*Agkistrodon halys*	32 kDa	Park *et al* (1998)	Baculovirus	Park *et al* (1998)
Staphylokinase	*Staphylococcus aureus*	136 residues 15.5 kDa	Collen *et al* (1993)	*Escherichia coli*	Sako (1985)
Streptokinase	*Streptococci* strains	48 kDa	Cederholm-Williams *et al* (1979)	-	-
Tissue-type plasminogen activator	*Homo sapiens*	530 residues 68 kDa	Pennica *et al* (1983)	*Escherichia coli*	Pennica *et al* (1983)
Urokinase-type plasminogen activator	*Homo sapiens*	411 residues 54 kDa	Husain *et al* (1983)	*Escherichia coli*	Zamarron *et al* (1984)

Two bacterial plasminogen activators, streptokinase and staphylokinase, are currently intensively researched to determine their suitability as thrombolytic agents. Streptokinase, produced by *Streptococci*, forms a 1:1 stoichiometric complex with plasminogen, which is in turn capable of converting other plasminogen molecules to plasmin. The streptokinase-plasminogen complex is resistant to inhibition by circulating α2-antiplasmin (Cederholm-Williams *et al*, 1979). Streptokinase causes temporary hypertension in many patients and significant allergic reactions in some patients. It is also highly antigenic, which makes repeated administration undesirable (Collen, 1997).

Staphylokinase is produced by certain strains of *Staphylococcus aureus*. Like streptokinase, staphylokinase forms a 1:1 stoichiometric complex with plasminogen. Unlike the streptokinase-plasminogen complex, the staphylokinase-plasminogen complex is inactive and requires activation to staphylokinase-plasmin. Activation of the plasminogen part of the molecule results in exposure of the active site and conversion of the complex to an effective plasminogen activator (Collen *et al*, 1993). In the absence of fibrin, the complex is rapidly degraded by α_2-antiplasmin (Lijnen *et al*, 1991), which is also in contrast to the resistant streptokinase-plamin(ogen) complex. Staphylokinase molecules released from the complex can be recycled to interact with other plasminogen molecules (Silence *et al*, 1993a). However, in the presence of fibrin, the inhibition rate is more than 100-fold reduced, making staphylokinase highly fibrin-specific (Lijnen *et al*, 1992; Silence *et al*, 1993b).

Staphylokinase does not activate plasminogen in the absence of fibrin, probably due to a weak affinity between plasminogen and staphylokinase in plasma (Sakharov *et al*, 1996). Furthermore, α_2-antiplasmin inhibits the generation of active staphylokinase-plasmin complexes. Trace amounts of plasmin are found at the fibrin surface and it forms an active staphylokinase-plasminogen complex. This complex is bound to fibrin via lysine binding sites on the plasmin molecule and it is thus protected from rapid inhibition by α_2-antiplasmin. Following digestion of the fibrin clot, the complex is released and inhibited. This leads to prevention of further plasminogen activation (Collen, 1997).

The tertiary structure of staphylokinase has been elucidated by Rabijns *et al* (1997). It has a flattened structure composed of a mixed five-stranded β-sheet, packed on a single α-helix of twelve residues. Mutagenesis of staphylokinase by Silence *et al* (1995) showed that two pairs of charged amino acids (46:50 and 65:69) are crucial for its interaction with plasmin. These four amino acids map to the side of the molecule, comprising the α-helix. Functional staphylokinase:plasmin complex formation also depends on a cluster of charged amino acids at the N-terminus (residues 11-14), which extends from the same side as the two pairs of charged amino acids (Silence *et al*, 1995). Furthermore, activation of plasminogen by the staphylokinase:plasmin complex requires processing of staphylokinase by plasmin. Ten N-terminal residues are removed by plasmin leading to the exposure of Lys^{11} as the new amino terminus (Schlott *et al*, 1997). Mutagenesis of the catalytic domain of plasmin showed that Arg^{719} mediates complex-formation and the plasminogen activation potential of staphylokinase:plasmin (Jespers *et al*, 1998). This critical residue is situated in an extended loop at the western edge of the active-site cleft (Lamba *et al*, 1996).

Jespers *et al* (1999) used alanine-scanning mutagenensis and phage display to study the structural and functional basis of plasminogen activation by staphylokinase. For the purpose of this study, the staphylokinase:μ-plasmin complex was investigated to deduce a coherent docking model of the crystal structure of staphylokinase on the homology-based model of μ-plasmin, the catalytic domain of plasmin. Staphylokinase binding is mediated by two surface-exposed loops, 174 and 215, at the edge of the active site cleft of μ-plasmin. The binding epitope of staphylokinase involves the N-terminus and the five-stranded mixed β-sheet. The α-helix and the β2 strand do not participate in the binding to μ-plasmin, but are essential to induce plasminogen activation by the staphylokinase:μ-plasmin complex. A topologically distinct activation epitope thus exists. Binding of staphylokinase to the catalytic domain of plasmin allows protrusion of the activation epitope into a broad groove near the catalytic domain. This process generates a competent binding pocket for μ-plasminogen, which buries approximately 2500 Å of the staphylokinase:μ-plasmin complex upon

binding (Jespers *et al*, 1999). While the manuscript of Jespers *et al* (1999) was in preparation, this deduced staphylokinase:μ-plasmin:μ-plasmin complex was fully confirmed by X-ray crystallography (Parry *et al*, 1998).

Staphylokinase has been studied intensively in several animal models and in humans (Collen and Lijnen, 1994). Like streptokinase, staphylokinase is also highly immunogenic, which precludes repeated administration. Staphylokinase contains three non-overlapping immunodominant epitopes. Two of these epitopes could be eliminated by site-directed mutagenesis, where clusters of two to three charged amino acids were replaced with alanine, (Collen *et al*, 1996a). The variants SakSTAR.M38 (Lys^{35}, Glu^{38}, Lys^{74}, Glu^{75} and Arg^{77} substituted by Ala) and SakSTAR.M89 (Lys^{74}, Glu^{75}, Arg^{77}, Glu^{80} and Asp^{82} substituted by Ala) were thrombolytically active and induced significantly less antibody formation than the wild type staphylokinase. This was found in animal models and in patients with peripheral arterial occlusion (Collen *et al*, 1996b). These results indicate that staphylokinase can be manipulated to produce a less immunogenic protein which has potential clinical value as a thrombolytic agent.

Tissue-type plasminogen activator (t-PA) is a human fibrinolytic protein and is mainly produced by the endothelial cells. t-PA is synthesized as a single chain molecule of 530 amino acids and has a molecular weight of 68 kDa (Pennica *et al*, 1983). It consists of a finger domain, an epidermal growth factor domain, two kringle domains and a catalytic domain. t-PA activates plasminogen by cleavage of the Arg^{561}-Val^{562} bond. The single chain form is converted to a two-chain form by plasmic cleavage of the Arg^{278}-Ile^{279} bond (Ichinose *et al*, 1984), which increases its fibrin-specificity (Husain *et al*, 1989). Plasminogen activation is thus localized to sites of vascular injury. The fibrin specificity of t-PA is situated in its kringle domains and finger domain (Pennica *et al*, 1983). The binding site of t-PA on fibrin has been localized to residues 149-161 (Bosma *et al*, 1988), although other evidence indicates that t-PA binds to both the D and E domains of fibrin (Hasan *et al*, 1992).

A single-chain variant (alteplase) and a double-chain variant (duteplase) of t-PA has been produced (Collen, 1997). A deletion mutant of t-PA, consisting of the kringle 2 and protease domain, was constructed and administered as a bolus for coronary artery thrombolysis in patients with acute myocardial infarction. This variant was found to be equipotent to streptokinase (INJECT Investigators, 1995). Potent mutants were also constructed by substitution or deletion of one or a few selected amino acids. TNK-rt-PA, in which Thr^{103} was replaced with Asn, Asn^{117} with Gln, and Lys^{296}-His-Arg-Arg with Ala-Ala-Ala-Ala, was found to have an 8-fold slower clearance and a 200-fold enhanced resistance to PAI-1. This mutant had an increased potency on platelet-rich clots and was more effective upon bolus administration at a lower dose that rt-PA in *in vivo* models (Keyt et al, 1994; Collen et al, 1994).

Urokinase-type plasminogen activator (u-PA) is found in large amounts in urine. u-PA contains 411 amino acids and has a molecular weight of 55 kDa (Husain *et al*, 1983). Like t-PA it also cleaves the Arg^{561}-Val^{562} bond of plasminogen. It has structural similarities to t-PA, but contains only one kringle domain and lacks the finger domain. The single chain form can be rapidly converted to the two-chain form by plamin or kallikrein (Zamarron *et al*, 1984; Ichinose *et al*, 1986). The two-chain forms have greater enzymatic activity, but are less fibrin-specific than the single-chain form (Zamarron *et al*, 1984). Both two-chain forms are inhibited by PAI-1 and PAI-2, while the single chain form is not (Stump *et al*, 1986; Kruithof *et al*, 1986). u-PA has been successfully used to treat thrombotic disorders, like venous thrombosis, pulmonary embolism, arterial thrombosis, acute myocardial infarction and acute reocclusion following percutaneous transluminal angioplasty (Loscalzo, .19

Park *et al* (1998) studied a novel plasminogen activator from *Agkistrodon halys* venom. First, they constructed a cDNA library and used a probe, based on the consensus sequence of serine protease from snake venom, for screening purposes. A positive clone was successfully expressed in a baculovirus system as a 32 kDa protein and was purified to homogeneity. Western analysis indicated that the band was indeed a snake venom component. The recombinant protein enhanced fibrinolysis by activation of plasminogen (Park *et al*, 1998).

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The Amazon leech, *Haementaria ghilianii*, produces a fibrinolytic enzyme called hemetin. It is a metalloprotease, which does not inhibit other coagulation factors. Hemetin is also not inactivated by plasma protein inhibitors (Malinconico *et al*, 1984).

A family of closely related plasminogen activators was isolated from the saliva of the vampire bat, *Desmodus rotundus* (Gardell *et al*, 1989; Gardell *et al*, 1990). They exist as three major forms with structural similarities to human t-PA. They seem to be more fibrin selective and more potent than human t-PA (Gardell *et al*, 1991; Kratschmar *et al*, 1991).

2.3.5 Combination proteins:

In some cases it is preferable to combine different activities in a single molecule to target different levels of haemostasis. Such a multifunctional agent could have enhanced efficacy and so lead to more effective treatment of a patient.

Knapp et al (1992) designed a novel recombinant protein with combined antithrombotic and antiplatelet activity. The disintegrin activity was obtained by placing an RGD-sequence at the tip of a finger-like loop of hirudin. Native hirudin contains SDGE at the tip of the protruding finger (residues 32-35). Variants were obtained by replacing SDGE with RGDS to obtain hirudisin and KGDS to obtain hirudisin-1. Inhibition studies indicated that hirudisin is a 2-fold more potent thrombin inhibitor than hirudisin-1 or r-hirudin. Hirudisin also prevented ADP-induced platelet aggregation. Hirudisin is an important example of the success of novel proteins with combined antithrombotic and antiplatelet activities.

Smith *et al* (1995) utilized the strategy of loop grafting, in which the amino acid sequence of a biologically active, flexible loop on one protein is used to replace a surface loop on an unrelated protein. HCDR3 from Fab-9, an antibody selected to bind the β_3-integrins with nanomolar affinity (Smith *et al*, 1994), was grafted onto the epidermal growth factor-like module of human t-PA. This variant of t-PA (LG-t-PA) was cloned and expressed in COS cells. LG-t-PA bound to platelet integrin

$\alpha_{IIb}\beta_3$ with nanomolar affinity, maintaining full enzymatic activity and was normally stimulated by its physiological co-factor fibrin. Like the donor antibody, binding of LG-t-PA to platelet integrin $\alpha_{IIb}\beta_3$ was dependent on divalent cations and was inhibited by an RGD-containing peptide, indicating that LG-t-PA binds specifically to the ligand binding site of the integrin (Smith *et al*, 1995). The use of combined profibrinolytic and antiplatelet activity can therefore enhance the fibrin-specificity of the thrombolytic agent.

Riesbeck *et al* (1998) fused a HLA class I leader sequence with hirudin, linked to domains three and four of human CD4 and the intracytoplasmic sequence of either CD4 or human P-selectin. Mouse fibroblasts, Chinese hamster ovary (CHO)-K1 cells, immortalized porcine endothelial cells (IPECs) and a pituitary secrectory cell line (D16/16) were transfected with the hirudin fusion constructs. Hirudin was expressed at the cell surface, where it bound thrombin and prevented the formation of fibrin in an *in vitro* assay using human plasma. Hirudin-CD4-P-selectin fusion proteins accumulated in storage granules of secretory cells. When activated with phorbol ester, the fusion proteins were relocated to the cell surface, where the exposed hirudin was fully active. These fusion proteins can be used proactively in situations where thrombotic complications are anticipated, such as vascular surgery and transplantation. Constitutive hirudin expression would be absent on normal endothelial cells, but could be rapidly expressed when the complement system is activated. This approach is designed to inhibit trace amounts of thrombin generation before clot formation (Riesbeck *et al*, 1998).

A monoclonal antibody, 59D8, recognises an epitope on fibrin that is only exposed after thrombin cleaves the β-chain of fibrinogen to release fibrinopeptide B (Hui *et al*, 1983). Bode *et al* (1994) utilised this exclusive fibrin-binding property and covalently linked recombinant hirudin to the Fab' of 59D8. The resultant fibrin-targeting molecule has a molecular weight of approximately 57 kDa, which corresponds to a 1:1 molar ratio of hirudin and 59D8Fab'. Hirudin-59D8Fab' was 10 times more effective than hirudin to inhibit fibrin deposition onto experimental clot surfaces in fibrin solution and human plasma. Furthermore, it inhibited

peptidolytic activity more effectively than free hirudin. Therefore, fibrin-targeting can thus greatly increase antithrombotic activity of known antithrombotic drugs (Bode *et al*, 1994).

