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Targets of Antithrombotic and Fibrinolytic Therapy

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INTRODUCTION

The delicate balance between thrombotic and anti-thrombotic activities in maintaining hemostasis while preventing thrombosis is regulated by a variety of cells and by specific cell-mediated mechanisms. The endothelial cell plays an active role in the negative control of thrombus formation. For example, the endothelial cell synthesizes both prostacyclin[1] and endothelial cell-derived relaxing factor[2] which inhibit platelet activation and vasoconstriction. Plasminogen activator, which initiates blood clot lysis, is also synthesized and released from endothelium[3].

However, when the vascular endothelium is damaged platelets become activated and undergo a series of changes: adhesion, shape change, secretion of constituents of their storage granule such as ADP, serotonin and arachidonate metabolites, thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2), further aggregation to form a hemostatic platelet plug[4]. With the changes in platelet membrane during secretion and aggregation, blood coagulation pathway is also activated[5]. The generated thrombin is a powerful platelet stimulant as well as having the ability to catalyze fibrin formation, which consolidates the plug with strands of fibrin finishing the hemostasis process.

On the other hand, the same process governing hemostasis is apparently involved in the progression of atherosclerosis, acute coronary artery syndromes such as unstable angina, myocardial infarction, and sudden ischemic death and also in cerebrovascular disease causing stroke[6, 7]. Thus, any damage to the vessel wall from atherosclerosis, thermal injury, or infectious agents, etc. is interpreted by this process in the same way as when a vessel is damaged. The resulting platelet activation may result in formation of mural or occlusive thrombi. When the location of the thrombus occurs in the heart or brain, it may be immediately fatal or seriously dehabilitating.

In order to prevent platelet activation in this pathological process, several approaches have been used as antithrombotic therapy, namely, inhibition of von Willibrand factor (vWF)-Glycoprotein (GP)I_b[8], fibrinogen-GPII_bIII_a[9], thrombin activation[10], and thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) activation[11]. Also, for the treatment of thrombosis, several plasminogen activators (PAs) such as tissue-type PA (t-PA) and urokinase-type PA (u-PA, two-chain and single-chain) and streptokinase (SK) have been applied to dissolve the fibrin clot as fibrinolytic therapy[12].

TARGETS OF ANTITHROMBOTIC THERAPY

Several receptors on platelet membrane mediate platelet responses upon stimulation. These include GPI_b for vWF, GPII_bIII_a for fibrinogen, TXA_2/PGH_2 receptor and thrombin receptor. Because of the importance of these receptors in platelet responses, inhibitors of these receptors can be developed as effective antithrombotic agents. Understanding of the molecular mechanism of the ligand-receptor interaction has greatly aided development of the new antit-

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hrombotic agents.

vWF-GPIb

The first event related to the participation of vWF in thrombogenesis is the recognition of surface-bound vWF by platelets through the GPIb receptor. This not only promotes the initial platelet contact with the thrombogenic surface but also mediates activation of GPIIbIIIa, which supports platelet aggregation[13].

Therefore, it is apparent that the selective inhibition of vWF binding to GPIb may be an appropriate early intervention likely to result in effective reduction of the thrombi formed on exposure of subendothelial vWF to flowing blood.

Progress has been made in identifying the binding domains of vWF and GPIb, which can be used for the rational design of the selective inhibitors of vWF-GPIb interaction. A tryptic fragment of 52/48 kD comprising residues Val^{449}-Lys^{728} has been demonstrated to interact with GP Ib[14]. Within the GP Ib binding domain of vWF, two discontinuous sequences corresponding to Cys^{474}-Pro^{488}, Leu^{694}-Pro^{708} are thought to be involved in GP Ib binding[15]. These peptides which are separated in the linear sequence by 205 residues and maintained in close spacial proximity in the folded molecule by disulfide bonding appear to be functionally optimal in combination (Fig. 1). In this regards, recombinant GPIb-binding domain of vWF ($rvWF^{445-733}$), synthetic peptides representing linear sequence of vWF involved in the GPIb binding (Cys^{474}-Pro^{488}, Leu^{694}-Pro^{708}) and monoclonal antibody against binding domains of vWF and GPIbα can be used to inhibit vWF-GPIb interaction. Further studies on the structure-function relations using site-directed mutagenesis is likely to provide the information necessary for the design of peptides and small organic molecules aimed at specifically interfering with platelet adhesion and aggregation.

