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Review Article

Recent advances in the status and targets of antithrombotic agents

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CONTENTS

Abstract

Cardiovascular diseases associated with intravascular thrombosis are the most common cause of death in both developed and developing countries. Clinical and experimental studies indicate that abnormalities in the normal blood flow, activation of platelets, coagulation cascade or fibrinolysis contribute to the pathogenesis of intravascular thrombosis/thromboembolism. Although the treatment strategy for thrombosis has improved with newer diagnostic and surgical tools, effective antithrombotic therapy with minimal side effects still poses a challenge to scientists around the globe. There has been an intense effort in the last decade to understand mechanisms involved in thrombosis and also to develop novel antithrombotic agents for the treatment and prevention of these disorders. Advanced understanding of the molecular mechanisms of thrombosis has helped in the development of newer and specific experimental models for evaluating the antithrombotic agents with high specificity. These developments are also expected to minimize time and the cost incurred on the newer potential antithrombotic drugs. This review describes various important targets for effective antithrombotic action. Methods for evaluating potential antithrombotic compounds against each target and status of the newer compounds developed in the respective classes are also covered.

Introduction

Cardiovascular diseases associated with intravascular thrombosis are the most common cause of death in both the developed and developing countries. Approximately 3 million individuals die each year in the U.S. from venous (deep vein thrombosis and pulmonary embolism) or arterial thrombosis (acute myocardial infarction and unstable angina).

Initiation of thrombosis is a complex phenomenon as evident from Figure 1. The final event, *i.e.*, thrombus formation, is however, primarily due to the activation of platelets and coagulation cascade. Etiology of each thrombotic disorder is unique, thus there have been intense efforts in the last decade to understand thrombosis and to develop novel antithrombotic agents for the treatment and prevention of these disorders.

The vascular endothelium actively maintains normal unperturbed blood flow by releasing nitric oxide (NO), prostacyclin, and also by expressing thrombomodulin (TM), a receptor for thrombin. Atherosclerotic plaque rupture or injury to blood vessels, however, exposes the subendothelial components such as collagen and von Willebrand factor (vWF), so that they may interact with platelet membrane receptors to promote adherence and activation. Effective understanding of the central role played by platelets in thrombus formation and new insights on the associated molecular mechanisms has provided an incentive for the development of site-specific antiplatelet/antithrombotic agents (1, 2).

Intravascular blood coagulation is intrinsically prevented in 3 steps. Firstly, thrombin is converted after binding to TM, the anticoagulant factor which leads to the rapid cleavage and activation of the anticoagulant protein C (APC). APC further downregulates formation of thrombin by inactivating the coagulation factor V (FV) and VIII (FVIII). Secondly, circulating proteinase inhibitors such as tissue factor pathway inhibitor (TFPI), C1 inhibitor (C1 INH) and antithrombin III (ATIII) retain the coagulation