Lijnen *et al* (1995) fused a C-terminal fragment (Ser^{47}-Leu^{411}) of recombinant single chain urokinase-type plasminogen activator (rscu-PA) to the C-terminus of hirudin (Asn^{53}-Gln^{65}) via a fourteen amino acid linker to achieve combined fibrinolytic and antithrombotic potential. The recombinant chimeric protein was produced in *E. coli.* The fibrinolytic potency of the chimera in plasma was maintained, when compared to the truncated form of rscu-PA and wild-type scu-PA. The chimera led to prolongation of thrombin time in normal human plasma and inhibited thrombin induced platelet aggregation. The amidolytic activity of thrombin remained unchanged, since the chimera left the active site of thrombin unblocked and available for small synthetic substrates. Their results also indicated that a higher concentration of the chimera was required for inhibition of coagulation than for fibrin clot lysis or for inhibition of thrombin-induced platelet aggregation. Therapeutically effective concentrations for clot lysis and prevention of platelet aggregation may thus be reached without influencing the systemic blood coagulation system (Lijnen *et al*, 1995).

Szarka *et al* (1999) analysed the suitability of staphylokinase as a fusion protein by fusing it to hirudin. Both N-and C-terminal fusions were constructed and the recombinants were expressed in *Bacillus subtilis*. Removal of the first ten amino acids by plasmin caused release and subsequent instability of the N-terminal fusion proteins in the presence of plasmin. Site-directed mutagenesis was performed at Lys^{10} and Lys^{11} to produce a plasmin-resistant variant. It could, however, not be done without interference with activation of staphylokinase by plasmin. Two putative plasmin cleavage sites (Lys^{135} and Lys^{136}) are located at the C-terminus of staphylokinase. However, both these sites are resistant to plasmin cleavage, leading to the production of stable C-terminal fusion. Staphylokinase activity in terms of plasminogen activation were tested using a chromogenic substrate for plasmin. Activity of the C-terminal fusion was

indistinguishable from that of wild-type staphylokinase. Staphylokinase is thus a potential protein to use in a fusion for the addition of fibrinolytic activity to antithrombotic agents (Szarka *et al*, 1999).

An interesting approach to inhibit thrombin generation was followed by Riesbeck *et al* (1997), where a recombinant tissue factor pathway inhibitor (TFPI) was utilised to inhibit FXa. Full length or a truncated TFPI, lacking the third Kunitz domain, was fused to domains three and four and the carboxy-terminal of human CD4. The fusion proteins were expressed in a mouse fibroblast cell line and both proteins were tethered to the cell surface, where they were able to bind FXa. Inhibition of FXa activity was verified using a chromogenic assay. Genetic manipulation of endothelial cells to express functional TFPI may inhibit the development of coronary artery heart disease following cardiac allotransplantation and may inhibit thrombosis in the context of xenotransplantation (Riesbeck *et al*, 1997).

Recently, a modified hirutonin was introduced. An Asp-Ser sequence was inserted between the active site blocking moiety and the anion binding site moiety of hirutonin (LeBlond *et al*, 1999). When washed platelets were stimulated with thrombin receptor activated peptide, the modified hirutonin displayed disintegrin activity and antithrombin acitivity was maintained. However, when platelets in platelet rich plasma was stimulated with ADP, no disintegrin activity was observed (Van Wyk et al, unpublished observations).

It is evident that the combination of antithrombin and fibrinolytic or antithrombin and disintegrin activity into one peptide can be done successfully. It is too early to assess whether this approach will be a success, since very few of these peptides were tested *in vivo*. In addition, the modified peptide usually lost some of the activity of the parent molecule and also some of the activity of the molecule that was added. It is therefore still an open question whether the less active combination peptide will be as effective as the more specific parent molecules.

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CHAPTER 3

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THROMBOSIS
RESEARCH

REGULAR ARTICLE

Production of a Recombinant Antithrombotic and Fibrinolytic Protein, PLATSAK, in Escherichia coli

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Abstract

The three main components involved in thrombosis and haemostasis are thrombin, platelets, and plasmin. Almost all inhibitors of thrombosis are focused either on the inhibition of thrombin or on the inhibition of platelets. We designed a construct using the fibrinolytic activity of staphylokinase, fused via a cleavable linker to an antithrombotic peptide of 29 amino acids. The peptide was designed to include three inhibitory regions: (1) the Arg-Gly-Asp (RGD) amino acid sequence to prevent fibrinogen binding to platelets; (2) a part of fibrinopeptide A, an inhibitor of thrombin; and (3) the tail of hirudin, a potent direct antithrombin. The amino acid sequence of the 29 amino acid peptide was reverse translated, and the gene was chemically synthesised and cloned into an expression vector as a 3′ fusion to the staphylokinase gene. Gene expression was induced in E. coli Top 10 cells and the fusion protein, designated PLATSAK, was purified using metal affinity chromatography. The purified fusion protein significantly lengthened the activated partial thromboplastin time and thrombin time and inhibited the amidolytic activity of thrombin. The fibrinolytic activity was almost equal to that of recombinant staphylokinase as measured with a thrombelastograph. Platelet aggregation was not markedly inhibited by PLATSAK, probably due to the unfavourable three dimensional structure, with the Arg-Gly-Asp sequence buried inside. Our results confirm that it is feasible to design and produce a hybrid multifunctional protein that targets various components of the haemostatic process. © 1998 Elsevier Science Ltd.

Key Words: Staphylokinase; Antiplatelet; Antithrombin; Recombinant

Platelets and thrombin both play pivotal roles in thrombogenesis whereas plasmin dissolves the thrombus. It is therefore not surprising that we have experienced a drastic increase in the design and development of new antithrombotic agents that can either inhibit the action of thrombin or prevent platelet aggregation, and fibrinolytic agents that can enhance fibrinolysis. There are potent new inhibitors of platelet function, whereas powerful inhibitors of thrombin function and production are currently under study. The platelet inhibitors include synthetic Arg-Gly-Asp (RGD)-peptides [1,2], snake venoms [3,4], and the $F(ab')_2$ fragment of the monoclonal antibody 7E3 [5]. Amongst the thrombin inhibitors are hirudin [6], hirudin fragments [7], the hirulogs [8], hirugen [9], and synthetic hirudin C-terminal peptides [10]. Inhibitors of thrombin production include recombinant tick anticoagulant peptide [11] and recombi-

Abbreviations: SAK, staphylokinase; Pr, primer; FXa, factor Xa; TT, thrombin time; aPTT, activated partial thromboplastin time; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; NTA, nitrilo-tri-acetic acid; ADP, adenosine diphosphate.

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nant activated protein C [12]. Collen and Lijnen [13] suggested that the effectivity of anticoagulant and antiplatelet agents could be enhanced by conjunctive use with thrombolytic agents like staphylokinase (SAK) or streptokinase. SAK is a 136 amino acid fibrinolytic protein produced by the bacterium Staphylococcus aureus which forms a 1:1 complex with plasmin(ogen). This complex then activates other plasminogen molecules [14].

For the purpose of this study, we designed an antithrombotic peptide of 29 amino acids that combines antithrombin and antiplatelet activity and used SAK to provide fibrinolytic activity. SAK was linked to the antithrombotic peptide through a sequence that can be cleaved by activated coagulation factor X. The factor Xa target sequence was introduced to enable release of the antithrombotic peptide in vitro and in vivo. The peptide was designed to include three inhibitory regions: (1) an amino acid sequence (RGD) derived from fibrinogen, which is essential for binding of fibrinogen to its receptor (Glycoprotein IIb/IIIa) on platelet membranes [15]; (2) a part of fibrinopeptide A (residues 8–16), an inhibitor of thrombin [16]; and (3) the C-terminal tail of hirudin (residues 54–65), a direct antithrombin [6]. We hypothesised that the construct, named PLATSAK (Platelet-Antithrombin-Staphylokinase), would prevent platelet aggregation, inhibit the action of thrombin and enhance fibrinolysis. In this paper we report on the construction, purification, and in vitro characterisation of this recombinant fusion protein.

1. Materials and Methods

1.1. Host Strain

Escherichia coli Top 10F′ [mcr A, Δ(mrr-hsdRMS-mcrBC), φ80ΔlacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara, leu), 7697, galU, galK, λ^-, rpsL, endA1, nupG, F′] was used for cloning the recombinant genes and for propagation of plasmids. The same strain was also used as host for gene expression.

1.2. Enzymes and Chemicals

Restriction endonucleases, T4 DNA ligase, Taq polymerase and Chromozym TH were purchased from Boehringer Mannheim. Synthetic fibrinopeptide A (residues 8–16) and hirudin C-terminus (residues 54–65) were obtained from Sigma. Chemicals for media were purchased from Difco. All chemicals used were of analytical grade.

1.3. Recombinant DNA Techniques

DNA techniques were performed according to the general criteria currently in use [17]. Fragments for cloning were purified from agarose gels using Qiagen purification kits. E. coli transformation were performed as described [18].

1.4. Cloning Strategy

The amino acid sequence encoding the antithrombotic peptide was reverse translated using optimal universal codons [19]. Two 60-mer oligonucleotides, designated primers 1 and 2 (Table 1), were chemically synthesised. These two primers overlap 22 base pairs and the lacking ends were filled in by PCR (Figure 1). The purified gene was subsequently cloned into the Sma I restriction site of pUCBM21 (Figure 2) and the resulting plasmid was designated pWB1. Using primers 2 and 3 (Table 1), the gene was amplified from pWB1 and cloned simultaneously with the SAK gene and the Factor Xa cleavage site into the Eco RI and the Sal I restriction sites of pWB6 (Figure 2). The Factor Xa cleavage site was constructed as two complementary oligonucleotides, primers 4 and 5 (Table 1). The newly constructed plasmid was designated pWBM3. To aid purification of the recombinant fusion protein, PCR was applied to add six consecutive histidine residues to the C-terminus of the protein, using primers 6 and 7 (Table 1). The newly constructed plasmid was called pWBM3H. A plasmid containing only the SAK gene was named pWBM and produced in parallel with pWBM3H. Gene expression was under control of the strongly inducible tac promoter, which can be induced by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To prevent the promoter from producing a low level of protein during the initial phases of growth, the cells were co-transformed with pREP4, which carries the lacI gene encoding the lac repressor. All constructs were sequenced to verify accurate gene synthesis.

1.5. Media and Cultivation

Following transformation, the cells were plated on SOB agar (2% tryptone, 0.5% yeast extract, 0.05%

Table 1. Summary of primers

Name	Sequence, 5′ to 3′	Use
Primer 1 (60-mer)	AGAGGTGACTTCTTGGCTGAA- -GGTGGTGGTGTTAGACCAGGT- -GGTGGTGGTAACGGTGAC	Upper strand of anticoagulant gene
Primer 2 (60-mer)	CGCTCGAGCTACAAGTATTCTT- -CTGGAATTTCTTCGAAGTCA- -CCGTTACCACCACCACCT	Lower strand of anticoagulant gene
Primer 3 (30-mer)	GCCCCGGGAGAGGTGACT- -TCTTGGCTGAAG	Primer 1+Sma I restriction site
Primer 4 (36-mer)	CAAGGTTGTTATAGAAAAG- -AAATCGAGGGAAGGAG	Upper strand of FXa linker
Primer 5 (32-mer)	CTCCTTCCCTCGATTTTCTT- -TTCTATAACAAC	Lower strand of FXa linker
Primer 6 (26-mer)	ACAGAATTCAGGAGGC- -CTCATATGTC	Forward primer for adding 6 his
Primer 7 (69-mer)	GCGGTCGACCTAGTGATG- -GTGATGGTGATGCAAGTA- -TTCTTCTGGAATTTCTTC- -GAAGTCACGTTACC	Reverse primer for adding 6 his

NaCl, and 2.5 mM KCl) containing the appropriate antibiotics. For cultivation purposes the cells were grown on TB medium (1.2% tryptone, 2.4% yeast extract, and 0.4% glycerol, pH controlled at 7 with 0.017 M KH_2PO_4/0.072 M K_2HPO_4), containing 25 µg/ml kanamycin and 100 µg/ml ampicillin for maintenance of the plasmids. A 3-ml culture was inoculated with a single colony and grown for three hours at 37°C in a shaking incubator. This starter culture was then used to inoculate a 200-ml working culture, which was allowed to grow to an OD_{600} of 0.5. Gene expression was subsequently induced by addition of 2 mM IPTG to the culture. Following growth for an additional hour, the cells were har-

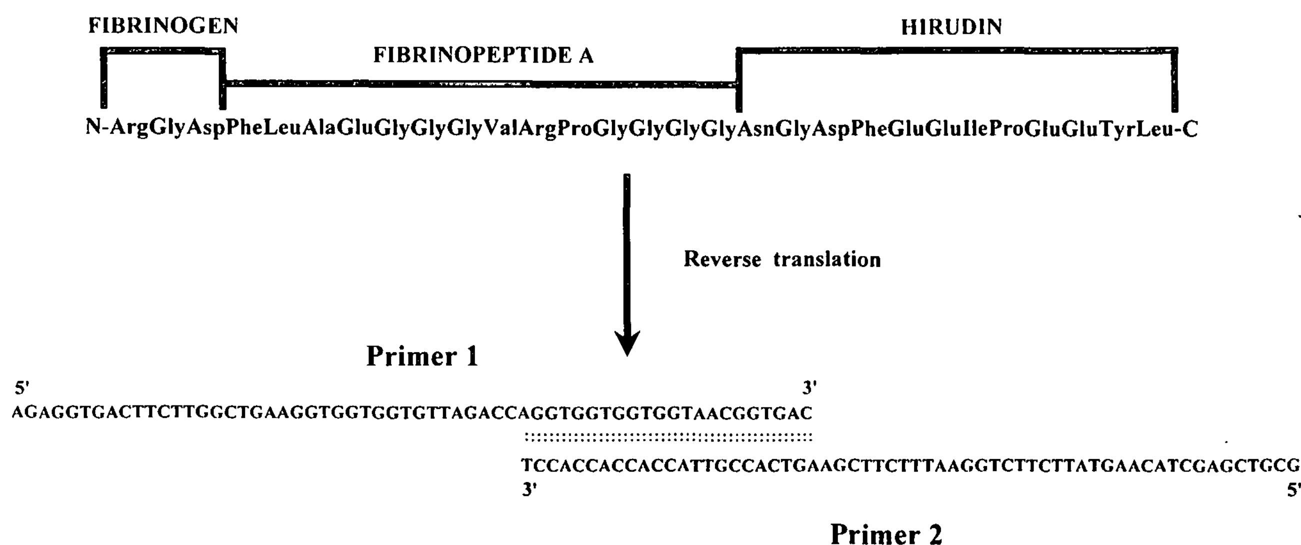

Fig. 1. The amino acid sequence of the antithrombotic peptide was reverse translated and two overlapping oligonucleotides (primers 1 and 2) were chemically synthesised. The gene was constructed by annealing the two primers and filling in the lacking ends by PCR. The resultant gene was subsequently cloned into pUCBM21 resulting in plasmid pWB1.