Fibrinogen-GPIIbIIIa

Fibrinogen binding to platelet GPIIbIIIa is activation dependent, which includes the conversion of the platelet glycoprotein GPIIbIIIa from latent state to active conformation[16]. As a consequence of fibrinogen binding to its receptor, platelet aggregation can ensue, which is a final common pathway shared by a variety of stimuli, such as ADP, thrombin or epinephrine[17]. Platelet aggregation is a key event in thrombus formation and under certain pathologic conditions, platelet aggregation can result in thrombus formation and ultimately stenosis of the vessel. Thus, antagonists of fibrinogen binding to GPIIbIIIa are potential therapeutic agents.

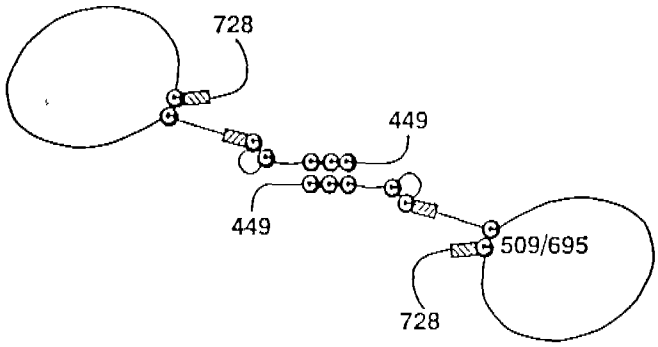


Fig. 1. Schematic representation of the glycoprotein Ib-binding domain of von Willibrand Factor

The first fibrinogen receptor antagonists were monoclonal antibodies, e.g., PAC-1[18] and 10E5[19] or F(ab')2 which bind to GPIIbIIIa with high affinity and inhibit platelet aggregation[20]. In view of the physiologic and pathophysiologic significance of ligand binding to GPIIbIIIa, major emphasis has been placed on identifying fibrinogen-GPIIbIIIa binding sites. Two sets of peptides, corresponding to specific amino acid sequences within fibrinogen referred as "recognition peptides", RGD[21] and gamma chain peptides[22], inhibit ligand binding to GPIIbIIIa and platelet aggregation.

Besides, potent platelet aggregation inhibitors termed "disintegrin" have been isolated from a wide variety of viper venom[23]. These peptides all contain RGD recognition sequence with one exception (KGD sequence in case of barbourin with apparent GPIIbIIIa specificity[24]) and high cysteine content conserved in primary sequence (Fig. 2). However, these are 500~1,000 times more potent inhibitors of platelet aggregation than linear RGDX peptides, which indicates that other structural determinants than recognition sequence are important for binding. The solution structure of kistrin[25] which is a member of disintegrin superfamily has been demonstrated a series of tightly packed

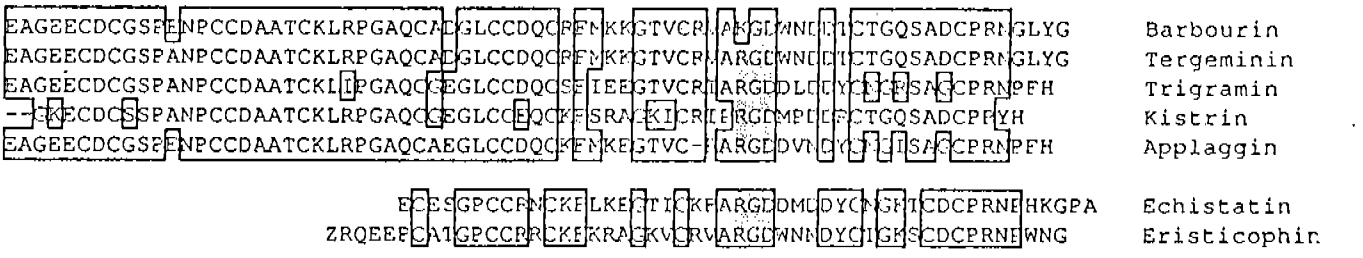


Fig. 2. Sequence alignment of viper venom GPIIbIIIa antagonists (disintegrin)