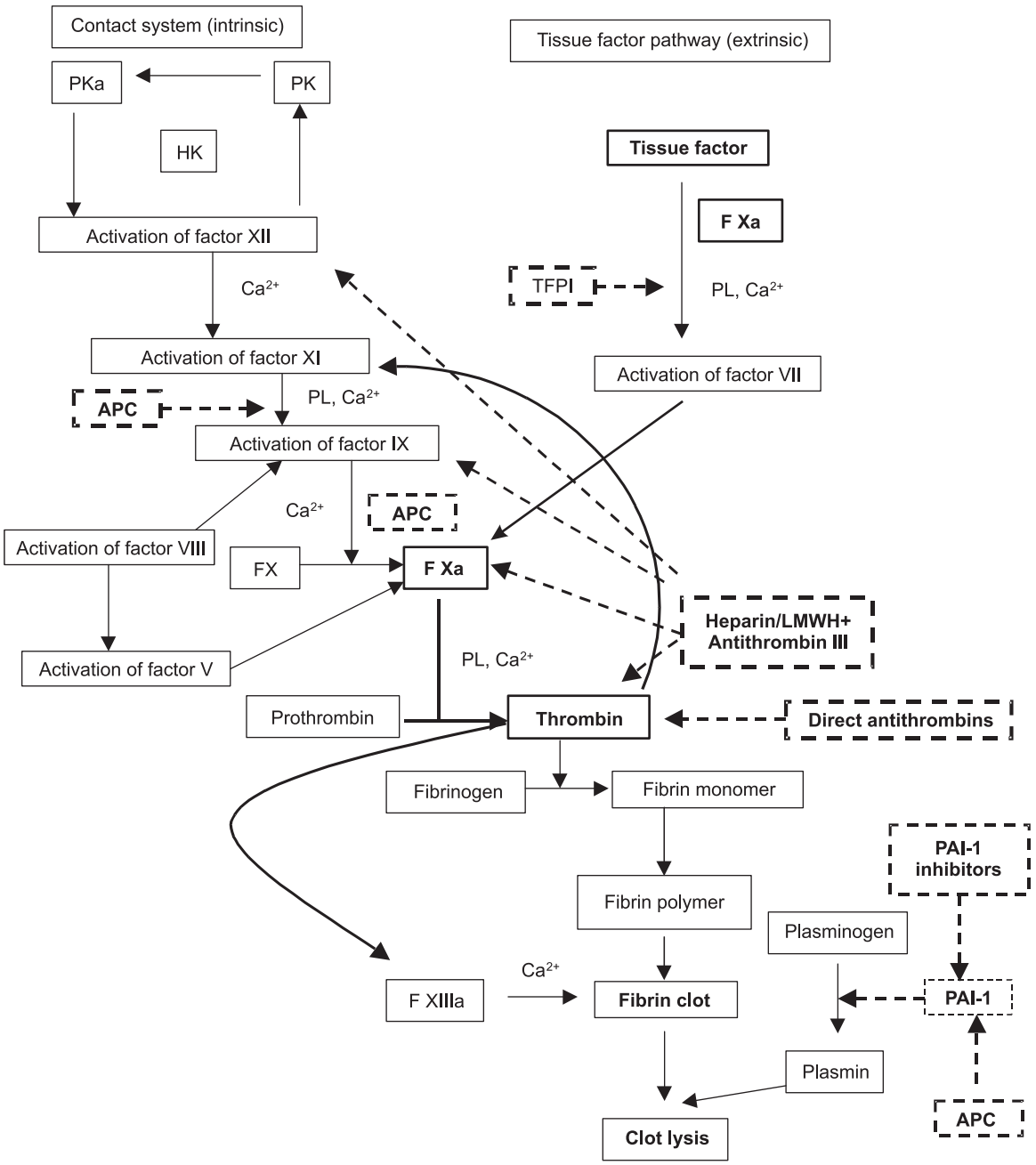

Fig 1. Blood coagulation cascade. The blood coagulation cascade consists of an extrinsic and intrinsic pathway. Inactive precursors in the pathway are activated in series and activated cofactors are designated as "a". Both pathways result in activation of factor X, which subsequently converts prothrombin to thrombin. The coagulation cascade is controlled by factors released from endothelium and circulating platelets. Activation is indicated by solid arrows and inhibition by dotted arrows. Various sites in the coagulation cascade that are inhibited by antithrombotic drugs are shown in bold boxes while the various antithrombotic classes are shown in bold, dotted boxes. F: factors, PL: phospholipase, APC: activated protein C, PAI-1: plasminogen activator inhibitor-1, LMWH: low-molecular-weight heparin, PK: plasma kallikrein.

factors in the inactivated state. Finally, activation of the plasma protein thrombin-activated fibrinolysis (TAFI) hinders fibrin inside the clot by removing carboxy-terminal lysine residues (3).

Plasmin has multiple actions including lysis of fibrin, inactivation of the FV and FVIII and activation of metalloproteinases (4). Conversion of plasminogen to plasmin initiates fibrinolysis. Tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are released from damaged endothelial cells to activate plasminogen (5). Plasminogen activity is regulated by plasmin inhibitors such as α_2-antiplasmin and α_2-macroglobulin

and also by type 1 plasminogen activator inhibitor (PAI-1), the primary physiological inhibitor of tPA and uPA. Even though the normal hemostasis maintains blood flow in the vessels, aberrations in platelet activation, coagulation cascade or fibrinolysis may lead to intravascular thrombosis, which must be prevented through pharmacological interventions.

Improved understanding of the conversion of dormant proteases to active serine proteases or coagulant factors and platelet activation mechanisms has made them suitable targets for the development of newer antithrombotic drugs. However, due to a vague understanding of the complex mechanisms involved in the maintenance of endothelial integrity, development of drugs directed to them still remains a distant hope. Availability of experimental animal models closely mimicking clinical situations, specific *in vitro* test systems and improved understanding of the mechanisms of thrombosis provides better opportunities to develop a potential antithrombotic drug with fewer side effects. An ideal antithrombotic compound should have the ability to inhibit propagation of thrombus and platelet aggregation and promote fibrinolysis with minimal side effects on coagulation parameters. A potential antithrombotic compound is evaluated during preclinical testing using different *in vitro* systems and various animal models.

The present review will discuss the targets of antithrombotic drugs with a brief description of the *in vitro* and *in vivo* tests to assess the antithrombotic efficacy as well as the status of new compounds under development.

Thrombin inhibitors

The serine protease thrombin is the key enzyme in the coagulation cascade. It converts soluble fibrinogen to insoluble fibrin and also activates clotting factors V, VIII, XI, XIII to further stimulate the coagulation cascade. In addition, proteolytic cleavage of thrombin receptors leads to platelet aggregation. Due to the multiple sites of action, thrombin has been recognized as an important target for drug development.

Intravenous heparin and oral coumarins, the indirect thrombin inhibitors, have been in use for the last 50 years and still remain the mainstays of anticoagulant therapy (6). Clot-bound thrombin, formed by the adsorption of thrombin to fibrin is resistant to indirect thrombin inhibition (7). Heparin administration also requires frequent monitoring to prevent hemorrhagic complications and is ineffective in patients with ATIII deficiency (8), while coumarins exhibit severe drug interactions. Limitations in the use of these drugs led to continuous research for the development of new antithrombotic agents with fewer side effects. First, low-molecular-weight heparins were developed which have more predictable dose responses, can be administered s.c. without laboratory monitoring and are effective against venous thrombosis and unstable angina (9, 10). Platelet membrane phospholipid accelerates the rate of thrombin production and a higher concentration of platelets in arterial thrombus compared to venous thrombi could lead to relatively greater local levels of thrombin. Thrombin inhibitors have shown better efficacy in blocking thrombus progression in veins and so far there is no clinical evidence for a superiority of direct thrombin inhibitors over heparin in platelet-rich arterial thrombosis (11, 12).

Mapping of thrombin domains by mutagenesis studies revealed the binding sites of the thrombin molecule. Crystallographic characterization of thrombin in its latent and fibrin bound state provided better insight into the design of molecules for effective thrombin inhibition and subsequently, direct site-specific inhibitors were developed (13, 14). Bifunctional antithrombins (hirudin, hirulog) bind to both the catalytic and the anion binding exosite while catalytic site antithrombins (RWJ-27755, argatroban) and exosite antithrombins (hirugen, aptamers) bind only to the catalytic or anion binding exosite, respectively (15). Hirudin, a thrombin inhibitor, was initially isolated from the saliva of leech. A hirudin-derived peptide, hirulog-1, has now reached advanced stages in clinical development (16, 17). However, these anticoagulants are expensive, need parenteral administration and have relatively short half-lives and thus are suitable only for acute care. The main advantage of these drugs is that their action is independent of circulating antithrombins and can effectively inhibit clot-bound thrombin (16). Another potential antithrombin agent is argatroban which has been recently launched for the treatment of peripheral arterial occlusive disease and acute ischemic stroke (18). It is a direct, potent and selective inhibitor of free and clot-bound thrombin and can inhibit thrombin-induced platelet aggregation (19-21). The potent antithrombotic effect of argatroban has been demonstrated in various animal models of venous and arterial thrombosis (Table I) and also in clinical trials as an adjunct to thrombolytic therapy in patients with myocardial infarction (22).