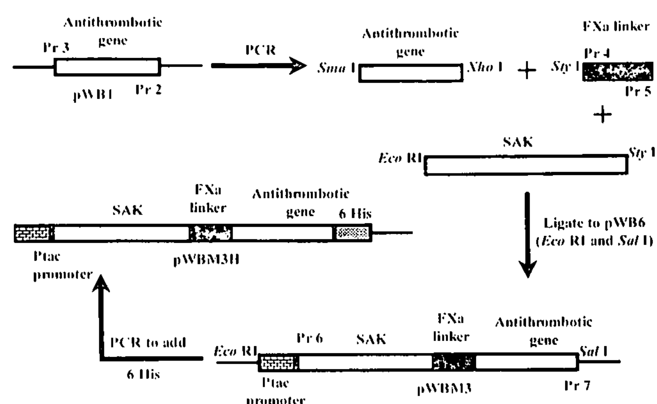

Fig. 2. A schematic representation of the strategy followed for construction of the PLATSAK gene. Cloning vectors are presented as linear figures. Pr, primers.

vested by centrifugation (3000×g for 10 minutes). This procedure was also followed in parallel to produce SAK, which was also analysed in all activity assays to ensure that the SAK portion did not contribute to any antithrombotic or antiplatelet activity (results not shown).

1.6. Protein Extraction and Purification

Recombinant proteins were isolated by repeated cycles of freezing and thawing [20]. The cell pellets were frozen for ten minutes in liquid nitrogen and thawed on ice for fifteen minutes. This procedure was repeated twice. Two ml of sonication buffer (50 mM Na-phosphate (pH 8.0), 300 mM NaCl) was added to the cell pellets and left on ice for another hour. Cell debris was removed by centrifugation at 4°C for ten minutes at 10000×g.

The protein extract was allowed to bind to Ni-NTA resin (Qiagen) for one hour at 4°C in a batchwise manner. The resin was left to settle in a column and was washed with sonication buffer until the OD_{280} was below 0.01. To remove other contaminating proteins, the resin was extensively washed with washing buffer [50 mM Na-phosphate (pH 6.0), 300 mM NaCl, 10% Glycerol] until the OD_{280} was below 0.01. The purified PLATSAK was eluted from the column with elution buffer (50 mM Na-phosphate (pH 4.0), 300 mM NaCl) and concentrated using Centricon-10 concentrators (Amicon). Protein concentrations were determined using the BCA Protein Assay system from Pierce. Proteins were separated on 15% SDS-PAGE gels and silver stained with the BDH Instaview Silver Staining Kit.

1.7. Measurement of Antithrombin Activity

Thrombin time (TT) and activated partial thromboplastin time (aPTT), measured with standard assay systems, were used to test for antithrombin activity [21]. An amidolytic assay of thrombin activity, performed at 30°C using Chromozym TH (Boehringer Mannheim) as thrombin substrate, was also used [22]. The reactions were done in 0.1 M Tris-HCl (pH 8.3), containing 0.2 M NaCl and 0.05% Triton-X100, with 0.25 nM enzyme, 31.25 μg Tos-Gly-Pro-Arg-p-nitroanilide (Chromozym TH), and different concentrations of PLATSAK and SAK in a total volume of 250 μl. The reaction was followed over 60 minutes at 405 nm in an EL 312e Bio-kinetics microplate reader (Bio-tek Instruments). For purposes of comparison, the effect of individual peptides of fibrinopeptide A (residues 8–16) and the tail of hirudin (residues 54–65) on TT, aPTT, and the amidolytic activity of thrombin was also tested.

1.8. Measurement of Antiplatelet Activity

Platelet aggregation in response to 2 mM ADP (final concentration) was turbidimetrically measured in a Monitor IV Plus Platelet Aggregometer (Helena Laboratories) at 37°C, as described in detail [23]. The platelet count in platelet rich plasma was 200×10^9/l. The aggregation response, measured at 4 minutes following addition of ADP, was related to the difference in light transmission between platelet poor plasma and platelet rich plasma and expressed as a percentage. Before the ADP was added, the platelet rich plasma was incubated with different concentrations of PLATSAK as well as SAK.

1.9. Measurement of Fibrinolytic Activity

The fibrinolytic activities of PLATSAK and SAK were determined using a Hellige thrombelastograph. Human venous blood (4.5 ml) was collected in polystyrene syringes and anticoagulated with 0.25 ml tri-sodium citrate (9% w/v stock solution). The anticoagulated blood (250 μl) was calcified by the addition of 100 μl $CaCl_2$ (0.645% w/v working solution). Different concentrations of purified SAK or PLATSAK were immediately added to the reaction mixture and clot formation and subsequent

lysis due to SAK and PLATSAK activity were followed over time. The L-value, which spans the distance measured by the thrombelastograph from the start of clot formation to the end of lysis, was determined for each concentration used.

2. Results and Discussion

The gene encoding the antithrombotic peptide was constructed by reverse translating the synthetic amino acid sequence to the corresponding nucleic acid sequence, keeping optimal codon use in mind [19]. SAK was added not only to improve the stability of the small peptide, but to include digestion of fibrin to the range of activities carried by the construct. The two genes were separated by a cleavable linker, the Factor Xa target site (Figure 2). The fusion construct was cloned into pWB6 and gene expression was controlled by the strongly inducible tac promoter, using E. coli as expression host. Purification was facilitated by adding six histidine residues to the C-terminal part of the fusion protein. Six consecutive histidine residues have a very high affinity for Ni-ions associated with the Ni-NTA purification resin [24]. Transformants carrying the gene were cultivated and gene expression was induced by addition of IPTG to logarithmically growing cells. It resulted in the appearance of a protein band of approximately 18 kDa on SDS-PAGE gels (results not shown). Maximal protein production was reached after one hour of induction, after which the cells were subjected to freeze-thaw lysis [20]. This resulted in the preferential release of recombinant proteins from the frozen cell pellets. This method has the advantage of having fewer contaminating proteins that could interfere with the subsequent purification steps. Furthermore, it is a very simple, inexpensive and exceptionally efficient method of enriching recombinant proteins produced intracellularly by E. coli.

Further purification was performed using Ni-NTA resin. Protein extracts and column fractions were analysed on 15% SDS-PAGE gels and silver stained. Figure 3 shows the efficiency of the purification procedure used. The standard purification experiment yielded about 1 mg of recombinant PLATSAK per 200 ml culture. Of interest was the finding that the yield of PLATSAK, under similar conditions, was markedly lower than in the case of SAK (results not shown). It may be possible

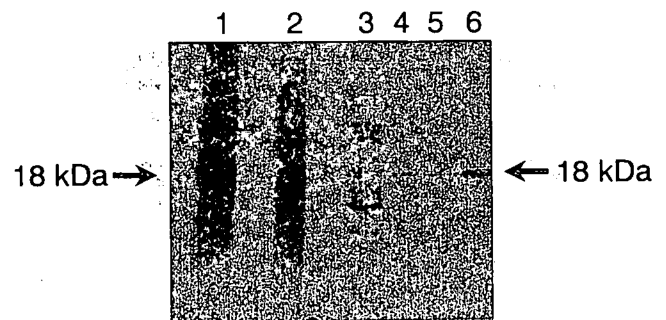

Fig. 3. SDS-PAGE analysis of protein purification procedure. *Lane 1:* total intracellular E. coli proteins; *Lane 2:* the enriched fraction obtained from the freeze-thaw lysis; *Lane 3:* the washing fraction from the Ni-NTA resin; *Lanes 4–6:* purified PLATSAK.

that the antithrombotic part of the fusion protein could have an inhibitory effect on protein production by E. coli. In spite of all precautions and the application of different elution procedures, a contaminating band of low concentration co-eluted with PLATSAK. The concentration of the contaminant is nevertheless so low that it is not visible in lanes 4 to 6 (Figure 3) and it apparently has not interfered with the subsequent assays.

The antithrombin properties of purified PLATSAK were investigated by measuring TT and aPTT (Figure 4). In both assays equal volumes of elution buffer served as control. Addition of 15 μM of PLATSAK markedly lengthened TT and aPTT, thereby indicating inhibition of thrombin activity. SAK had no effect on TT and aPTT. The lengthening in TT and aPTT must therefore be due to the two antithrombin sequences present in the peptide fused to SAK. The mechanism of interaction of our peptide with thrombin is not clear and we have no data to show whether the antithrombin activity is due to the hirudin or to the fibrinopeptide A components. It can be assumed that the tail of hirudin binds to the anion binding exosite of thrombin [25,26] and the fibrinopeptide A sequence to the active site [16]. Since these two motifs are adjacent to each other in our construct (Figure 1), one would not expect a single molecule to bind simultaneously to the exosite and the active site. Residues 7 to 16 of fibrinopeptide A are critical to efficient peptide hydrolysis, while residues outside this region are critical for thrombin binding [27]. It appears that the amino acids outside the antithrombotic fragments of PLATSAK contribute to thrombin binding. In the chromogenic assay,

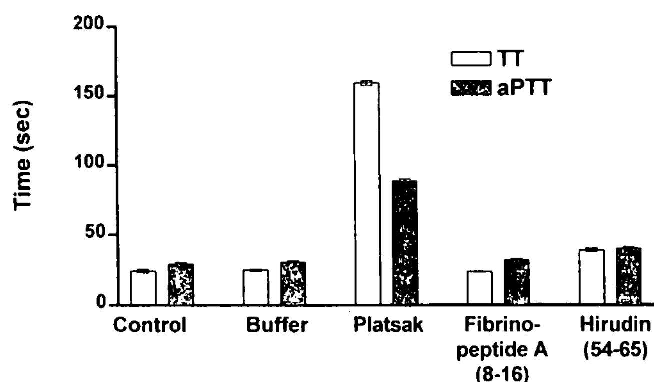

Fig. 4. Determination of antithrombotic activity using TT and aPTT. In both cases 15 μM of PLATSAK, fibrinopeptide A (residues 8–16) or hirudin (residues 54–65) was used to assay the antithrombotic activity. Results are the average of three individual experiments.

5.5 μM of the protein was sufficient to inhibit the action of thrombin significantly (Figure 5A). It is known that the C-terminal domain of hirudin is unable to inhibit the amidolytic activity of thrombin towards small substrates [9,28]. Inhibition of thrombin towards substrates like Chromozym TH is, thus, achieved by blocking the active site. In this experiment, thrombin inhibition was, thus, achieved either by the fibrinopeptide A derived part of the antithrombotic peptide binding to the active site or by blocking of the active site by the N-terminal part of PLATSAK, while the C-terminus was bound to the exosite.

It is important to note that PLATSAK markedly lengthened the TT and aPTT (Figure 4) and inhibited the amidolytic activity of thrombin (Figure 5) when compared to equimolar concentrations of the antithrombin part of fibrinopeptide A and the tail of hirudin. This strongly suggests that combination of the fibrinopeptide A part and the hirudin-tail with SAK greatly enhances the antithrombin activity of PLATSAK. We have no ready explanation for this finding.

The RGD sequence is an essential part of all integrins, since it is responsible for binding of the integrin to the glycoprotein IIb/IIIa receptor on the membranes of activated platelets [29], and RGD peptides and monoclonal antibodies containing the RGD motif are effective inhibitors of platelet dependent thrombus formation [30,31]. We introduced the RGD motif at the N-terminus of our peptide (Figure 1) to facilitate antiplatelet activity. The effect of PLATSAK on platelet aggregation was negligible (Figure 5B). At a concentration of 33 μM, PLATSAK could not inhibit ADP induced platelet aggregation. On the contrary, 20 μM of a synthetic RRRRRRRRRGDV could prevent platelet aggregation induced by thrombin, ADP, collagen, and epinephrine [1]. We could not overcome this problem by prior in vitro digestion of PLATSAK with Factor Xa. There are several possible explanations for the inability of PLATSAK to inhibit platelet aggregation. It could be due to the inaccessibility of the RGD in the tertiary structure of the protein. It is also possible that the RGD motif is situated in non-optimal surroundings [1]. An alternative would be to place the RGD motif on the tip of a loop to increase its accessibility to activated platelets.

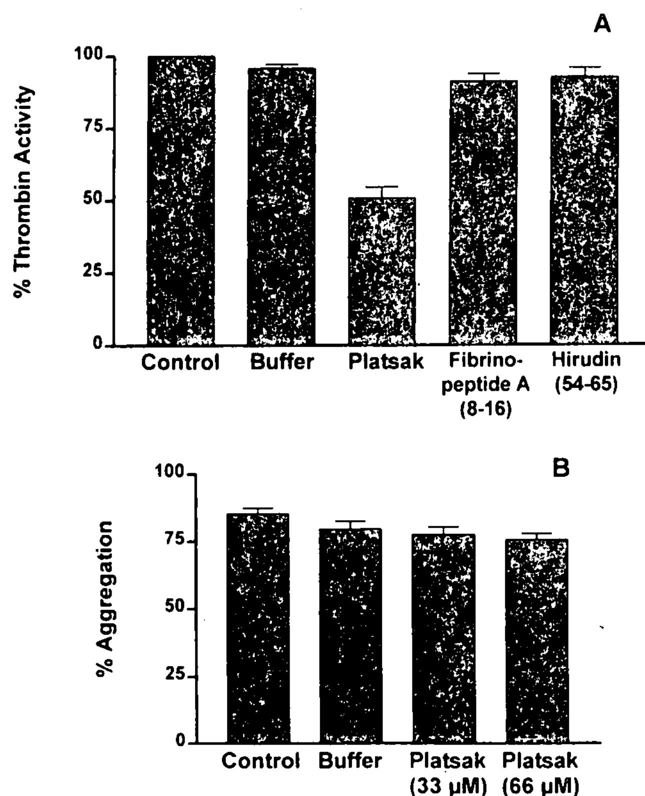

Fig. 5. A. Determination of thrombin inhibition using Chromozym TH as substrate for thrombin. The antithrombotic activity of different concentrations of PLATSAK is expressed as the percentage of thrombin activity retained following incubation of 60 minutes with PLATSAK. B. Determination of the inhibition of ADP induced platelet aggregation. Results are the average of three individual experiments.