loops held together by disulfide bonds and RGD recognition sequence located at the apex of a long loop across the surface of the protein. These results indicate favorable conformational restraints of the RGD recognition sequence is required for enhanced binding to GPIIbIIIa and aided in the design of small synthetic peptide GPIIbIIIa antagonists.

Modification of linear recognition sequences has yielded several peptide antagonists and their structure-activity relationship has been studied[26, 27]. Only modest improvements in potency were achieved with linear analogues of pentapeptide, Ac-Gly-Arg-Gly-Asp-Val-OH, while dramatic results were achieved with constraining the conformation of peptides by cyclization, culminating in the cyclic disulfide Ac-Cyclo-S,S-(Cys-(N-Me)Arg-Gly-Asp-Pen)-NH_2 and Cyclo-S-Ac-(D-Tyr)-Arg-Gly-Asp-Cys-OH (sulfoxide bridge) which yielded equipotent antagonists as kistrin (Fig. 3).

Therefore, these "recognition peptides" and potent mimetics of these peptides have a potential as antithrombotic drugs.

TXA_2/PGH_2 activation

The arachidonic acid metabolites PGH_2 and TXA_2 released by platelets in response to a variety stimuli are potent vasoconstrictor and platelet activating agents, which serve as an amplification mechanism in the platelet activation sequence[28].

The profound biological actions of TXA_2/PGH_2 suggest its putative role as a pathological mediator of cardiovascular disease[29]. Several clinical studies have suggested that TXA_2 is involved in a number of cardiovascular diseases as demonstrated by measuring the metabolite, TXB_2. Clearly,

Ac-Arg-Gly-Asp-Val-NH_2
Ac-Cyclo-S, S-(Cys-Arg-Gly-Asp-Cys)-NH_2
Ac-Cyclo-S, S-(Cys-(N-Me)Arg-Gly-Asp-Pen)-NH_2

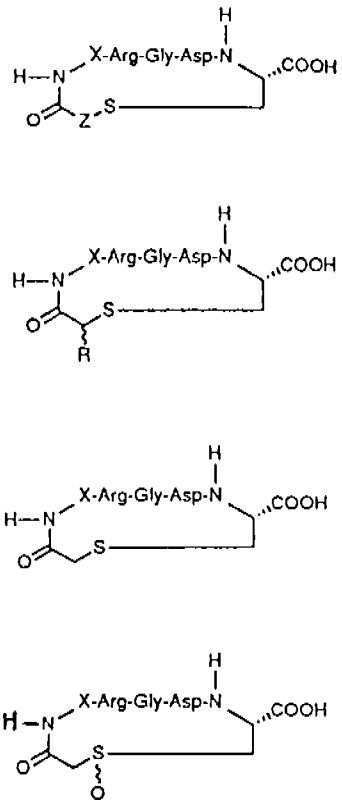


Fig. 3. Structure of small peptide GPIIbIIIa antagonists

the regulation of TXA_2/PGH_2 synthesis or its interaction at a specific receptor has potential clinical implications.

There have been several approaches to the control of PGH_2/TXA_2 activation: cyclooxygenase inhibition, thromboxane synthase inhibition, and PGH_2/TXA_2 receptor antagonism[30]. However, cyclooxygenase inhibitors such as