The above mentioned antithrombins need to be administered i.v. or s.c., thus warranting the development of orally active novel antithrombins. Direct thrombin inhibitors represent an emerging class of antithrombotic agents (Table II) that may have many of the qualities of an ideal antithrombotic and are currently being evaluated as an alternative strategy for treating and preventing thrombotic diseases (23, 24). Orally active thrombin inhibitors might be more effective with better therapeutic prospects and, hence, are the most sought after class of antithrombotics.

A thrombin inhibitor is required to block the actions of thrombin on both fibrin formation and platelet aggregation. High-throughput screening can provide a preliminary evaluation of the direct catalytic activity of an inhibitor of thrombin through examination of inhibition of amidolytic activity in both the fluid phase as well as in plasma clot-bound thrombin (25-27). Compounds are also tested for their specificity by simultaneously monitoring inhibition of other serine proteases such as trypsin, factor Xa, plasmin, *etc*. Potential leads may be subsequently evaluated in various *in vitro* and *in vivo* models as indicated in

Table I: In vitro *and* in vivo *models for evaluating antithrombotic drugs.*

Class of antithrombotic	Method	Ref.
Thrombin inhibitors	*Specific tests:*	
	Amidolytic activity of free and clot-bound thrombin *in vitro*	23, 24, 104
	$FeCl_2$/$FeCl_3$-induced thrombosis	105
	Coronary cyclic flow reduction	
	Nonspecific tests:	
	Venous thrombosis involving stasis/stasis plus injury	106
	Electric current/electrolytically induced thrombosis	107
	Vena cava occlusion model	
	Thrombin/TRAP-induced platelet aggregation	33
Factor Xa inhibitors	*Specific tests:*	
	Quantitative estimation of factor Xa inhibition by kinetic assays *in vitro*	108
	Arteriovenous shunt model	109
	Nonspecific tests:	
	Electrical stimulation-induced thrombus formation	110
	Photoirradiation-induced arterial thrombosis	111
Inhibitors of platelet mediated thrombus formation	*Specific tests:*	
	Inhibition of gpIIb/IIIa by high-throughput screening	
gpIIb/IIIa receptor inhibitors	Inhibition of TxA_2 receptor/synthase by competitive binding or kinetic assays	
Thromboxane receptor inhibitors and synthase inhibitors	Purinergic receptor binding	
	Elevation of cGMP levels and GC activity estimation	
ADP receptor antagonists	*Nonspecific tests:*	11
GC activators	$FeCl_3$-induced thrombosis	11
NO-aspirins	Electrolytically induced thrombosis	113-119
	Coronary cyclic flow reduction	66, 67
	Carotid artery thrombosis	118
	Photoirradiation-induced arterial thrombosis	119
	Arteriovenous shunt model	93
	$^{[111]}$In-oxine labeling of platelets	120
	Fluorescent dye-induced thrombosis	119
	Arterial thrombosis involving electrolysis and stasis	

Table I. These compounds are further characterized for their site specificity by X-ray crystallography of thrombin bound to the inhibitor. The most commonly used experimental model for thrombin inhibition is $FeCl_2$/$FeCl_3$-induced carotid artery thrombosis in rodents which involves occlusive arterial thrombosis due to the interactions between platelets, coagulation cascade and fibrinolytic systems (27, 28).

Since thrombin inhibition is likely to increase bleeding time, these compounds need to be monitored for various commonly used coagulation parameters such as activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT). These parameters help to optimize the dose of the test compound and are widely used as an index for monitoring the antithrombotic and adverse effects of thrombin inhibitors in animal models and clinical trials (29, 30). The sequence of steps in the development of a potent antithrombotic drug is outlined in Figure 2.

Thrombin, in addition to its procoagulant nature, also possesses anticoagulant properties. These anticoagulant properties are regulated by TM and/or sodium ion binding to the thrombin molecule (31). Thrombin's interaction with TM on the endothelial cell surface activates protein C and S, which inhibit factors Va and VIIIa and decreases PAI-1. It also modulates the inflammatory response associated with venous thrombosis (32). The anticoagulant effect of APC infusion has been investigated in a mouse inferior vena cava occlusion model (33). With the improved understanding of the vascular endothelial biology and coagulation factor function, APC concentrates appear to be promising antithrombotic and anticoagulant agents; sodium binding to the thrombin augments its procoagulant and prothrombotic potential (34). An ideal anticoagulant would thus be an agent that specifically inhibits only the procoagulant activity of thrombin (31). Small molecules that could modulate the allosteric nature of thrombin to avert its intrinsic procoagulant property and at the same time augment the anticoagulant efficacy to enhance the protein C pathway might be an attractive and future strategy to prevent thrombosis.

In addition to direct thrombin inhibition or augmenting the anticoagulant nature of the molecule, another efficient approach would be the inhibition of thrombin receptors by thrombin receptor antagonists. Thrombin activates various cell types such as platelets and vascular smooth

Table II: Developmental status of various antithrombotic agents.

Mechanism of action	Preclinical	Clinical trials	Discontinued	Launched
Direct thrombin inhibitors	>31	BMY-433921 C-18665 IK-HIR02 MCC-977 S-18326 BIBR-1048 CRC-200 CX-397 Efegatran Hirudin Melagatran	>16	Argatroban Bivalirudin Desirudin Antithrombin II
gpIIb/IIIa receptor inhibitors	>37	DMP-802 G-7453 S-1197 SDZ-GPI-562 TP-9201 ME-3277 TS-943 Lefradafiban YM-337 Elarofiban FK-633 Fradafiban Roxifiban Prourokinase	>17	None
Factor Xa inhibitors	>37	DPC-423 Tick anticoagulant peptide ZD-4927 DPC-906 DX-9065a ZK-807834	>5	Enoxaparin sodium Tinzaparin sodium
Other platelet related targets Thromboxane synthase inhibitors Thromboxane receptor inhibitors Platelet aggregation antagonists PAF antagonists gpIb receptor antagonists	>30	Aprosulate sodium CS-747 AT-1015 KT-2962 Samoxigrel MED-27 NCX-4016 Ifetroban Terbogrel Camonagrel TLC-C53 Foropafant KC-764	>32	Ramotroban