Table 2. Comparison of L-values obtained from thrombelastography

Protein concentration	SAK L-value (mm)	PLATSAK L-value (mm)
27 μM	12.67±0.47	16.5±0.41
22 μM	13.67±0.47	17.25±0.82
16 μM	17.00±0.82	25.67±0.47
11 μM	26.67±1.25	36.5±1.48

The fibrinolytic activity of the SAK portion of the protein was determined using thrombelastography, as well as purified recombinant SAK as control. A surprising result was that PLATSAK was slightly less effective than recombinant SAK (Table 2). In theory PLATSAK should inhibit clot formation, due to the antithrombin peptides at its C-terminal end, and activate fibrinolysis, and so have a more pronounced effect on the assay than SAK on its own. A possible explanation for our finding may be that fusion of additional amino acids to the C-terminus of SAK distorts the structure in such a way that it slightly impairs protein activity. However, one would expect that factor Xa would release the antithrombotic peptide from SAK and that the peptide would not have had an effect on SAK. Alternatively, the fact that the fibrinolytic activity was slightly lower and that the inhibition of platelet function was low, strengthens the suggestion that the N-terminal part of the antithrombotic peptide is buried inside the protein, or sterically hindered in some other way. This may also hinder release of the peptide by factor Xa-activity. We have however no data to support this. The C-terminus, with its antithrombin activities, are seemingly more accessible. Unfortunately, no crystal structure for SAK is available at the moment, which could have helped to explain our observations. A possible way of testing the hypothesis would be to rearrange the different motifs of our peptide so that the RGD is at the C-terminal end, or even at the N-terminus of SAK itself.

In conclusion, we were able to produce a hybrid protein in E. coli that was able to inhibit thrombin activity and enhance fibrinolysis in vitro. Antiplatelet activity was negligible, but can possibly be improved by redesigning or repositioning the RGD motif. The important contribution of this study, we believe, is that we have shown that it is possible to design a multifunctional peptide that can modulate haemostasis and thrombosis. The next step would be to test the construct in a suitable in vivo animal model.

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CHAPTER 4

OPTIMIZATION OF ANTIPLATELET ACTIVITY

4.1 INTRODUCTION:

In spite of good thrombin inhibition by PLATSAK, it did not inhibit platelet function as expected (Chapter 3), perhaps because of the inaccessibility of the RGD-motif in the tertiary structure of PLATSAK. Since platelets play a pivotal role in thrombogenesis, I consider it important to enhance the antiplatelet activity of PLATSAK. I attempted to overcome this lack of antiplatelet activity by introducing an additional RGD-motif at either the N-terminus or the C-terminus of PLATSAK.

4.2 MATERIALS AND METHODS:

4.2.1 Host strain:

Escherichia coli Top 10F' [mcr A, Δ(mrr-hsdRMS-mcrBC), ϕ80ΔlacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara, leu), 7697, galU, galK, λ^-, rpsL, endA1, nupG, F'] was used for screening of the newly established recombinants and for propagation of plasmids. The same strain also served as host for gene expression.

4.2.2 Enzymes and chemicals:

Restriction endonucleases, T4 DNA ligase and Taq DNA polymerase were purchased from Boehringer Mannheim (Germany). All chemicals used were of analytical grade.

4.2.3 Recombinant DNA techniques:

General DNA techniques, such as PCR, agarose electrophoresis, restriction digests and ligation reactions were performed as described by Sambrook *et al* (1989). Qiagen purification kits were used to purify DNA fragments from agarose gels to use for cloning purposes. *E. coli* transformations were performed according to the method of Tang *et al* (1994).

4.2.4 Cloning strategy:

PCR was applied to add an RGD-motif to the N- and the C-terminus of PLATSAK, respectively. Primers 1 and 2 (Table 4.1) were designed to amplify the entire gene and to introduce an additional RGD-sequence immediately downstream from the His-tag. The resultant protein was designated PLATSAK2. Primers 3 and 4 (Table 4.1) were used to add an additional RGD-sequence to the N-terminus immediately downstream of the initiating Met-residue of PLATSAK, resulting in PLATSAK3. Fig. 4.1 schematically represents the strategy that was followed in both cloning procedures. The resultant recombinant plasmids were designated pWBM3H4 and pWBM3H5 for the modified C- and N-termini, respectively. Repression of constitutive gene expression was performed as described in section 3.2.4. All constructs were sequenced to verify accurate gene synthesis and fidelity of PCR.

4.2.5 Cultivation, protein purification and measurement of antiplatelet activity:

Cultivation of recombinant cells and purification of recombinant proteins were performed as described in section 3.2.5 and section 3.2.6, respectively. The antiplatelet activity was determined as described in section 3.2.8.

Table 4.1 Summary of primers.

Name	Sequence, 5' to 3'	Use
Primer 1 (26-mer)	ACAGAATTCAGGAGGCCTCATATGTC	Forward primer for adding RGD to the C-terminus
Primer 2 (51-mer)	CGCGTCGACCTAGTCACCTCTGTGATGG- -TGATGGTGATGCAAGTATTCTTC	Reverse primer for adding RGD to C-terminus
Primer 3 (52-mer)	ACAGAATTCAGGAGGCCTCATATGAG- -AGGTGACTCAAGTTCATTCGACAAAG	Forward primer for adding RGD to N-terminus
Primer 4 (69-mer)	GCGGTCGACCTAGTGATGGTGATGG- -TGATGCAAGTATTCTTCTGGAATTT- -CTTCGAAGTCACGTTACC	Reverse primer for adding RGD to N-terminus

4.3 RESULTS AND DISCUSSION:

It is evident that the position of the RGD-sequence in PLATSAK was such that its antiplatelet activity is suboptimal. In view of the fundamental role of the RGD-sequence in the prevention of platelet aggregation (Marguerie *et al*, 1980), it was necessary to optimize the antiplatelet activity. PCR was used to perform *in vitro* modifications to the chimera in an attempt to enhance its antiplatelet activity. PLATSAK2 is the product of this modification. It consists of the original PLATSAK molecule with an additional RGD-sequence added downstream from the six consecutive histidines at the C-terminus. The gene, encoding PLATSAK2, was cloned into pWB6 (Fig. 4.1) and sequenced to confirm the correct nucleic acid sequence. The verified construct was expressed in *E. coli* cells.

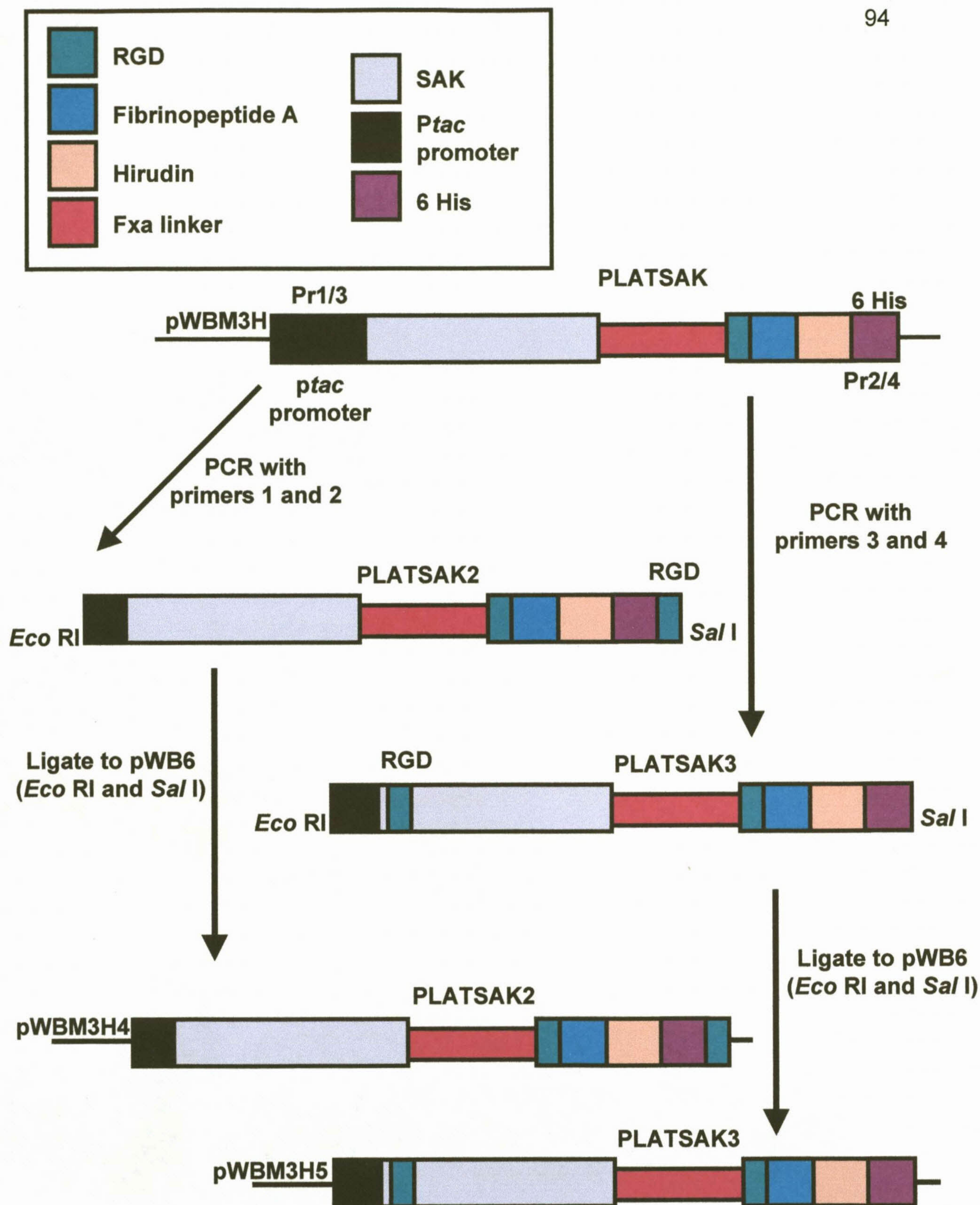

Fig. 4.1 Cloning strategy followed to produce PLATSAK2 and PLATSAK3. Primers 1 and 2 are used to add the RDG-motif to the C-terminus to produce PLATSAK2. Primers 3 and 4 are used to add the RGD-motif to the N-terminus to produce PLATSAK3.

Purification of the recombinant protein was performed as described in section 3.2.6. Although a strong band of approximately 18 kDa appeared upon induction of protein expression (Fig. 4.2), it seemed that the newly introduced RGD-motif downstream from the His-tag interfered with the purification process. A meaningful decrease in the yield of purified protein was observed. The standard purification procedure yielded about 1 mg of PLATSAK per 200 ml culture (Chapter 3), while a yield of 0.2 mg PLATSAK2 per 200 ml culture was obtained. The addition of three extra amino acids downstream from the histidines could have led to an unfavourable three-dimensional folding of the protein, with some distortion of the histidine residues.

The low yield of PLATSAK2 made it difficult to test its antiplatelet activity at similar molarities to that used for PLATSAK. The antiplatelet activity obtained at 10 μM of PLATSAK2 was unsatisfactory as no inhibition of platelet aggregation could be observed (results not shown). A possible explanation is that the concentration of PLATSAK2 was too low to have a detectable effect on platelet aggregation. Furthermore, it is difficult to predict the tertiary structure of this new variant of PLATSAK and the RGD-motif might be unable to bind to the Gp IIb/IIIa receptor on activated platelet membranes.

This observation forced us to construct a new variant, PLATSAK3, by introducing the RGD-motif at the N-terminus. PCR was used to insert the three amino acids directly downstream from the initiating methionine residue. The cloned gene was sequenced and transformed to *E. coli* cells. Cell cultivation was performed as described for PLATSAK2.

In contrast to the strong gene expression of PLATSAK and PLATSAK2 observed on SDS-PAGE, no expression of PLATSAK3 could be detected. It is a known phenomenon that in many cases the initiating methionine residue of prokaryotic proteins is removed post-translationally. Furthermore, according to the N-end rule postulated by Varshavsky (1992), an N-terminal arginine renders a protein that is highly susceptible to proteolytic degradation. The total lack of PLATSAK3 production can possibly be explained by the removal of the initiating methionine and the subsequent high instability of proteins with an arginine at

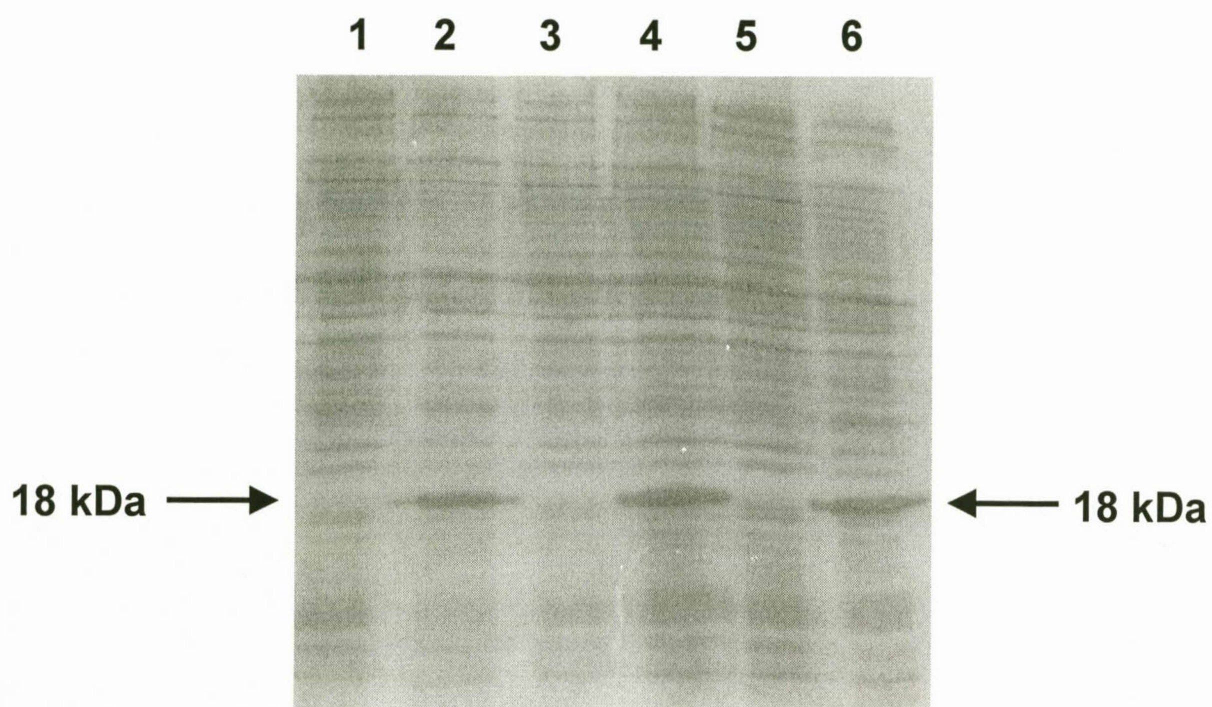

Fig 4.2 SDS-PAGE analysis of intracellular proteins to follow the induction of protein synthesis. **Lanes 1, 3 and 5:** Intracellular protein profile before induction. **Lanes 2, 4 and 6:** Intracellular protein profile after one hour of induction.

their N-terminus. Furthermore, removal of the first ten amino acids of staphylokinase in the presence of plasmin (Schlott *et al*, 1997), can cause the co-removal of the RGD-sequence when administered in an *in vivo* system.