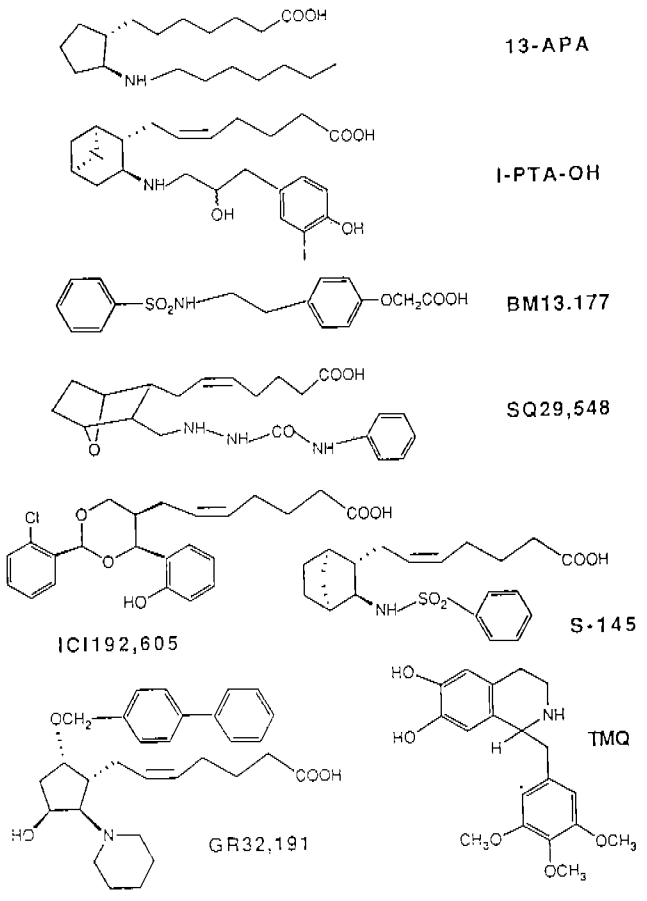


Fig. 4. Structure of TXA_2/PGH_2 receptor antagonists

aspirin can have the dual effect of reducing proaggregatory PGH_2/TXA_2 and antiaggregatory PGI_2. Also, in clinical trials thromboxane synthase inhibitors have been disappointing when compared to their efficacy *in vitro*. An explanation for the weak activity for thromboxane synthase inhibitors *in vivo* is that prostaglandin endoperoxide may substitute for the proaggegatory effect of TXA_2.

In this regard, TXA_2/PGH_2 receptor antagonism is the most plausible approach to selectively inhibit platelet activation induced by TXA_2/PGH_2. A number of TXA_2/PGH_2 receptor antagonists of diverse structure and potency have been reported (Fig. 4). The majority of TXA_2/PGH_2 receptor antagonists may be considered a typical alpha-side chain, but often with substantial modification of the omega -side chain. Of the many TXA_2/PGH_2 receptor antagonists, a few have been studied in humans, which include AH 23, 848, GR 32,191, BM 13,177, BM 13,505, SQ 31,491 and ICI 192,605[31]. Another approach to inhibit TXA_2/PGH_2 activation is dual inhibitors of thromboxane synthase and TXA_2/PGH_2 receptor by combining thromboxane synthase inhibition with thromboxane receptor antagonism in one drug[32]. These may provide benefits from the redirection of the prostaglandin metabolism to prostacyclin and PGD_2, which further increase the inhibitory potential on platelet and vasospasm.

Thrombin activation

In addition to its well-known role in cleaving fibrinogen to fibrin, thrombin is able to evoke biological responses from a variety of cells, including platelets, endothelial cells, and fibroblasts[33]. These multiple cell activating functions of thrombin may lead to orchestrate the response to vascular injury, mediating not only hemostatic but inflammatory and reparative responses. Platelet activation by thrombin at the sites of vascular injury, which is the most potent stimulator of platelet aggregation, is believed to be a critical event in arterial thrombosis[34]. Identification of the thrombin receptor revealed a unique proteolytic mechanism of receptor activation (Fig. 5)[35, 36] and led to the development of a novel agonist peptide capable of activating the thrombin receptor independent of thrombin and thrombin's protease activity[50] and will allow the development of thrombin receptor antagonists.