muscle cells via proteolytic processing of specific cell-surface receptors known as proteinase activated receptors (PARs), the prototype of which is PAR-1. Since the fibrin pathway is unaffected by thrombin receptor antagonism, thrombin antagonists might have minimal bleeding liability, an advantage over thrombin inhibitors. The currently available thrombin receptor antagonists fall into 3 categories: (i) peptide antagonists, (ii) peptidomimetics and (iii) nonpeptide thrombin receptor antagonists, and they have recently been extensively reviewed by Chackalamannil (2). Thrombin receptor antagonists exert antiinflammatory in addition to antiplatelet actions. These compounds are evaluated *in vitro* using radioligand binding assays and thrombin receptor activating peptide (TRAP)-induced platelet aggregation assays and *in vivo* in rat restenosis models (35). These agents are in preclinical stage of development and availability of compounds with oral activity and high affinity will provide better understanding of the future prospects of thrombin receptor antagonists.

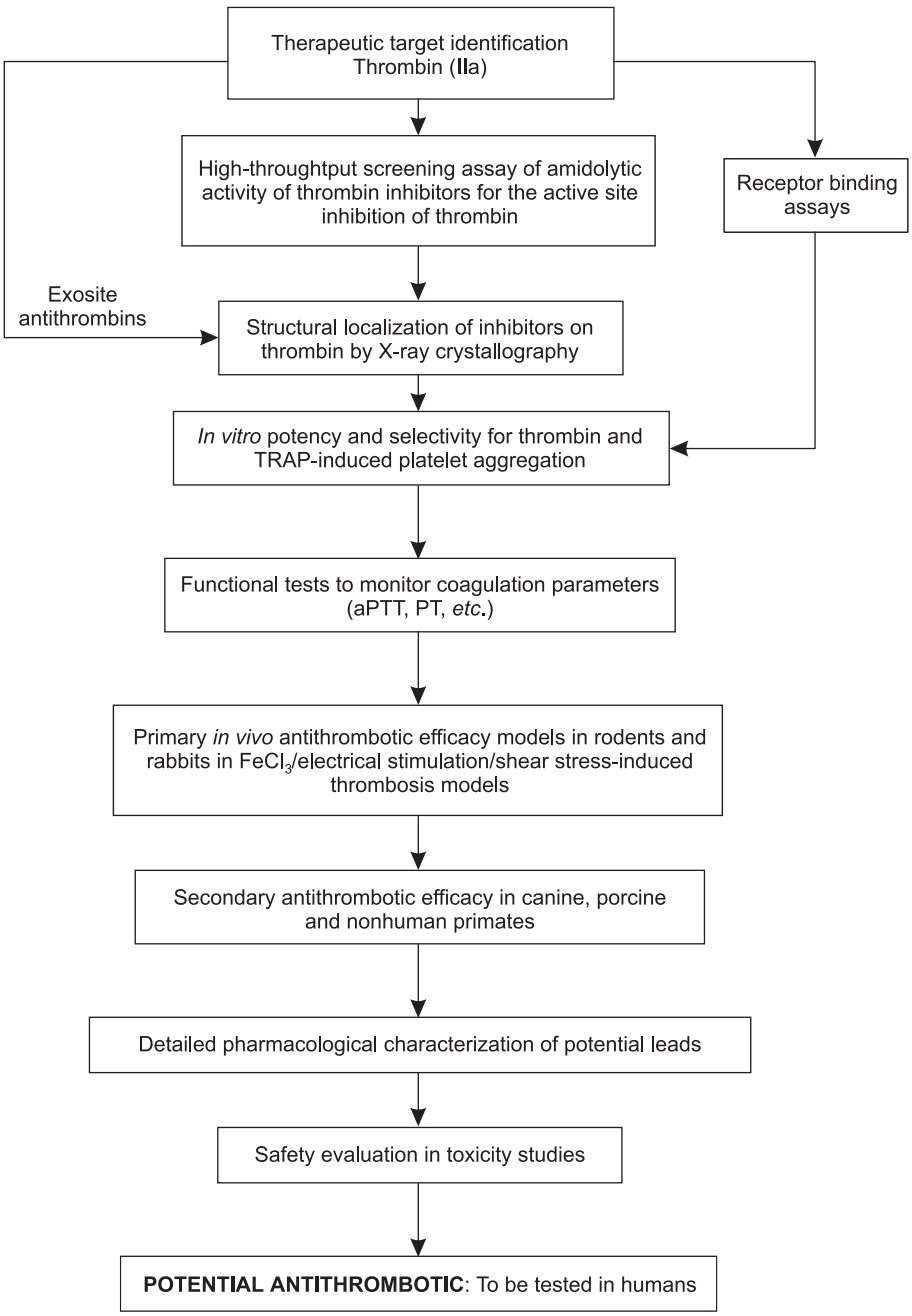

Fig. 2. Representative scheme of events in the preclinical development of an antithrombotic in a New Drug Development program. The development of an antithrombin is explained in detail as an example. aPTT: activated partial thromboplastin time, PT: prothrombin time, TRAP: thrombin receptor activating peptide.

Factor Xa inhibitors

Because one factor Xa molecule produces 138 molecules of thrombin, inhibition of factor Xa is an attractive approach in comparison to thrombin inhibition for preventing restenosis following thrombolysis. Inhibition of factor Xa seems to be the most logical step to effectively reduce thrombin production by extrinsic or intrinsic pathways without interfering with basal thrombin activity (36). Recent reports suggest that there is an increased interest in developing synthetic and selective Xa inhibitors (37-39).