4.4 CONCLUSIONS:

Although we could not accomplish the production of a variant with increased antiplatelet activity, we decided to abandon any further attempts. Optimization of the purification procedure to yield higher and possibly more practical concentrations of PLATSAK2 proved to be unsuccessful. The potentially unfavorable post-translation modification and subsequent instability of PLATSAK3 and the undesirable *in vivo* removal of the first 13 amino acids (including the RGD) in the presence of plasmin, makes the latest variant unsuitable for our specific needs. Production of more variants would be a time-consuming and costly process and we decided to use the PLATSAK variant for *in vivo* studies.

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CHAPTER 5

OPTIMIZATION OF PURIFICATION OF PLATSAK

5.1 INTRODUCTION:

Testing of PLATSAK in an *in vivo* model was deemed essential to evaluate its full potential as an antithrombotic agent. However, to do such an assay, one would require a large quantity of protein extract in the purest possible form. I therefore analyzed two different purification systems to attempt to produce PLATSAK of the highest possible purity at large scale. Both systems involved metal affinity chromatography based on the interaction between the His-tag and different divalent metals.

5.2 MATERIALS AND METHODS:

5.2.1 Conventional metal affinity chromatography using Ni-NTA resin:

Ni-NTA (nickel-nitrilotriacetic acid) resin was chosen for small scale purification purposes. The resin was obtained from Qiagen. NTA binds four of the six ligand binding sites in the coordination sphere of the nickel ion. Two sites are free for interaction with the His-tag (Fig. 5.1). NTA binds metal ions with higher stability than other chelating ions (Hochuli, 1989) and is capable of retaining the ions under stringent wash conditions.

The recombinant cells were grown as described in section 3.2.5 and intracellular proteins were extracted by the freeze-thaw technique (section 3.2.6). Protein purification, protein electrophoresis, concentration of protein fractions and determination of protein concentrations were performed as described in section 3.2.6.

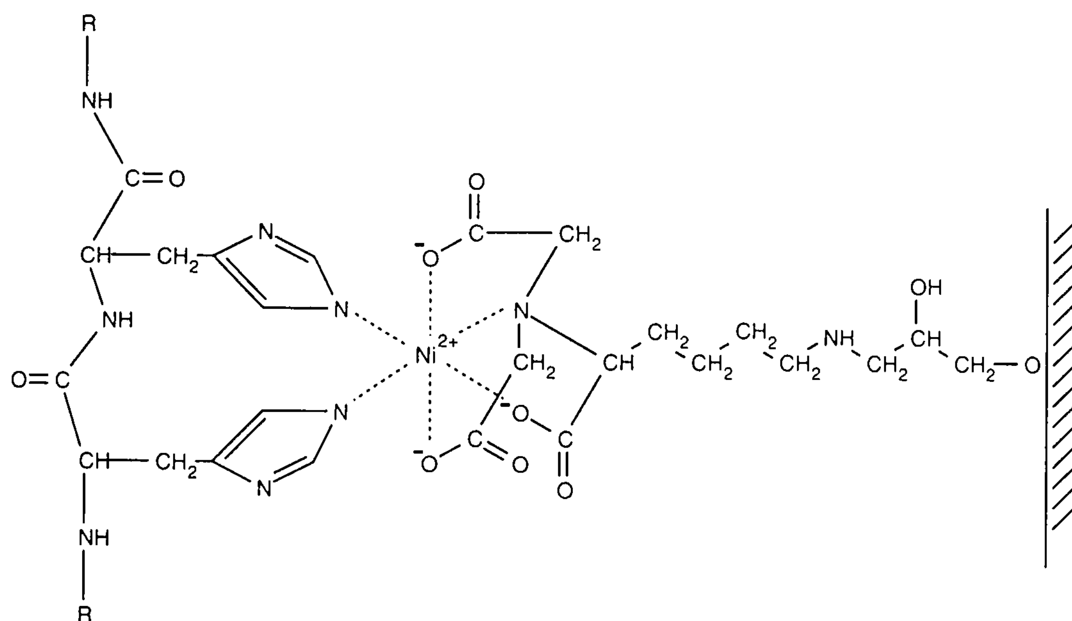

Fig. 5.1 Interaction between the His-tag and the Ni-NTA-resin.

5.2.2 Metal chromatography using an FPLC-column:

A Poros MC prepacked FPLC metal affinity chromatography column (i.d. 4 mm, length 100 mm, column bed volumn 1.7 ml) was purchased from Boehringer Mannheim (Germany). The column was designed to be charged with the metal of choice. The resin consists of cross-linked polystyrene/divinylbenzene flow-through particles with a patented bimodal pore size distribution for rapid mass transport. The particles are surface-coated with a crosslinked polyhydroxylated polymer functionalized with imidodiacetate groups. It allows binding through bidentate ligation of a wide range of transition metals. Binding of proteins is accomplished through the formation of coordination complexes between the remaining metal coordination sites and certain surface amino acids, such as histidine, cysteine and tryptophan. The purification procedure was performed as prescribed by the manufacturer and will be discussed briefly.

The crude protein extract was obtained as described in section 3.2.6 according to the method of Johnson and Hecht (1994). For purification, the proteins were dissolved in sonication buffer [50 mM Na-phosphate (pH 8.0), 300 mM NaCl]. A Gilson 712 chromatographic system was used and protein binding and elution was followed using UV-detection with a Gilson 112 UV detector. A flow rate of three ml per minute was maintained throughout the procedure and the column was operated in a pressure range of 400 to 800 psi. The column was shipped in 0.1 M Na_2SO_4, 20% ethanol and was rinsed with 5 column bed volumes of water before initial operation. Prior to initial use, the column was stripped with 20 column bed volumes of 100 mM EDTA in 1 M NaCl to discharge any metal ions from the resin. Ten column bed volumes of water were used to remove all the EDTA from the column. The imidoacetate sites on the resin were fully saturated with the appropriate metal of choice by applying 100 ml of 0.1 M of the sulphate or chloride salt of the metal. Excess metal was washed away with 10 column bed volumes of water. Ionically bound metal was removed by washing the column with 10 column bed volumes of 0.5 M NaCl. The column was subsequently equilibrated in 10 column bed volumes of sonication buffer and the protein extract was loaded onto the column. The column was extensively washed with sonication buffer until the OD_{280} was below 0.01.

Washing buffer [50 mM Na-phosphate (pH 6.0), 300 mM NaCl, 10% glycerol] was then applied to remove any contaminating proteins and it was continued until the OD_{280} was below 0.01. Proteins were eluted with different elution buffers and collected as one ml fractions. All elution fractions were analyzed on SDS-PAGE and gels were stained with Coomassie Blue. Fractions containing the highest concentration of PLATSAK were pooled and concentrated with Centricon-10 concentrators. Protein concentrations were determined using the BCA Protein Assay system from Pierce.

Proteins can also be eluted with solutes that compete with the proteins for binding to the metal binding sites. As an alternative for reduction in pH, proteins were eluted from the Poros MC column with washing buffer containing imidazole. In this method of elution, the imidazole would compete with PLATSAK for the metal binding sites and would eventually replace it. PLATSAK would subsequently be eluted. Different concentrations of imidazole were investigated to ascertain the most applicable concentration.

5.3 RESULTS AND DISCUSSION:

Six consecutive histidine residues were added to the C-terminus of PLATSAK to aid purification of this recombinant protein. This histine-tag has a very strong affinity for metal ions associated with the purification resin (Janknecht *et al*, 1991). Ni-NTA resin was used for the purpose of conventional purification. The efficiency of the purification procedure is presented in Fig. 3.3, where the elution fractions from the column show a single band of 18 kDa. Furthermore, it was a highly reproducible method and a standard purification procedure yielded about 1 mg of PLATSAK per 200 ml culture. However, scaling up of the method seemed problematic. It was not reproducible, since binding and elution of PLATSAK appeared to be different. The other option was to repeat a standard purification procedure several times in order to purify enough PLATSAK to test in an *in vivo* model. However, this was not practical, since it would result in

many small-scale purification procedures, over several months. A reduction in the activity of the first versus the last batches of purified PLATSAK could occur. The total activity of PLATSAK would thus be questionable.

As an alternative we investigated a method that would result in higher concentrations of PLATSAK and one that would be quicker and less time-consuming. An FPLC-column, Poros MC, for purifying larger amounts of PLATSAK using a single purification step was investigated. This particular column was designed for charging with the metal ion of the researcher's own choice. Furthermore, it has a large capacity and would result in a rapid purification procedure at large scale. The first variable to be investigated was charging of the column with the most effective metal ion. I tested two other metal ions in addition to nickel. Charging of the column with cobalt was unsuccessful. The cobalt was discharged from the column during the 0.5 M NaCl washing step. Although washing of the cobalt-charged column with a lower salt concentration, such as 0.1 M NaCl, resulted in maintenance of the metal ions, PLATSAK bound only slightly to the cobalt and was easily eluted from the column during initial washing steps. Copper was also investigated. The binding of copper to the resin was very successful and the bound metal was not easily removed from the column. However, the interaction between the His-tag of PLATSAK and the copper ions were very strong and PLATSAK could not be eluted under the mild conditions of the elution buffer. An alternative would have been to co-elute the copper ions and PLATSAK bound to it with EDTA. Since EDTA is also an anticoagulant, it would have been essential to completely remove all EDTA molecules from the purified protein extract. I decided against this avenue, since it would necessitate a subsequent dialysis step and because it would be very difficult to ascertain that all EDTA was indeed removed.

Since both cobalt and copper were unsuitable, nickel was evaluated. The nickel ions bound well to the resin and were not easily discharged from the column. Elution of PLATSAK with a decrease in pH of the buffer appeared not to be reproducible and different elution profiles were obtained with each run. Furthermore, much contaminating proteins co-eluted with PLATSAK (Fig. 5.2).

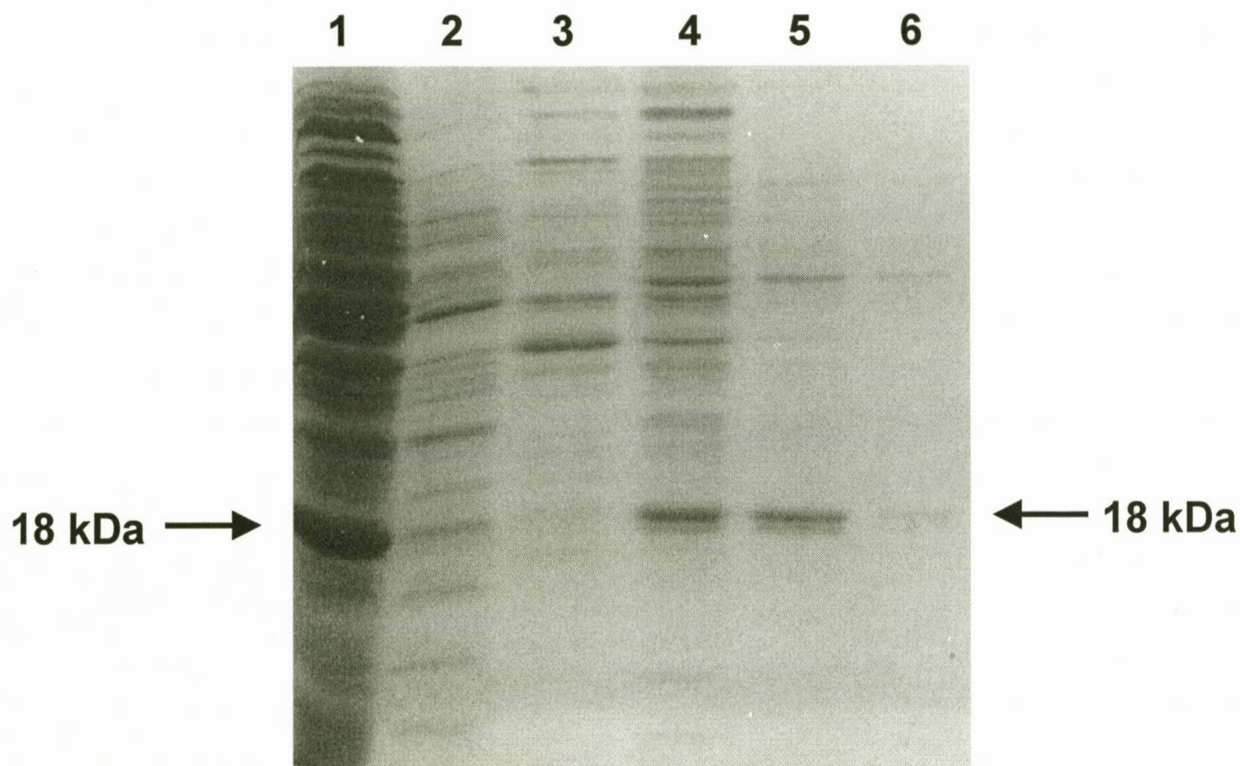

Fig. 5.2 SDS-PAGE of purification using Poros MC column. The column was charged with Ni-ions and the protein was eluted with washing buffer (pH 4.0). **Lane 1:** 10 μl of crude extract. **Lane 2:** 10 μl of flow-through fraction. **Lane 3:** 10 μl of washing fraction. **Lanes 4-6:** 10 μl of first three elution fractions.

Additionally, although the amount of starting material was three times that of the material used for conventional chromatography, the purification still yielded only 1 mg per run. SDS-PAGE of the flow-through fraction showed that all the PLATSAK that was loaded, did bind to the resin. Unfortunately, using this specific column under these conditions did not yield higher concentrations of PLATSAK when compared to the conventional Ni-NTA procedure.