The anticoagulant effect of unfractionated heparin and most low molecular weight heparin is mediated primarily by enhancing the inactivation of thrombin by antithrombin III (AT III)[37]. However, thrombin which is bound to fibrin clot remains fully active and is resistant to inhibition by the AT III-dependent thrombin inhibitors[38]. In this regards, the AT III-independent thrombin inhibitors[39] have been developed. These include: hirudin, hirulog, synthetic thrombin inhibitors, activated protein C and thrombin receptor antagonists.

Hirudin, the most potent natural inhibitor of thrombin, was isolated from the European medicinal leech *Hirudo medicinalis*[40], which forms a stable noncovalent 1:1 stoichiometric complex with thrombin (K_i 10^{-11} to 10^{-14} M). Hirudin consists of a compact NH_2-terminal head and a long polypeptide COOH-terminal tail, which has been shown to interact with active site and anion binding exos-

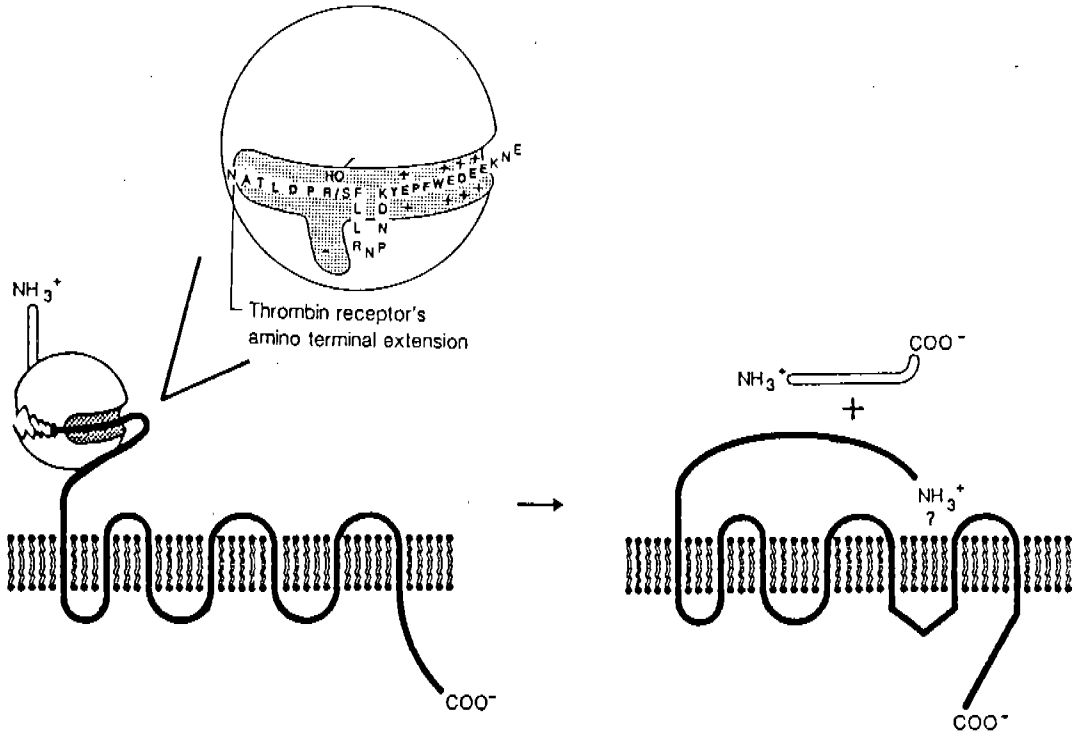


Fig. 5. Thrombin's binding interaction with its receptor and model of thrombin receptor activation

ite of thrombin, respectively (Fig. 6)[41, 42]. Based on the structure of hirudin, bivalent synthetic peptide called hirulogs have been designed[43]. Hirulogs consist of a catalytic-site inhibitor moiety ((D-Phe)-Pro-Arg-Pro), a flexible spacing segment ((Gly)n) and an anion binding exosite recognition moiety (COOH-terminal hirudin peptides) (Fig. 7). Studies on the optimal length of the oligoglycyl spacer showed that at least 3-4 glycines were necessary for optimal inhibitory activity. The anticoagulant activity of Hirulog-1 ((gly)$_4$) has shown to be comparable to that of hirudin. Besides the sequence ((D-Phe)-Pro-Arg-Pro), other structures such as nonpeptide groups or other peptide sequences could be employed for a catalytic-site binding moiety.