Pharmacological characterization of molecules such as factor Xa inhibitors is done in a simple chromogenic assay involving examination of the serine protease activity of factor Xa. Factor Xa inhibitors like thrombin

inhibitors are also tested for specificity against other prevalent serine proteases. These inhibitors are subsequently subjected to testing in various animal models as shown in Table I and simultaneously for coagulation parameters. Both peptide and nonpeptide compounds have been evaluated for factor Xa inhibitory activity. Tick anticoagulant peptide and its recombinants exhibit Xa inhibition and anticoagulant effect in various venous and arterial models of thrombosis (40-42). The status of factor Xa inhibitors is outlined in Table III. Currently, focus is on the development of nonpeptide and orally active direct inhibitors of human factor Xa. With the clear understanding of the crystal structure of factor Xa (43), site-selective inhibitors are now under development (44) and some of them have shown significant selectivity over the reported compounds for the targeted factor Xa (45).

Factor Xa inhibitors inhibit the source of thrombin generation rather than its catalytic activity and have no direct effect on thrombin-activated platelet aggregation. They have minimum risks of bleeding, thrombotic reactivation rebound and associated ischemic events (46). A better picture of the therapeutic efficacy of this class of compounds over other classes will be known only after the clinical data is available.

Tissue factor inhibitors

Tissue factor (TF), a member of the cytokine receptor superfamily, is an integral membrane protein present on the surface of certain cell types outside the vasculature. Vascular injury exposes TF to the blood born factors VII/VIIa to which it binds with very high affinity and specificity and, in turn, generates thrombin. Factor VIIa is an extremely weak serine protease, but its enzymatic activity is enhanced more than a million fold following binding to TF (47). Since TF is an integral membrane protein, the TF:VIIa complex is always tethered to the membrane surface. Once the TF:VIIa complex forms, it triggers the blood coagulation cascade in 2 ways. Firstly, it converts factor IX to IXa via limited proteolysis. The newly generated IXa assembles on a phospholipid surface with its protein cofactor, factor VIIIa, to catalyze the conversion of factor X to Xa. Secondly, TF:VIIa can directly activate factor X to Xa to generate thrombin. A central role of TF has been demonstrated in models of disseminated intravascular coagulation induced by sepsis, arterial thrombosis overlying an atherosclerotic plaque and after coronary angioplasty (47, 48). Increased TF mRNA expression and activity has been observed throughout the arterial wall after balloon injury.

No synthetic inhibitors of TF have been reported to date. However, TF pathway inhibitor (TFPI), the principal physiologic inhibitor of the TF-factor VII/VIIa complex is found on endothelial cells and it prevents thrombosis (49). Recombinant TFPI (rTFPI) therapy prevents acute intravascular thrombosis, arterial reocclusion after fibrinolysis and intimal hyperplasia in models of arterial injury (50, 51). The protective effects of rTFPI were substantially potentiated in the presence of heparin and aspirin. Interestingly, rTFPI does not cause any prolongation of bleeding time, unlike direct thrombin inhibitors, and hence, it appears to be a promising approach for the development of better antithrombotic therapies alone or as adjuncts in the near future.

Inhibitors of plasminogen activator inhibitor-1

A critical component in thrombus formation and clearance is the balance between tPA and PAI-1 (52, 53). tPA-mediated generation of plasmin is a key event in the cleavage of fibrin and the subsequent restoration of normal blood flow. PAI-1 levels are higher in various disorders such as deep vein thrombosis, unstable angina, coronary artery disease and acute myocardial infarction (54). Administration of thrombolytics like streptokinase, urokinase or tPA have some inherent problems of inducing immunogenic response and significant increases in the bleeding time which leads to secondary complications. Thus, inhibition of PAI-1, the primary physiological regulator of tPA activity in plasma, seems to be a much safer strategy (52). Inhibition of PAI-1 is therapeutically beneficial as well, since it potentiates fibrinolysis. PAI-1 activity can be reduced by either decreasing PAI-1 production using antisense oligonucleotides to specific sequences of the PAI-1 gene (55) or by the inhibition of PAI-1 activity by generating inhibitory antibodies (56). Low-molecular-weight inhibitors of PAI-1 have been recently developed from the secondary metabolites that inhibit PAI-1 binding to tPA (57). These compounds are evaluated *in vitro* by a simple chromogenic assay (58) and also in simple rabbit venous thrombosis models (59). A few compounds are currently being developed as PAI-1 antagonists as well as plasminogen activator stimulants and this class is currently under use as thrombolytics.

Platelet gpIIb/IIIa receptor antagonists

The final step in platelet activation is the exposure of the platelet fibrinogen integrin receptor, gpIIb/IIIa, regardless of the nature of stimuli involved (Fig. 3). The peptide sequence arginine-glycine-aspartic acid (RGD) on the alpha chain of fibrinogen mediates binding to the receptor (60). Inhibitors of gpIIb/IIIa receptors were originally thought to be the most potent way to inhibit platelet activation *in vivo*. A monoclonal antibody directed against the gpIIb/IIIa receptor, c7E3 Fab or abciximab, was the first type of inhibitor of this receptor, effectively reducing ischemic complications after percutaneous intervention and attenuating platelet activation in patients after myocardial infarction (61). Other selective gpIIb/IIIa antagonists include the cyclic heptapeptide, eptifibatide (62, 63) and a nonpeptide mimetic of the RGD sequence, tirofiban (64). These agents are beneficial in the short-term management of acute coronary states but do not