Alternatively, a different elution method was investigated. The column was washed in washing buffer containing 0.5 mM imidazole. Elution of PLATSAK was attempted by addition of 250 mM imidazole to the washing buffer. However, elution of PLATSAK was again incomplete. Even an increase in the concentration of imidazole to 500 mM could not solve the problem and an insufficient amount of PLATSAK was obtained from the column (Fig. 5.3). The suppliers suggested that the column should be saturated with imidazole prior to equilibrating the column in washing buffer. However, this procedure completely prevented the binding of PLATSAK to the resin.

5.4 CONCLUSIONS:

Different metals were used to charge the column and different elution methods were attempted. None of these attempts were sufficient to purify large amounts of PLATSAK. In view of this and because it is time-consuming to search for other protein purification methods, I decided to abandon further attempts until later.

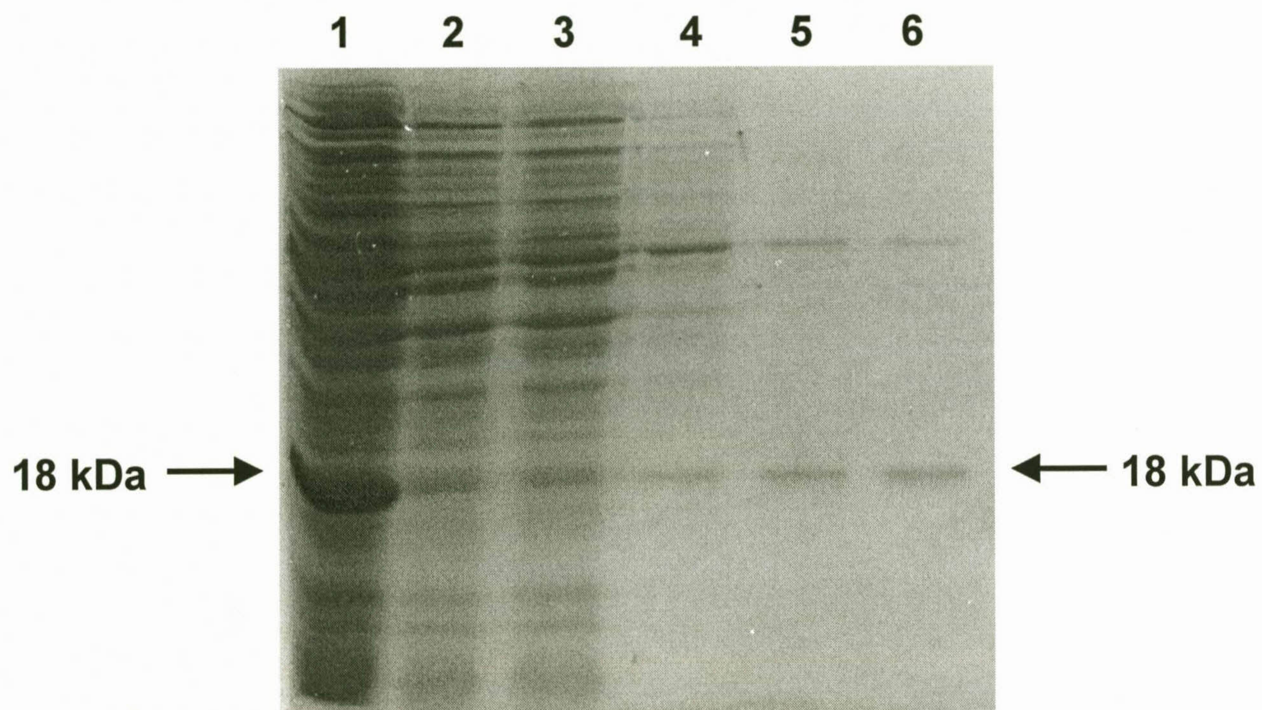

Fig. 5.3 SDS-PAGE of purification using Poros MC column. The column was charged with Ni-ions and the protein was eluted with washing buffer, containing 0.5 M imidazole. **Lane 1:** 10 μl of crude extract. **Lane 2:** 10 μl of flow-through fraction. **Lane 3:** 10 μl of washing fraction. **Lanes 4-6:** 10 μl of first three elution fractions.

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Johnson BH, Hecht MH. Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. Biotechnology 1994;12:1357-60.

CHAPTER 6

PLATSAK, A POTENT ANTITHROMBOTIC AND FIBRINOLYTIC PROTEIN, INHIBITS ARTERIAL AND VENOUS THROMBOSIS IN A BABOON MODEL.

(Submitted to Thrombosis Research)

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Summary

PLATSAK is a chimeric protein that was recombinantly produced in *Escherichia coli* cells. The protein was designed to target haemostasis at three different levels. It consists of staphylokinase (SAK) for activation of fibrinolyis, the Arg-Gly-Asp (RGD) sequence for the prevention of platelet aggregation and an antithrombotic peptide for the inhibition of thrombin. The *in vivo* activity of PLATSAK was evaluated by assessing its effect on platelet deposition in a baboon model of arterial and venous thrombosis. Dacron vascular graft segments and expansion chambers, inserted as extensions into permanent femoral arteriovenous shunts, were used to simulate arterial and venous thrombosis, respectively. PLATSAK (3.68 mg/kg) was administered as a bolus ten minutes before placement of the thrombogenic devices. Platelet deposition onto the graft surface and in the expansion chamber was imaged in real time with a scintillation camera as the deposition of ^{111}In-labelled platelets. After two hours, platelet deposition in the graft segments and expansion chambers was inhibited by 50% and 85% respectively when compared to control studies. The

aPTT was lengthened to >120 seconds. Interestingly, the level of FDP in plasma did not increase after administration of PLATSAK. These results demonstrate that PLATSAK effectively inhibited platelet deposition in both arterial- and venous-type thrombosis in an animal model.

Introduction

Several studies in animal models have emphasized the importance and essence of blood platelets and thrombin in thrombogenesis. The pivotal role of the adhesive protein receptor, glycoprotein (Gp) IIb/IIIa, in platelet-platelet interactions, has been demonstrated in canines (1-3) and non-human primates (4-6), by using selective antagonists. Of particular importance is the fact that Arg-Gly-Asp (RGD) containing peptides and proteins effectively block platelet-platelet interactions (5,7). The crucial role of thrombin in thrombogenesis has been demonstrated by using direct thrombin inhibitors (8,9). In other studies the formation of thrombin was inhibited with activated protein C (10), tissue factor pathway inhibitor (11) or recombinant tick anticoagulant, which inhibits activated factor Xa (12).

In view of the importance of the RGD binding domain on the platelet adhesive proteins and the role of thrombin in thrombogenesis, together with the central role of fibrinolysis, multifunctional peptides have been designed and developed. The approaches were to combine antiplatelet and antithrombin activity, to combine antithrombin and fibrinolytic activity, to combine antiplatelet and fibrinolytic activity and to combine antithrombin, antiplatelet and fibrinolytic activity in the same molecule. Peptides that combine antiplatelet and antithrombin activity include the hirudisins (13), a chimeric antithrombin peptide (14) and a modified hirutonin (15). These peptides markedly inhibited thrombin, but had variable affectivity in inhibiting platelet-platelet interactions. Examples of peptides where antithrombin activity were combined with fibrinolytic activity, include one where r-hirudin were coupled with streptokinase (16) and one where

a 40 kDa part of recombinant single chain urokinase-type plasminogen activator were coupled to the tail (Hir^{53-65}) of hirudin (17). Both proteins retained their fibrinolytic and antithrombin activity. A protein where an RGD binding domain was introduced into tissue-type plasminogen activator binds to Gp IIb/IIIa and retains its fibrinolytic activity (18).

PLATSAK is a protein that combines antiplatelet, antithrombin and fibrinolytic activity (19). The protein consists of staphylokinase linked, via a factor Xa cleavage site, to an antiplatelet and antithrombin peptide (Fig. 1). Staphylokinase (SAK) is a highly fibrin-specific fibrinolytic protein (20) and should transport the chimera to a fibrin clot and thus to a factor Xa rich environment. The antiplatelet and antithrombin peptide can then be released by factor Xa to inhibit additional platelet aggregation and fibrin formation. The peptide was designed to contain three inhibitory regions. Firstly, on its N-terminus it has the RGD-sequence for binding to the fibrinogen receptor (Gp IIb/IIIa) and so prevents platelet aggregation (21). That is followed by a part of fibrinopeptide A (residues 8-16) to inhibit the active site of thrombin (22) and the C-terminus of hirudin (residues 54-65) to bind to and block the anion binding site of thrombin (23). *In vitro* the chimera significantly inhibited the action of thrombin and activated plasminogen, but had no marked effect on platelet aggregation (19).

In this study we evaluated the antithrombotic effect of PLATSAK in a baboon model of arterial and venous thrombosis (24). The baboon was used because the composition and function of its hemostatic apparatus closely resembles that of humans (25). Thrombogenic devices consisting of Dacron vascular graft material and an expansion chamber were inserted as extension segments into chronic arteriovenous (AV) shunts (26). The effect of a high dosage of PLATSAK on platelet deposition was investigated in real time by scintillation camera imaging of the deposition of ^{111}In-labeled platelets.

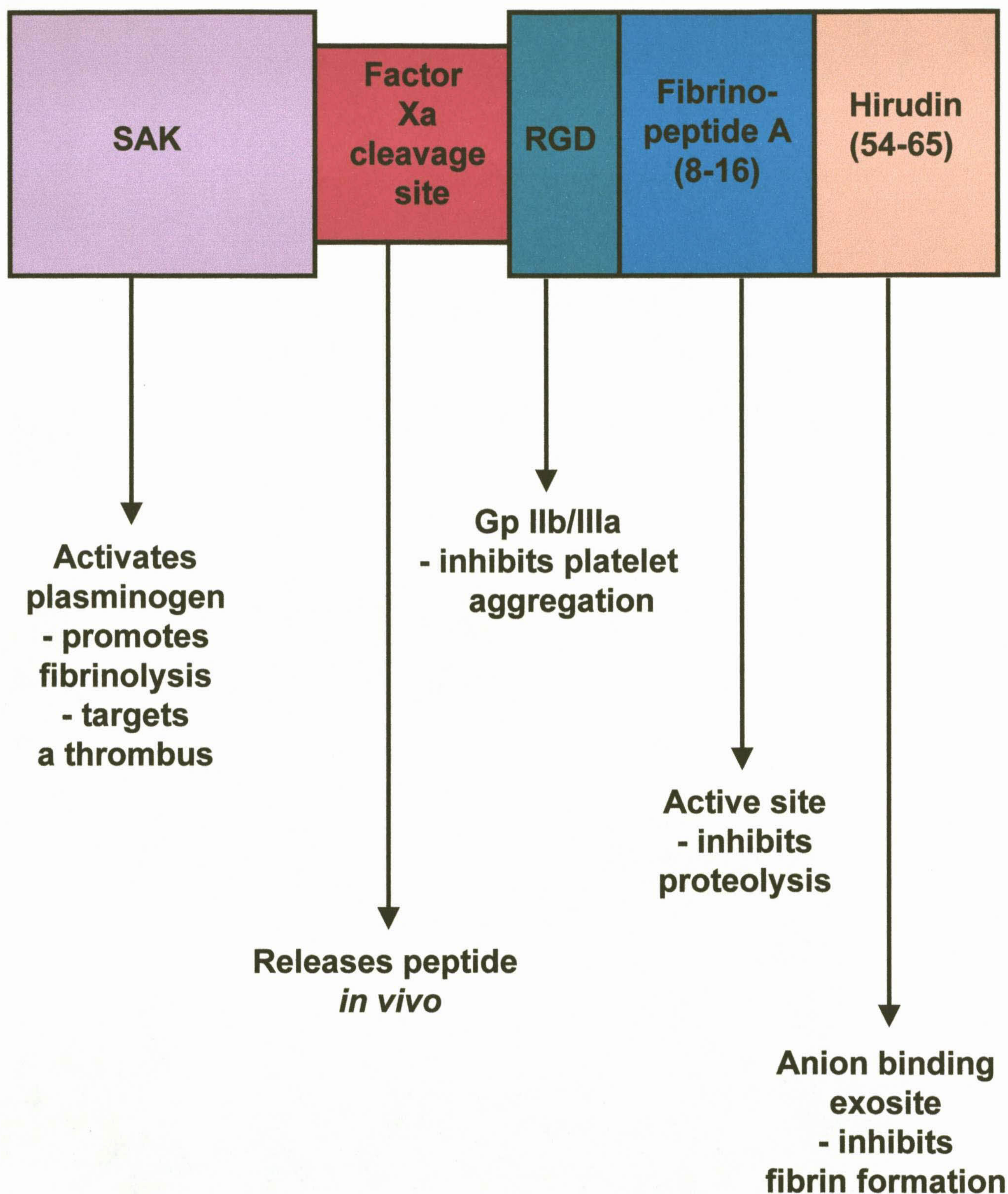


Fig. 1 Schematic representation of the composition of PLATSAK and the function of the individual components. The staphylokinase part is linked to the antiplatelet and antithrombotic peptide via the recognition sequence of factor Xa.

Materials and Methods

Preparation of PLATSAK

PLATSAK was recombinantly produced in *E. coli* cells and cultivation was performed as described in detail (19). The cells were harvested by centrifugation (3000xg for ten minutes) and proteins were extracted by repeated cycles of freezing and thawing (27). This procedure led to an enrichment of the recombinant proteins and the proteins could be extracted in any buffer of choice. In this study the proteins were dissolved in saline. The enriched fractions were run on SDS-PAGE gels and the percentage of PLATSAK present was determined by densitometry (Hoefer Scientific Instruments, San Francisco, USA). The extract contained 46% PLATSAK. In order to exclude possible effects of contaminating *E. coli* proteins on thrombogenesis, cells that were transformed with the empty vector were treated in exactly the same manner as the PLATSAK producing cells. These extracts were then used in the control baboons. Protein concentrations were determined using the BCA Protein Assay system from Pierce (Rockford, Illinois, USA).

Animals studied

Three normal male baboons (*Papio ursinus*) were used in this study. The animals weighed approximately ten kg and were disease-free for at least six weeks prior to use. To enable handling, the baboons were sedated with approximately 1 mg/kg ketamine hydrochloride (Premier Pharmaceuticals, Johannesburg, SA). The peripheral blood platelet count was normal (371 ± 112 $X\ 10^9/l$). All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa. The baboons supported

permanent Teflon-Silastic AV shunts implanted in the femoral vessels (24). These shunts do not detectably shorten platelet survival or produce measurable platelet activation (25).