The synthetic low molecular weight thrombin inhibitors act by blocking the active site of the thrombin to eliminate its enzymatic activity. The catalytic mechanism of the serine protease, thrombin, and its substrate specificity allow the design of several types of inhibitors[44]. These can be classified as substrate-derived competitive inhibitors and mechanism-based inhibitors according to the kinetics of inhibition and the types of binding. A key structural feature of these inhibitors is arginine or a group mimicking this

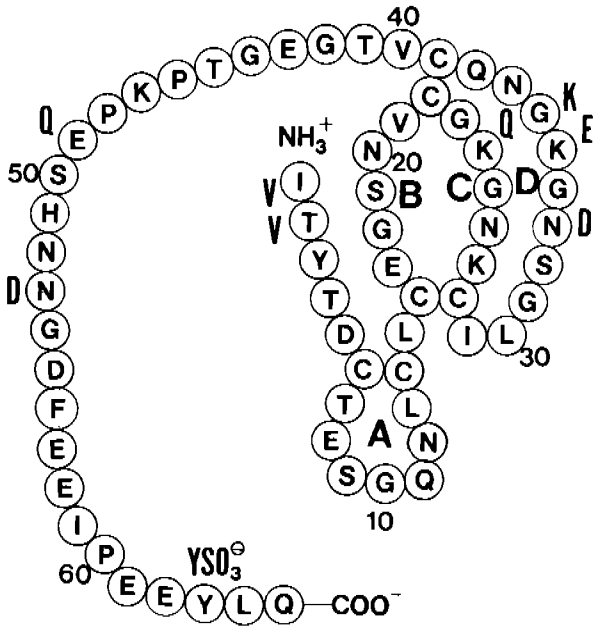
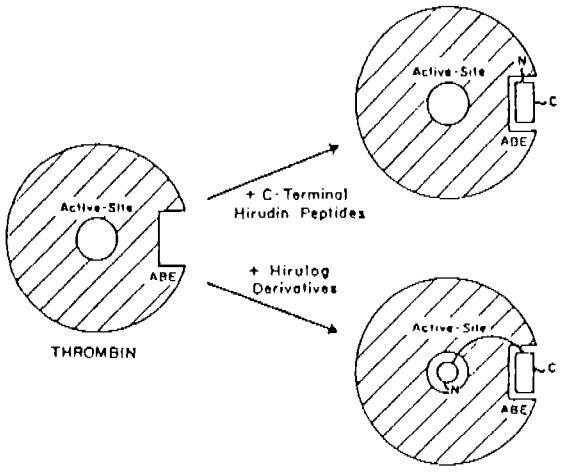


Fig. 6. The sequence of recombinant hiruding varient 2-Lys^{47} (rHV2-Lys^{47})