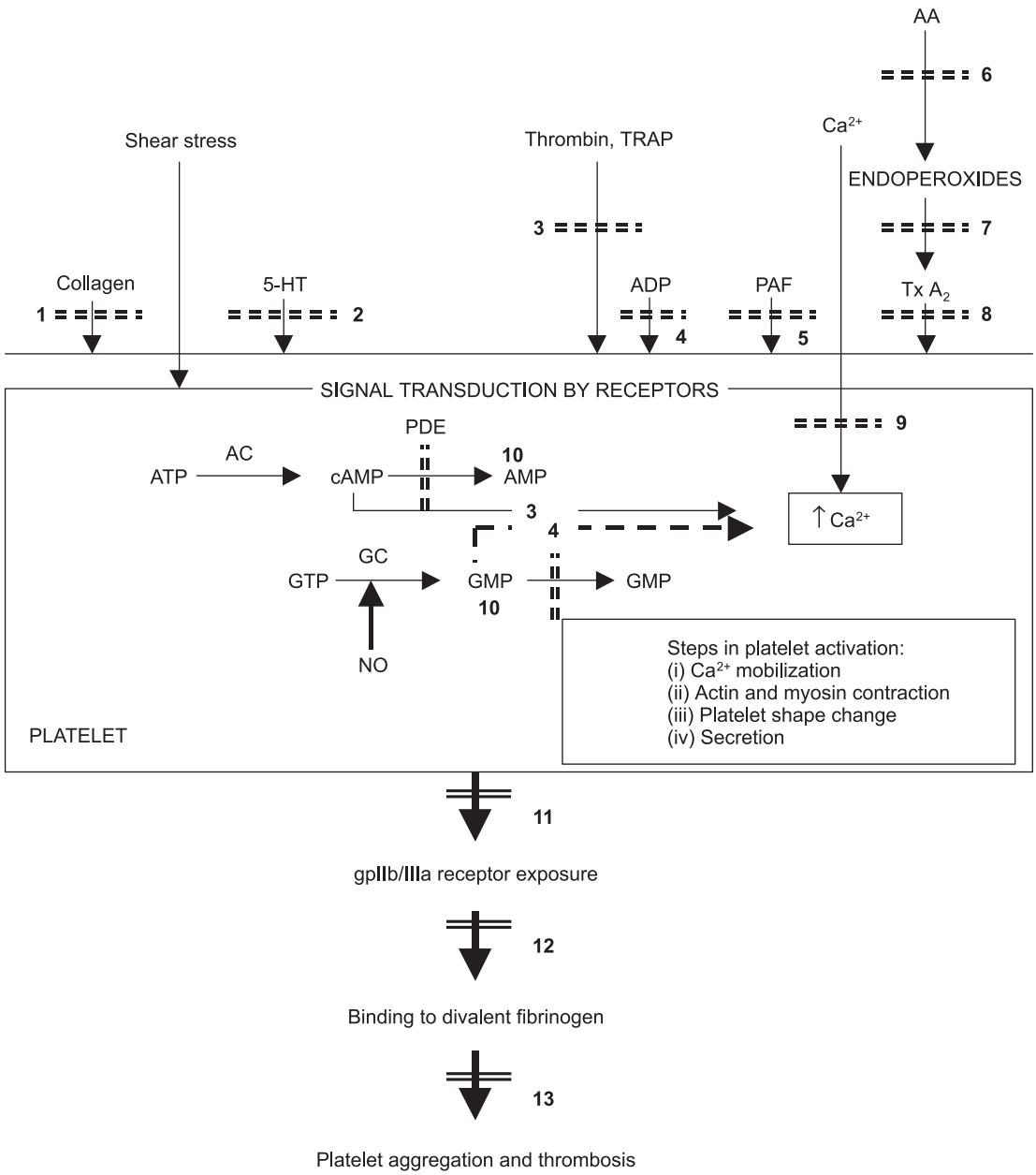

Fig. 3. Sequence of steps leading to platelet activation subsequently leading to thrombus formation. These stimuli act by different signal transduction mechanisms to increase intracellular Ca^{2+} level in platelets. Increase in Ca^{2+} leads to the exposure of platelet gpIIb/IIIa receptor which subsequently binds fibrinogen and causes aggregation. The various sites of intervention of antiplatelet/antithrombotic drugs are indicated by bold, double dotted arrows. The different classes of antiplatelet compounds in the figure are 1: collagen receptor inhibitors, 2: 5-hydroxytryptamine receptor inhibitors, 3: thrombin and thrombin receptor activating peptide (TRAP) inhibitors, 4: ADP receptor antagonists, 5: platelet activating factor antagonists, 6: cyclooxygenase inhibitors, 7: TxA_2 synthase inhibitors, 8: TxA_2 receptor blockers, 9: calcium channel antagonists, 10: cGMP/cAMP phosphodiesterase inhibitors, 11: von Willebrand factor receptor antagonists, 12 and 13: gpIIb/IIIa receptor antagonists.

exhibit prolonged inhibition. Hence, orally active gpIIb/IIIa receptor antagonists are aimed at providing benefits in the long-term treatment of chronic thrombotic events such as myocardial infarction and acute coronary syndromes (65).

Oral gpIIb/IIIa receptor inhibitors have limited bioavailability and many of them have a short half-life with an intermediate to fast receptor dissociation rate. Moreover, none have been shown to be superior over aspirin and heparin. On the other hand, low plasma drug concentrations may paradoxically lead to induction of aggregation because of a conformational change of gpIIb/IIIa receptors from a nonactivated state, as shown by the higher

expression of CD63 (P-selectin) and formation of thromboxane A_2 (TxA_2). Large-scale clinical trials with different oral gpIIb/IIIa inhibitors in atherosclerotic patients have not shown any significant advantage of these compounds over other classes. A new compound, roxifiban, is under trial and is postulated to be the last chance for oral gpIIb/IIIa inhibitors (66). gpIIb/IIIa receptor antagonists are also associated with an increased risk of bleeding as evidenced in the various coagulation assays. Moreover, these compounds do not inhibit TxA_2 production (67, 68), which thus formed will exert vasoconstriction even if platelet aggregation was suppressed. gpIIb/IIIa antagonists have been combined with antiplatelet agents like the TxA_2 synthase inhibitors or TxA_2 receptor antagonists to minimize the bleeding risk in recent studies (69). Overall, gpIIb/IIIa inhibitors significantly influence the quantitative and qualitative expressions of the procoagulant complexes leading to thrombin generation (70), and inhibition of these receptors will remain a logical approach to achieving significant antithrombotic activity.

gpIIb/IIIa receptor antagonists are evaluated *in vitro* by using a simple ELISA, inhibition of PAC-1 binding to platelets and *in vivo* in different models of platelet-dependent thrombosis (Table I). The potential compounds are also evaluated for their binding to the 2-ligand binding pockets of the integrin $\alpha_{IIb}\beta_3$ (gpIIb/IIIa) (71, 72).