Study protocol

The experimental setup is schematically presented in Fig. 2. Thrombogenic devices were prepared as previously described (24,26). A piece of Dacron vascular graft material (1.26 cm^2) served as a generator of platelet-dependent arterial-type thrombosis. An expansion chamber (3.77 cm^2) was used to generate coagulation-dependent venous thrombosis. Initial wall shear rates were approximately 318 sec^{-1} for the graft section and less than 10 sec^{-1} for the expansion chamber. Under these conditions the devices cause arterial-type platelet-dependent and venous-type coagulation-dependent thrombosis, respectively (26). The thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment in the permanent AV shunt, using Teflon connectors (24).

The control baboons were treated with an equimolar bolus of the freeze-thaw extract from *E. coli* cells that did not produce PLATSAK to show that the proteins did not affect thrombogenesis. The bolus was administrated ten minutes before control devices were placed. The devices were kept in place for 60 minutes or until it occluded, where after they were removed and blood flow through the permanent AV shunt re-established. Sixty minutes later the baboons were treated with a bolus of 3.68 mg/kg PLATSAK to attain a plasma concentration of approximately 0.10 mg/ml plasma. After ten minutes the thrombogenic devices were placed for 120 minutes, whereafter they were removed to re-establish blood flow through the permanent AV shunt.

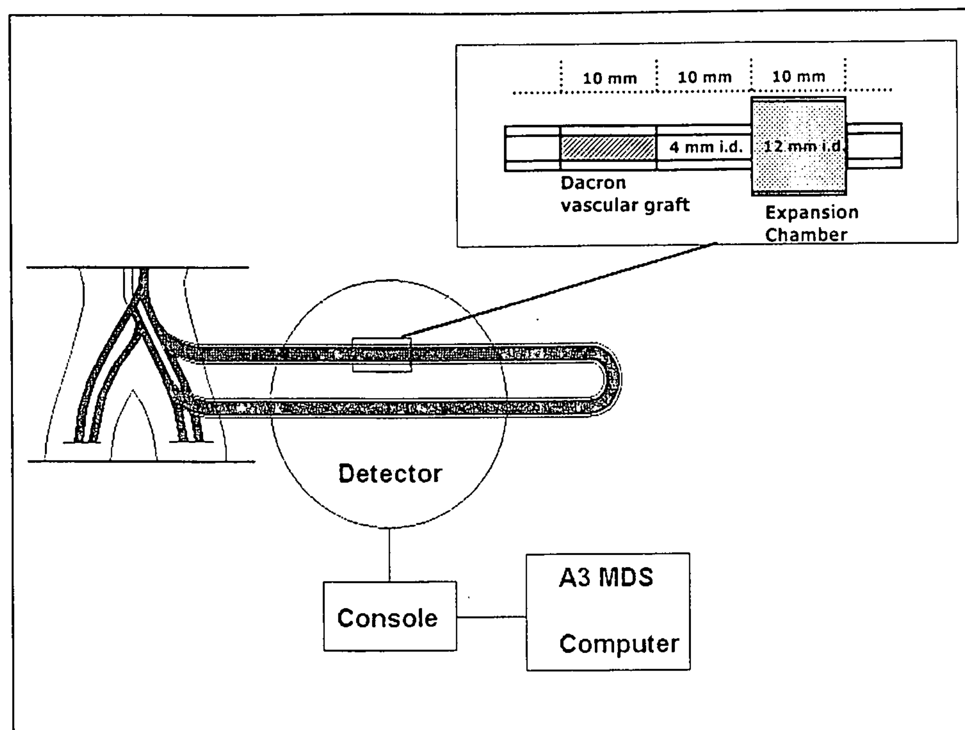


Fig. 2 A schematic representation of the experimental set-up to study platelet deposition. The enlarged Dacron graft was used to simulate arterial thrombosis, while the enlarged expansion chamber was used to simulate venous thrombosis.

Graft imaging and quantification of platelet deposition

Autologous platelets were labeled with ^{111}In-tropolone as previously described (28). Imaging and quantification of ^{111}In-platelets were done as described (24). In short, image acquisition of the grafts was done with a Searle Pho scintillation camera fitted with a high resolution collimator. The images were stored on and analyzed with a Medical Data Systems A^3 computer (Medtronic, Ann Arbor, MI) interfaced with the scintillation camera. Dynamic image acquisition, three minute images (128 X 128 byte mode) for one hour in the control studies and two hours in the PLATSAK studies, was started simultaneously with the placement and start of blood flow through the devices. A three-minute image of a three ml autologous blood sample was also acquired each time the grafts were imaged to determine circulating blood radioactivity (blood standard). This blood was transferred to a thrombogenic device and imaged in the same geometry as the thrombogenic device incorporated into the AV shunt. Regions of interest of the graft and expansion segments were selected to determine the deposited and circulating radioactivity in the dynamic image. Radioactivity in regions of similar sizes was determined for the graft and extension segments of the blood filled device to determine circulating radioactivity. This was subtracted from the radioactivity in the graft and extension regions to calculate deposited radioactivity. The total number of platelets deposited was calculated as described (9,12,24).

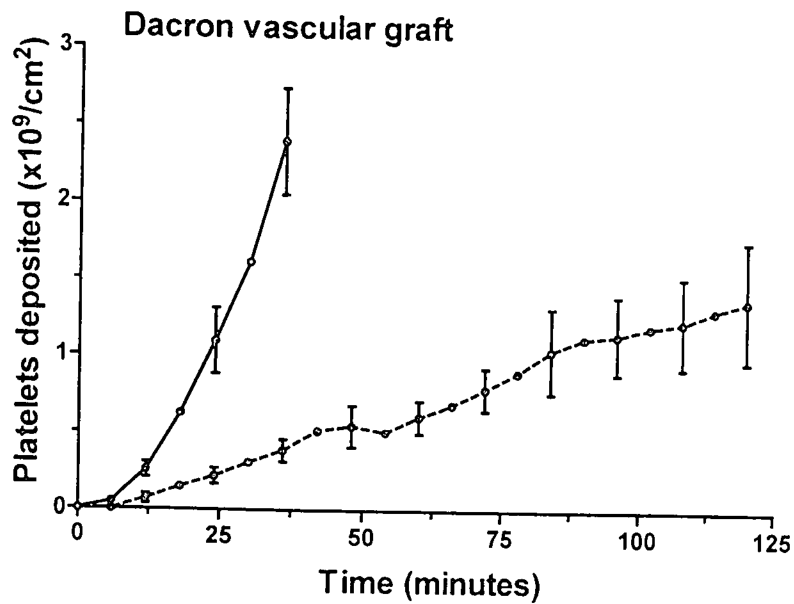
Laboratory measurements

The times at which blood samples were collected can be seen in the results section. The platelet count of blood samples collected in 2 mg/ml disodium EDTA were determined with a Technicon H2 blood cell analyser (Bayer Diagnostics, Tarratown, NY). The same blood sample was also used to determine whole blood and plasma radioactivity. Blood radioactivity was corrected by subtracting plasma radioactivity in order to calculate platelet

radioactivity (24). The radioactivity in the different samples was determined in a well-type scintillation counter. Blood was also collected in 3.8% sodium citrate (nine volumes blood to one volume citrate) to determine activated partial thromboplastin time (aPTT), plasma levels of the thrombin-antithrombin III (TAT) complex and fibrinogen degradation products (FDP). The aPTT was measured on a fibrinometer (Clotex II, Hyland Division, Travenol Laboratories, Costa Mesa, CA) using reagents supplied by the manufacturer. TAT complexes were determined using an ELISA-method (Enzygnost Combipack, Behring, Marburg, Germany) as recommended by the suppliers. FDP in plasma was determined semi-quantitatively using a latex agglutination method following the manufacturer's instructions (Diagnostica Stago, Asnieres-Sur-Seine, France).

Results

The results on platelet deposition are summarized in Fig. 3. In the three control studies the thrombogenic devices occluded after 42, 39, 42 minutes. Whereas platelet deposition onto the graft segment started almost immediately, there was a delay of approximately six minutes before they started accumulating in the expansion chamber. In addition, the graft segments accumulated approximately six times more platelets than the expansion chambers at the end of the control studies. Treatment with PLATSAK inhibited platelet deposition onto the Dacron vascular graft by approximately 84% after 39 minutes, i.e. at the time of occlusion of the control devices. Platelet deposition was however not completely interrupted, but deposited at a much slower rate than in the control studies, i.e. $1.43 \pm 0.71 \times 10^7$ platelets/cm^2/min versus $8.85 \pm 1.83 \times 10^7$ platelets/cm^2/min. The inhibitory effect of PLATSAK on platelet deposition in the expansion chambers was more pronounced, i.e. 94% less platelets deposited at 39 minutes than in the control studies. In addition, the start of deposition was delayed by approximately twelve to fifteen minutes. The rate of platelet deposition following treatment was also much slower than in the control studies, $0.22 \pm 0.12 \times 10^7$



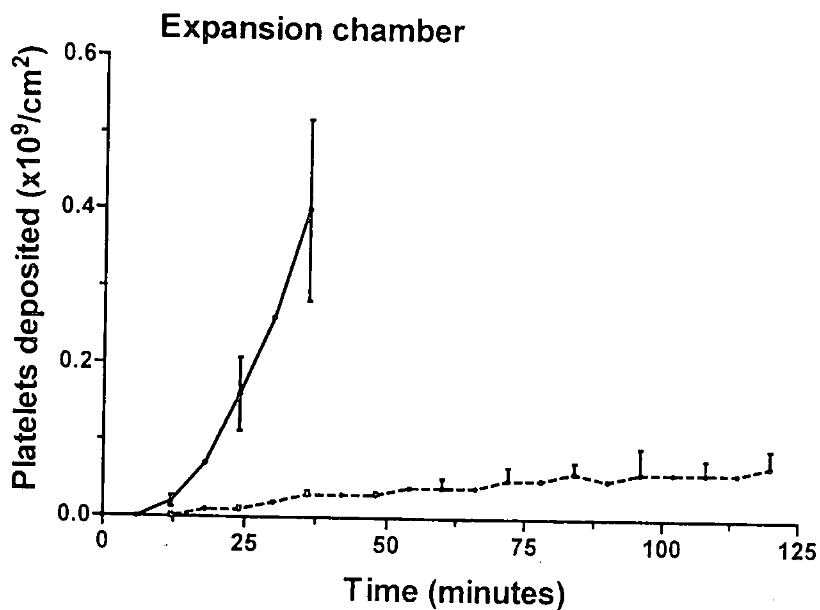


Fig. 3 The effect of PLATSAK on platelet deposition in control animals (—) and after treatment (----). Values are given as a mean ± SEM.

platelets/cm^2/min versus 4.39 ± 1.97 x 10^7 platelets/cm^2/min. None of the thrombogenic devices occluded during the 120 minutes of the study after treatment with PLATSAK.

The changes in platelet count, aPTT, TAT complexes and FDP levels are summarized in Table 1. Infusion of the freeze-thaw extract from PLATSAK non-producing *E. coli* decreased the platelet count slightly to approximately 80%. Placement of the thrombogenic devices caused a further decrease of approximately 21% during the 39 to 42 minutes that the devices remained potent. After treatment, placement of the thrombogenic devices for 120 minutes, decreased the platelet count by 8%. The aPTT was not affected by thrombogenesis in the control studies. It was markedly prolonged by treatment with PLATSAK, especially towards the end of the study. The TAT complexes increased approximately twenty-fold in the control study. After the thrombogenic devices were removed, and before placement of the second thrombogenic device 30 minutes later, it decreased to normal values. After treatment with PLATSAK, placement of the thrombogenic devices increased the TAT complexes two- to three-fold. The changes that we observed in the plasma levels of FDP are of interest. The test only became positive toward the end of the control study. Thereafter, the FDP levels remained constant in spite of treatment with PLATSAK.

Discussion

The thrombogenic devices that were used in this study typically represents conditions under which an arterial-type (graft segments) and a venous-type (expansion chambers) thrombus forms. The wall shear rate in the graft segment, $\approx 320\ sec^{-1}$, approximates that found in medium to large sized arteries, while the wall shear rate in the expansion chamber, $< 10\ sec^{-1}$, represents that found in the venous blood vessels (26,29). The finding that platelet deposition onto the graft segment was approximately six-fold more than in the expansion chamber (Fig. 3)

Table 1: Summary of the changes that were observed in platelet count, aPTT, TAT complexes and FDP levels. (Mean ± SD; ND = not done)

Study	Time (min)	Platelet count (x 10^9/l)	aPTT (sec)	TAT (μg/l)	FDP (μg/ml)
Control	Pre-bolus	371 ± 112	33 ± 3	13.1 ± 4.4	Neg
	Pre-graft	340 ± 94	34 ± 3	21.5 ± 15.5	Neg
	10	ND	34 ± 4	36.6 ± 16.7	Neg
	39 - 42	268 ± 55	40 ± 5	251.5 ± 26.9	20
PLATSAK	Pre-graft	267 ± 55	65 ± 8	27.6 ± 16.8	20
	10	ND	72 ±12	25.5 ± 13.4	20
	120	244 ± 62	> 120	61.0 ± 21.0	20

*p<0.05, students t-test. End versus pre-graft.

further indicates arterial-type, platelet-dependent thrombogenesis versus venous-type coagulation-dependent thrombogenesis (29). One must also bear in mind that anticoagulants such as heparin and low molecular weight heparin markedly inhibit platelet deposition in the expansion chamber, but has little efffect on platelet deposition onto graft or collagen surfaces (4,5,8,26). On the other hand, blockade of the Gp IIb/IIIa receptor markedly inhibits platelet deposition onto the graft surface (4,6), but not in flow expansion chambers (4).

We have no indication that the extract from PLATSAK non-producing *E. coli* cells inhibited platelet deposition in the control studies. Firstly, platelet deposition onto the graft segments and in the expansion chambers was rapid (Fig. 3) and all three devices occluded within 42 minutes following their placement. Secondly, the number of platelets deposited onto the graft surface and in the expansion chamber compares favourably with the results obtained in studies where similar

devices were used (26,29). Thirdly, there was no significant lengthening in the aPTT (Table 1). The injected proteins also did not cause thrombocytopenia since the peripheral blood platelet count was not markedly affected by infusion (Table 1).