(D-Phe)-Pro-Arg-Pro-$(Gly)_n$-NGDFEEIPEEYL

Fig. 7. The sequence of hirulog and illustrative scheme of thrombin inhibition

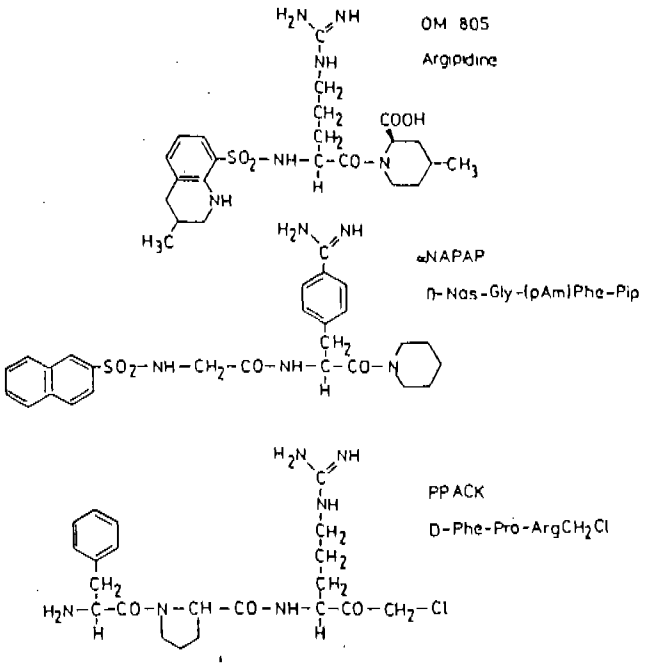


Fig. 8. Structure of prototype compounds of the arginine derivative, benzamidine derivative, and tripeptide-type synthetic thrombin inhibitors

amino acid, which governs the primary affinity of thrombin substrates. Figure 8 illustrates the structures of the most potent compounds of arginine- and benzamidine-derived inhibitors, namely, argipidine[45], αNAPAP[46] and PPACK[47].

Thrombin also triggers anticoagulant pathway by binding to thrombomodulin[48]. This initiate rapid protein C activation (Fig. 9). Activated protein C then complexes with protein S on the surface of either platelets or the endothelium. These complexes catalyzed the proteolytic inactivation of factors Va and VIIIa, which results in inhibition of thrombin formation. Besides, thrombomodulin accelerate the inhibition of thrombin by antithrombin III[49]. Therefore, activated protein C or regulation of thrombomodulin activity would prevent the coagulation responses.

Thrombin receptor blockers would be the most useful to prevent cell activation initiated by thrombin. Monoclonal antibodies against receptor's hirudin-like sequence, thrombin cleavage site or yet unidentified agonist peptide binding site can be obtained to block receptor activation[51]. In addition, uncleavable peptides mimicking thrombin receptor domain or competitive antagonists of the agonist peptide and their structure-activity relationship should be explored[52]. Further studies are required to elucidate arterial wall properties leading to thrombin generation and characteristics of thrombin inhibition for the development of a new class of antithrombotic drugs.

Targets of Fibrinolytic Therapy

The blood fibrinolytic system essentially consists of proteolytic enzyme activation processes leading to the formation of the fibrin degrading enzyme plasmin, and thus is the counterpart of the blood coagulation system. The proenzyme plasminogen can be converted to the active enzyme plasmin by plasminogen activators (PAs), which both are counter balanced by inhibitors in order to protect the body from systemic proteolysis. The fibrinolytic and antifibrinolytic activity are determined primarily by the balance between PAs and plasminogen activator inhibitor (PAI), which are under control of hormones and growth factors[53].

One approach to the treatment of thrombosis is the pharmacological dissolution of the blood clot via the intravenous infusion of PAs. Two physiologic PAs, t-PA and u-PA have been identified[54, 55] and recombinant t-PA (rt-PA), recombinant single-chain u-PA (rscu-PA), two-chain u-PA (tcu-PA), streptokinase (SK) and anisoylated plasminogen streptokinase activator complex (APSAC) are either approved for clinical use or under clinical investigation[56].