Thromboxane A_2 synthase inhibitors and receptor antagonists

Thrombotic occlusion, a multifactorial event, involves several factors such as TxA_2, thrombin, serotonin, endothelin and platelet activating factor (PAF). These substances facilitate thrombus formation as well as cause vasoconstriction. TxA_2 synthase inhibition leads to an increase in prostacyclin levels and this process is known as a "prostaglandin H_2 steal" (73). Moreover, when TxA_2 synthase is blocked, prostaglandin H_2 accumulates in platelets and is converted to prostacyclin by the endothelial cells. It was observed that prostacyclin formation rather than TxA_2 inhibition was more important in preventing thrombus formation by TxA_2 synthase inhibitor (74). Inhibition of TxA_2 synthase has been proved to be useful in combination with platelet integrin gpIIb/IIIa receptor antagonists for the prevention of vascular thrombosis (69).

Thromboxane receptor antagonists like vapiprost or ifetroban have been found to be more helpful than antithrombins (hirudin) and aspirin in preventing cyclic flow variations (68). The efficacy of thromboxane receptor antagonists in thromboxane-dependent platelet activation suggests that TxA_2 *per se* is important and stimulates platelet secretion and aggregation. Thromboxane receptor antagonists selectively prevent TxA_2/PG endoperoxide-dependent reocclusion induced by clot-bound thrombin after successful lysis (13). These compounds also antagonize TxA_2/PG endoperoxide-induced vasoconstriction as well as release of serotonin from activated platelets. Selective inhibition of thromboxane synthase, in particular if combined with blockade of thromboxane receptors, seems to be a useful approach to prevent rethrombosis after clot lysis.

TxA_2 synthase inhibitors and receptor blockers are primarily evaluated for their ability to inhibit platelet aggregation *in vitro* and *ex vivo* in platelet-rich plasma by the stable analogue of TxA_2. Experimental models used for detailed evaluation of these compounds as potential antithrombotics are shown in Table I.

ADP receptor antagonists

Adenosine diphosphate (ADP), present in high concentrations in the dense granules of the platelet, is released during platelet aggregation (75, 76). ADP is also rapidly generated from adenosine triphosphate (ATP) released from different sources, particularly red blood cells (77). Platelets exposed to ADP undergo shape changes and exhibit an increase in intracellular calcium secondary to the opening of receptor-modulated cation channels. The increase in intracellular calcium mediates a conformational change and activation of surface gpIIb/IIIa receptors that comprises the final common pathway culminating in platelet aggregation. ADP also facilitates recruitment of platelets during aggregation.

Human platelets possess 3 types of ADP receptors: $P2Y_1$ is coupled to phospholipase C and mobilizes intracellular calcium, while stimulation of $P2Y_{12}$ inhibits adenylate cyclase; these 2 are G-protein linked P_2 receptors. $P2X_1$ is a ligand-gated channel that causes calcium influx (78). Inhibition of either $P2Y_1$ or $P2Y_{12}$ with selective antagonists prevents ADP-induced TxA_2 generation, indicating that coactivation of the $P2Y_{12}$ and $P2Y_1$ receptors are essential for this event (79). The $P2Y_1$ receptor is present on both platelets and blood vessels, while the $P2Y_{12}$ receptor is found only on platelets (80). Thus, $P2Y_{12}$ receptor antagonists can attenuate platelet aggregation without affecting ADP-mediated vascular responses. $P2Y_{12}$ receptor antagonists, clopidogrel, ticlopidine and the recently developed CS-747, possess significant antithrombotic activity (81).

$P2Y_1$ null mice display strong resistance to thromboembolism induced by i.v. ADP infusion, a mixture of collagen and adrenaline or thromboplastin (82, 83). $P2Y_1$ receptor antagonists could also be potential drugs for antithrombotic therapy. Adenosine-2',5'-bisphosphate (A2P5P) and adenosine-3',5'-bisphosphate (A3P5P) are selective $P2Y_1$ receptor antagonists having significant antithrombotic potential (84). MRS-2179, another compound having structural similarities with A2P5P and A3P5P, so far is the most potent and selective known antagonist of the $P2Y_1$ receptor (85). ADP antagonists have been found to be effective in conditions of shear stress-induced platelet activation and insufficient endothelial function *in vivo*. However, they do not affect arachidonic acid metabolism in the platelet, are not active *in vitro* and require 3-5 days of administration for

complete antiplatelet action to occur *in vivo*. Moreover, these compounds cannot be used for immediate inhibition of platelet function and hence are used for secondary prevention of stroke, myocardial infarction and peripheral arterial occlusive disease. However, their efficacy has been found to be comparable to that of aspirin (86). This class of compounds is currently in preclinical phase of development but definitely holds promise as a potential target for antithrombotic action.

Recently, an ecto-ADPase, which rapidly metabolizes ADP released from activated platelets present on endothelial CD39 cells, has been reported to represent a novel antithrombotic agent to treat high-risk patients (87). Patients with coronary artery occlusion and thrombotic stroke are known to have activated platelets in the circulation. The efficacy of these compounds in preventing the activation of platelets in these patients is yet to be explored in detail.

The efficacy of $P2Y_{12}$ and $P2Y_1$ receptor antagonists are evaluated *ex vivo* against ADP-induced platelet aggregation. Hematological tests and bleeding time are also monitored in parallel during their evaluation (Table I). Subsequent studies are performed to understand the mechanism of inhibition of aggregation, mainly by measuring intracellular calcium and adenylate cyclase activity.

NO-aspirins

Aspirin is an effective antithrombotic agent for the secondary prevention of ischemic cardiovascular disorders (88, 89). However, it does not provide significant protection in some animal models of thrombosis such as high shear stress-induced thrombosis and also in those models where several platelet agonists play a pathogenic role. To suppress platelet activation more extensively than aspirin, the preferred approach is to combine the antiplatelet properties of aspirin with those of another drug class such as an ADP receptor antagonist (ticlopidine, clopidogrel) or a gpIIb/IIIa receptor antagonist (90, 91). NO, a potent platelet inhibitor and vasodilator, also exhibits protective effects against thrombosis in human as well as in animals (92). Recently, a nitro-aspirin derivative that has NO-releasing capabilities was found to be effective in different animal models of platelet-dependent and -independent pulmonary thromboembolism. NO-aspirin elevated intraplatelet cGMP with no significant hypotensive effect, minimal gastric irritations and a better antithrombotic profile than aspirin.