PLATSAK markedly inhibited platelet deposition onto the graft segment and in the expansion chamber when the thrombogenic devices were placed ten minutes after a bolus infusion (Fig. 3). In both cases the onset of platelet deposition was delayed when compared to the control studies. Furthermore, none of the devices occluded during the 120 minutes of the study. The differences that were observed in the inhibition of platelet deposition are of interest. At 39 minutes, when the control devices occluded, platelet deposition onto the graft segment was inhibited by approximately 84% and in the expansion chamber by approximately 94%. At 120 minutes inhibition was approximately 50% and 85%, respectively, when compared to the number of platelets deposited at 39 minutes in the control studies. These results suggest that the extent of inhibition of platelet deposition onto the graft segment was markedly less than in the expansion chamber. However, when inhibition of the rates of platelet deposition are compared, it seems that inhibition onto the graft surface and in the expansion chamber was equivalent. In the case of inhibition of platelet deposition onto the graft surface, the rate of deposition was inhibited by 83 ± 8%. The rate of platelet deposition in the chamber was inhibited by 94 ± 4%, i.e. slightly, but not significantly, more than in the case of the graft surface. This is difficult to explain, especially in view of our *in vitro* results with PLATSAK (19), where we have shown that the antithrombin activity was much more pronounced than the antiplatelet activity. One must also bear in mind that direct inhibition of thrombin with, for example hirudin, requires a much higher dose to inhibit platelet-dependent thrombosis than coagulation-dependent thrombosis (30). Therefore, one would have expected that the effect of PLATSAK should have been more pronounced in the expansion chamber since the *in vitro* studies suggest it (19) and *in vivo* results with direct thrombin inhibition support it (30). A plausible

explanation for our finding that platelet-dependent and coagulation-dependent thrombosis was equivalently inhibited by PLATSAK may be that its antiplatelet effect is more pronounced *in vivo* than *in vitro*. Alternatively, one may conclude that aggregation studies *in vitro* is not entirely suited to test antiplatelet effects of the blockade of the Gp IIb/IIIa receptor, especially when the antiplatelet effect is not very pronounced. It was clearly shown that not all platelets have to be functional to cause a normal platelet aggregation in response to agonists added *in vitro* (31).

Ten to twenty minutes after the infusion of PLATSAK the aPTT lengthened from approximately 33 seconds to 65 seconds to 72 seconds. It only lengthened to >120 seconds towards the end of the study (Table 1). In general, when an antithrombin is infused, the aPTT lengthens to near steady state levels within ten to fifteen minutes (9,32). We do not know if the design of PLATSAK can explain the results of treatment on the aPTT. The antithrombin/antiplatelet peptide is coupled to SAK with a factor Xa cleavage site (Fig. 1). It is plausible that when coupled to SAK, the antithrombin activity of PLATSAK is not optimal. Initially, before placement of the thrombogenic device and during the first ten minutes after the device was placed, most of the antithrombin part could still be attached to SAK, because not enough factor Xa is available to free the peptide. At later stages, on the other hand, factor Xa cleavage could provide enough of the peptide to inhibit thrombin and so lengthen the aPTT.

Treatment with PLATSAK either decreased the production of thrombin, or PLATSAK formed complexes with thrombin to make it inaccessible for binding to antithrombin III, or both. After treatment with PLATSAK, appreciably less TAT complexes formed (Table 1). FDP in plasma could only be measured at the end of the control studies, indicating some activation of fibrinolysis through normal mechanisms. It does not appear that SAK resulted in additional activation of

fibrinolysis, since the FDP levels in plasma does not seem to increase in the studies were PLATSAK was used (Table 1). One must however not overinterpret these results since the method to measure FDP is semi-quantitative.

We have not measured indices that could assess if PLATSAK increased the risk of developing a bleeding tendency. There were indications that it was the case. When we removed the permanent AV shunts after each study, we had some difficulty to stop bleeding at the sutures where the shunt access sites were closed. We do not know whether this was the result of inhibition of thrombogenesis by the peptide or activation of fibrinolysis by SAK. Indications are that it was activation of fibrinolysis. Firstly, the half-life of small peptides is very short (5,8), while that of SAK is relatively long (20). Secondly, one baboon developed a haematoma at the site of surgery 24 hours after the AV shunt was removed and bleeding effectively stopped. If it was indeed the case, one must redesign the thrombus targeting characteristic of PLATSAK.

In conclusion, we have shown that PLATSAK inhibited platelet deposition in both arterial- and venous-type thrombosis. Our results show that it is feasible to combine different antithrombotic strategies with success into the same molecule. We could not distinguish which strategy was responsible for inhibition. The results do, however, indirectly suggest that both the inhibition of thrombin and blockade of Gp IIb/IIIa played a part. It does not appear that fibrinolysis contributed to the lower number of platelets that were deposited. One must bear in mind that these conclusions are based on the results obtained in three baboons. In view of the difficulties to produce sufficient amounts of PLATSAK, and because the effect of PLATSAK on platelet deposition was so dramatic, we decided not to study more baboons. The results on the three baboons were regarded as sufficient proof that combination of different inhibitory moieties into one molecule is a viable strategy for the development of antithrombotic drugs.

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CHAPTER 7

CONCLUSIONS

My aim was to design and express a synthetic gene encoding an antithrombin and antiplatelet peptide and to evaluate its *in vitro* activity and *in vivo* potential. The chemically synthesized gene was fused downstream from the gene encoding staphylokinase (SAK), separated only by the recognition sequence of factor Xa (Fig 1.1). SAK was fused to the peptide to enhance its stability towards proteolytic degradation during production in *Escherichia coli* and furthermore, to add fibrinolytic activity to the range of activities of the chimera. The resultant chimeric protein was designated PLATSAK (PLatelet-AntiThrombin-StAphyloKinase) and had fibrinolytic, antithrombotic and antiplatelet activity. Since SAK is highly fibrin-specific, it can transport the chimera to a fibrin clot and thus also to a factor Xa-rich environment. The high fibrin-specificity of SAK enabled the development of PLATSAK as a local drug delivery system. Six consecutive histidine residues were added immediately downstream of the C-terminus of the peptide to use as an affinity tag during metal affinity chromatography to aid purification of PLATSAK.

Production of the chimeric protein in *E. coli* was successful and PLATSAK comprised 46% of the intracellular protein content after one hour of the induction of gene expression. Metal affinity chromatography was used to purify PLATSAK to homogeneity. *In vitro* tests showed that PLATSAK was able to inhibit thrombin activity markedly. The mechanism of interaction of PLATSAK with thrombin is not clear and it is not certain whether the antithrombin activity is due to the hirudin or to the FPA components. It can be assumed that the tail of hirudin will bind to the anion binding exosite of thrombin (Naski *et al*, 1990; Rydel *et al*, 1990) and the FPA sequence to the active site (Martin *et al*, 1992). However, it is unlikely that one molecule of PLATSAK would be able to inhibit both the exosite and the active site of a single thrombin molecule. *In vitro*, the antiplatelet activity was negligible, most likely due to an unfavourable three-

dimensional folding of the protein. Attempts at repositioning the RGD-motif to the C-terminus or the N-terminus of PLATSAK were made, but there was no success in improving the *in vitro* antiplatelet activity. However, more attempts at improving the *in vitro* antiplatelet effect need to be performed. More RGD-motifs could be placed at strategic sites on the molecule. The three-dimensional structure of SAK can be utilized to determine protruding loops for placing RGD-motifs at the tips of these loops. This strategy will hopefully result in the positioning of RGD-motifs at optimal sites for interaction with its receptors on activated platelet membranes. Data obtained by thrombelastography showed that the fibrinolytic activity of PLATSAK was slightly lower than that of recombinant SAK. It is possible that the addition of the antithrombotic peptide to the C-terminus of SAK led to a slight distortion of its tertiary structure or may somewhat interfere in the binding of PLATSAK to plasmin(ogen).

To test the activity of a drug in an animal model, it is preferable to use a preparation of the highest possible purity. For the *in vivo* evaluation of PLATSAK, the purification procedure used at that stage had to be optimized for large-scale purification. Although different resins, different metals and different elution procedures were tested, none proved to be sufficient for purifying the large amounts of purified proteins that were required for the *in vivo* studies. This lack of a suitable purification method left no other choice than to use an enriched intracellular extract of PLATSAK-producing *E. coli* cells for the animal studies.

Three baboons were studied in a well-established model of arterial and venous thrombosis (Cadroy *et al*, 1989) to evaluate the *in vivo* effectivity of PLATSAK on the inhibition of platelet deposition. The thrombogenic devices that were used typically represent conditions under which an arterial-type (using graft material) and a venous-type (using expansion chambers) thrombus form. In the control studies an equimolar extract of native *E. coli* proteins were administered as a bolus and haemostasis was not markedly affected. The same baboons were subsequently treated with a bolus of 3.68 mg/kg PLATSAK. After 120 minutes inhibition was approximately 50% on the graft material and 85% in the expansion chamber, when compared to the number of platelets deposited at 42

minutes in the control studies. Although no significant inhibition of platelet aggregation could be observed in the *in vitro* studies, it appeared that platelet aggregation was markedly inhibited by PLATSAK in the *in vivo* model. This illustrated that results of *in vitro* platelet aggregation studies not necessarily predict the *in vivo* antiplatelet effectivity as is also suggested by Cerskus et al (1980). aPTT was lengthened to more than 120 seconds, indicating significant inhibition of thrombin. Furthermore, appreciably less TAT complexes were formed, indicating less thrombin available for complex formation with antithrombin III. FDP appeared in the plasma towards the end of the control studies and did not increase after treatment of PLATSAK. It thus appeared that PLATSAK did not result in additional activation of fibrinolysis during the two hours of the study. However, removal of the permanent AV-shunts at the end of the study was accompanied by some bleeding, suggesting that activation of fibrinolysis by PLATSAK was taking place.

The combination of different activities into one molecule is not in concert with the current pharmacological approach. Most agents presently studied for their potential pharmacological use, are focused on only one specific activity, like hirudin for the inhibition of thrombin (Markwardt, 1994), annexin V for inhibition of factor Xa (Thiagarajan and Benedict, 1997), integrelin for the prevention of platelet-platelet interactions (Tcheng *et al*, 1995) and staphylokinase for the activation of plasminogen (Collen et al, 1996). Furthermore, due to its size and composition, PLATSAK is highly immunogenic and can only be administered once. To enhance its ability as a potential antithrombin and fibrinolytic agent the SAK portion need to be reduced to the minimum structure required for activity. The published structure of SAK in complex with μ-plasmin can be used to determine the parts of SAK involved in binding (Jespers *et al*, 1999). A minimal molecule with sufficient fibrinolytic and a low immune response can thus be constructed. A multifunctional agent like PLATSAK is not likely to be used as a routine antithrombin and will only be used under very specific and strictly controlled circumstances. The strict monitoring of the effect of PLATSAK on haemostasis can only be done in hospital circumstances. Due to its potent

activity it will not be used as precaution, but rather as treatment during procedures like endarterectomy and angioplasty. Furthermore, it may also be used for the treatment of thrombotic events like deep venous thrombosis.

In order to ascertain the exact *in vivo* potential of PLATSAK the large scale purification should be optimized, probably by using alternative fusion systems. The evaluation of other fusion, expression and purification systems, however, falls outside the boundaries of the current study. Effective large scale purification of PLATSAK will enable me to study more animals to determine a dose dependent response and to evaluate possible side-effects and bleeding tendencies.

Since this study was aimed at the construction, production and determination of the pharmacological value of a novel antithrombotic agent, it did not give new insights into the complex interactions of haemostasis and thrombosis. However, the interesting finding that both arterial and venous thrombosis was equivalently inhibited by PLATSAK might shed some light on the respective thrombotic mechanisms. This needs to be investigated with further *in vivo* studies. The important contribution of this study is that it proved possible to design a multifunctional recombinant chimeric protein that can modulate haemostasis and thrombosis in animal models. The study verified that it is feasible to combine different haemostatic strategies with success into the same molecule. The results obtained on three baboons were regarded as sufficient proof that combination of different inhibitory moieties into one molecule is a viable strategy for the development of antithrombotic agents.

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SUMMARY

Platelets and coagulation both play a pivotal role in thrombosis, one of the major life-threatening diseases in our society. We have thus experienced a drastic increase in the development of potent and secure antithrombotic, antiplatelet and fibrinolytic agents during the past decade. Recently, much research has been devoted to the development of chimeric proteins, where haemostasis is simultaneously targeted at different levels. For the purpose of this study, such a chimera, named PLATSAK, was designed. A 29 amino acid antithrombotic and antiplatelet peptide, comprising three inhibitory regions, was linked to staphylokinase via a cleavable factor Xa recognition sequence. The overall activities of PLATSAK should include inhibition of thrombin, prevention of platelet aggregation and activation of fibrinolyis.

The gene encoding PLATSAK was expressed in *E. coli* cells under controlled conditions. PLATSAK was produced as a strongly expressed protein of 18 kDa and was purified form native *E. coli* proteins using metal affinity chromatography. *In vitro* analysis of PLATSAK activity revealed strong inhibition of thrombin and potent fibrin degradation. However, no effect on platelet aggregation could be observed. Several attempts at producing more potent antiplatelet variants were unsuccessful.

According to its *in vitro* activity, PLATSAK appeared to be a potent novel haemostatic agent and was prone to be evaluated in an *in vivo* system. The *in vivo* activity of PLATSAK was evaluated by assessing its effect on platelet deposition in a baboon model of arterial and venous thrombosis. Dacron vascular graft segments and expansion chambers, inserted as extensions into permanent femoral arteriovenous shunts, were used to simulate arterial and venous thrombosis, respectively. PLATSAK (3.68 mg/kg) was administered as a bolus. Platelet deposition onto the graft surface and in the expansion chamber was imaged in real time with a scintillation camera as the deposition of ^{111}In-labelled platelets. After two hours, platelet deposition in the graft

segments and expansion chambers was inhibited by 50% and 85% respectively when compared to control studies. The aPTT was lengthened to >120 seconds. Interestingly, the level of FDP in plasma did not increase after administration of PLATSAK. These results demonstrate that PLATSAK effectively inhibited platelet deposition in both arterial- and venous-type thrombosis an animal model. This is in contrast to the lack of the antiplatelet activity of PLATSAK *in vitro*. This illustrates that *in vitro* platelet aggregation results can not be directly applied to an *in vivo* situation.

In summary, the recombinant production of a multifunctional haemostatic fusion protein, PLATSAK, was successful. *In vitro* PLATSAK showed significant antithrombin and fibrinolytic activity, but trivial antiplatelet activity. *In vivo* studies revealed that PLATSAK is a potent antithrombin and also prevented platelet deposition on thrombogenic material. The strong immuun response of PLATSAK however needs to be investigated and a variant with a weak immunogenic nature needs to be produced.