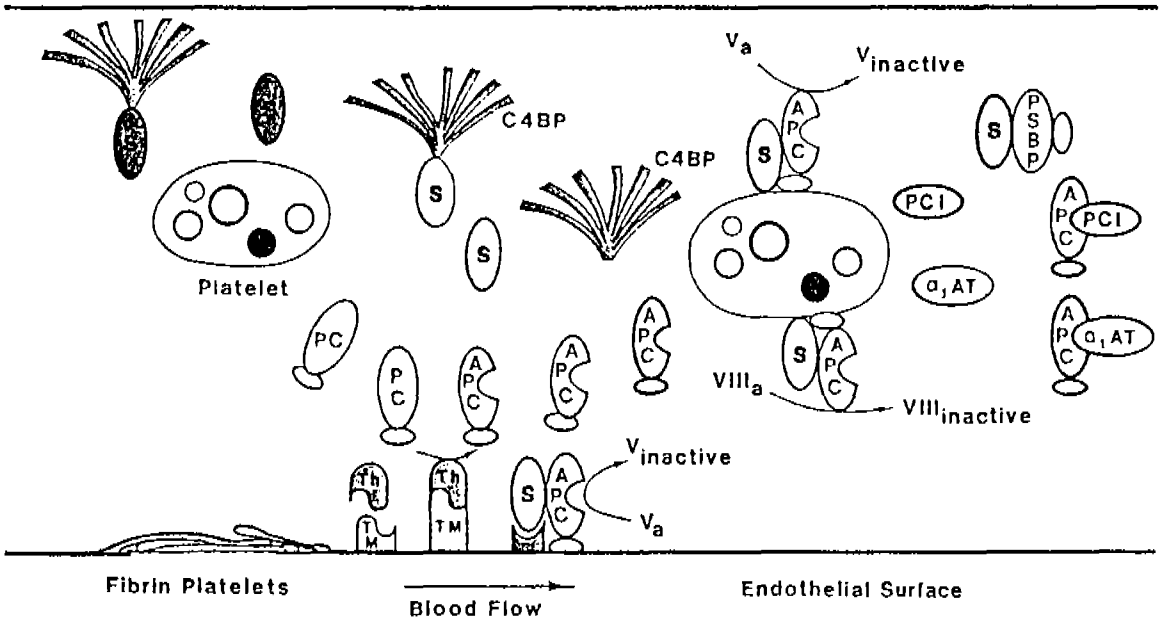


Fig. 9. Formation and function of activated Protein C

However, all available fibrinolytic agents still suffer significant limitations including large therapeutic doses, a limited fibrin specificity, a very short plasma half-life as a result of rapid hepatic clearance, and bleeding complication.

New developments towards further improved efficacy and fibrin specificity of fibrinolytic agents and, on the other hand, thrombolytic strategies to overcome resistance to clot lysis, to prevent reocclusion and to reduce bleeding tendency are being explored[57]. These include mutants and variants of rt-PA or rscu-PA, chimeric t-PA/u-PA plasminogen activators, and conjugates of plasminogen activators and either antifibrin or antiplatelet monoclonal antibodies. Several mutants of rt-PA obtained by deletion/substitution of functional domains of single amino acids have markedly reduced plasma clearance, but usually also reduced specific thrombolytic activity, resulting in unchanged or only marginally improved thrombolytic potency[57]. The t-PA of saliva from the vampire bat is highly homologous with human t-PA, but lacks K_2 and the plasmin cleavage site for conversion to a two-chain form[58]. It was found to be a potent and fibrin-specific thrombolytic agent, the activity of which was stimulated 45,000-fold in the presence of fibrin compared to 200-fold for human t-PA. The structural characteristics of bat-PA might serve to improve specificity of thrombolytic therapy.

A low molecular derivative of human scu-PA, scu-PA-32k, which lacks the amino terminal 143 residues, was found to be less pronounced systemic fibrinogen breakdown with comparable clot lysis[59]. Several chimeric PAs consisting of various portions of t-PA and u-PA have been constructed and characterized. These chimeras have maintained the enzymatic properties of u-PA or of t-PA but their fibrin specificity was very similar to those of scu-PA[60]. PAs conjugated with antifibrin or antiplatelet antibodies such as monoclonal antibodies against NH_2-terminal of the Bbeta-chain of fibrin[61] or against glycoprotein II_bIII_a[62] are targeted to blood clot, since thrombus contains both fibrin-rich and platelet-rich material. These conjugates resulted in a significantly increased thrombolytic potency. The use of conjugates of PAs and specific monoclonal antibodies may allow a significant reduction of the total dose of PAs and systemic side effects.

Concluding Remarks

Thrombosis plays a major role in acute ischemic syndromes of cardiovascular and cerebrovascular disease. Several approaches can be used to treat these disease, which might be antiplatelet, antifibrin, anticoagulant or combi-

nation of these. However, the choice of therapy should be based on the type of thrombus and its pathogenesis. Several lines of research towards improvement of antithrombotic and fibrinolytic agents should be explored based on the molecular mechanism of target molecules.

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