Although NO-aspirins have a better antithrombotic profile than aspirin, a detailed investigation in arterial and venous thrombosis remains to be carried out.

Guanylate cyclase activators

NO-releasing compounds and PDE (3/5) activators (Fig. 2) exhibit significant antithrombotic effects. NO-releasing compounds activate guanylate cyclase to elevate the intracellular levels of cGMP, while PDE inhibitors prevent degradation of cGMP. An increase in the intracellular level of cGMP reduces the free cytosolic Ca^{2+} in platelets by 2 mechanisms: through preventing the entry of Ca^{2+} from the plasma membrane and by inhibiting its release from the dense tubules. These compounds also significantly regulate the vascular tone by relaxing arteries to increase the blood supply to the ischemic organs (92).

Recently, a novel activator of guanylate cyclase was found to inhibit platelet activation (*e.g.*, ATP release, TxA_2 formation, phosphoinositide breakdown and intracellular Ca^{2+} mobilization) and platelet aggregation *in vitro* through NO-independent, cGMP-dependent mechanisms (93).

Like NO-aspirins, the efficacy of guanylate cyclase activators has not yet been demonstrated in different animal models of thrombosis or in humans.

In vitro evaluation of these compounds can be performed by direct activation of the guanylate cyclase or inhibition of phosphodiesterase. Detailed investigation can be carried out in the various commonly used experimental models.

von Willebrand factor inhibitors

The first step in the pathophysiologic process of arterial thrombosis involves binding of vWF to sites of endothelial damage in the vasculature. Circulating platelets bind to the immobilized vWF through a specific membrane receptor Ib (gpIb). This interaction is critical for the initiation of platelet deposition and enables other glycoprotein receptors, gpIIb/IIIa on the platelet membrane, to be exposed to fibrinogen and vWF binding resulting in platelet aggregation. Recently, a recombinant vWF segment AR545C exhibited antithrombotic properties due to its ability to inhibit platelet adhesion (94). S-nitroso derivative of the cysteine residue of AR545C showed more potent antiplatelet activity than the unmodified AR545C. This class of compounds is evaluated in ristocetin-induced platelet aggregation assays and subsequently in classical arterial thrombosis models (Table I).

Results of preliminary preclinical studies suggest that vWF and its receptor could be a potential target for antithrombotic therapy.

P-selectin inhibitors

Vessel wall inflammation appears to contribute substantially to the pathophysiology of venous thrombosis. Various experimental studies have also demonstrated the role of leukocytes in thrombosis and reperfusion injury (95-97). The initiating event in this cascade is the adhesion of leukocytes to the vascular endothelium through selectins and other adhesion molecules. P-selectins are upregulated on endothelial cell and platelet phospholipid surfaces by thrombin, tumor necrosis factor and

cytokines. Cytokines also potentiate leukocyte rolling, adhesion and extravasation. Effective protection from thrombus formation and clot propagation may be achieved by blocking the inflammatory component of the thrombotic process which may prove to be an efficient alternative mechanism of anticoagulation (98).

Recently, inhibition of the human P-selectin inhibitor PSGL-1, with an antagonist rPSGL-Ig, resulted in accelerated thrombolysis in arterial and venous thrombosis and effective prophylaxis for deep vein thrombosis in animal models (99). This new class is expected to reduce morbidity and mortality resulting from deep vein thrombosis and other thromboembolic disorders. However, it is still too early to derive any conclusion regarding the therapeutic efficacy of these agents. Identification of P-selectin antagonists requires estimation of the binding potential of the compounds on platelets and is done mainly by flow cytometry using monoclonal antibodies.

Platelet collagen receptor antagonists

Regulation of platelet activation is an important step in distinguishing normal hemostasis from pathological thrombosis. Hence, collagen interactions with platelets are important targets for pharmacological control. Collagen is present in the vascular subendothelium and vessel wall and is among the important platelet activators. Platelets have 2 major receptors for pharmacological control, the integrin $\alpha_2\beta_1$ receptor (100), which plays a major role in adhesion and platelet anchoring, and the Ig superfamily member GPVI (101), which is mainly responsible for signaling and platelet activation. In addition, gpIb-V-IX is an indirect collagen receptor acting via vWF as a bridging molecule and is essential for platelet interactions with collagen at high shear rates (102, 103). Although the involvement of these platelet receptors in thrombosis is currently understood, collagen receptor antagonists are still in preclinical stages of drug development. Thus, arriving at a conclusion regarding the significance of this target in relation to antithrombotic action is premature.

Conclusions

Treatment and prevention of thrombotic disorders is possible with the various classes of antithrombotic drugs like direct thrombin inhibitors, low-molecular-weight heparins, tissue plasminogen activators, gpIIb/IIIa receptor antagonists and ADP receptor antagonists due to the improved understanding of the mechanisms of thrombotic occlusion. The next generation of antithrombotic compounds, namely direct FXa inhibitors, direct FVIIa inhibitors, tissue factor pathway inhibitors, have also shown promising potential as antithrombotics. However, no specific antithrombotic agent has currently displaced heparin, coumarins and aspirin as the agent of choice for different thrombotic complications. Widespread innovation of new parenteral agents and improved understanding of the coagulation cascade and vessel wall biology has given investigators the opportunity to target early steps in the thrombus formation. Moreover, the high cost of anticoagulant and antithrombotic therapy necessitates the search for new effective chemical entities. This in turn should be aided by newer improved specific animal models mimicking the conditions associated with various thromboembolic diseases, and subsequently reduce the time and cost incurred in development of an antithrombotic drug.

With the dawn of the 21st century, the physician is armed with new and exciting developments in diagnosis and surgical interventions for thrombotic disorders, but effective antithrombotic therapy with fewer side effects is still lacking. Improved animal models, better chemical entities, as well as gene therapy and properly conducted clinical trials will, however, facilitate the development of potential antithrombotic therapy required for millions of people worldwide to prevent and treat thrombotic disorders.

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