

New drugs series

Platelet thrombin receptor antagonism and atherothrombosis

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Clinical manifestations of atherothrombotic disease, such as acute coronary syndromes, cerebrovascular events, and peripheral arterial disease, are major causes of mortality and morbidity worldwide. Platelet activation and aggregation are ultimately responsible for the progression and clinical presentations of atherothrombotic disease. The current standard of care, dual oral antiplatelet therapy with aspirin and the P2Y₁₂ adenosine diphosphate (ADP) receptor inhibitor clopidogrel, has been shown to improve outcomes in patients with atherothrombotic disease. However, aspirin and P2Y₁₂ inhibitors target the thromboxane A₂ and the ADP P2Y₁₂ platelet activation pathways and minimally affect other pathways, while agonists such as thrombin, considered to be the most potent platelet activator, continue to stimulate platelet activation and thrombosis. This may help explain why patients continue to experience recurrent ischaemic events despite receiving such therapy. Furthermore, aspirin and P2Y₁₂ receptor antagonists are associated with bleeding risk, as the pathways they inhibit are critical for haemostasis. The challenge remains to develop therapies that more effectively inhibit platelet activation without increasing bleeding complications. The inhibition of the protease-activated receptor-1 (PAR-1) for thrombin has been shown to inhibit thrombin-mediated platelet activation without increasing bleeding in pre-clinical models and small-scale clinical trials. PAR-1 inhibition in fact does not interfere with thrombin-dependent fibrin generation and coagulation, which are essential for haemostasis. Thus PAR-1 antagonism coupled with existing dual oral antiplatelet therapy may potentially offer more comprehensive platelet inhibition without the liability of increased bleeding.

Keywords

Platelets • Thrombosis • Antiplatelet therapy • Thrombin • PAR-1 antagonists

Introduction

Platelets play a key role in preventing blood loss after injury, but they are also central in the formation of pathological thrombi responsible for the acute clinical manifestations of atherothrombotic disease. Platelet activation is crucial to haemostasis and the development of pathological thrombosis and can be mediated by multiple pathways. Key among these are the von Willebrand factor, adenosine diphosphate (ADP), thromboxane A₂ (TXA₂), and thrombin-mediated platelet activation pathways.¹ Current oral antiplatelet agents inhibit the TXA₂ (aspirin) and ADP (P2Y₁₂ ADP receptor antagonists) pathways, but not thrombin-mediated platelet activation via protease-activated receptor-1

(PAR-1) binding. Thus despite receiving current standard-of-care dual oral antiplatelet therapy (aspirin and clopidogrel), patients continue to experience ischaemic events due to other platelet activation pathways. Furthermore, aspirin and P2Y₁₂ ADP receptor antagonists are associated with increased bleeding risk, as they impair platelet pathways essential for primary haemostasis. Thrombin receptor antagonists (TRAs or PAR-1 antagonists) represent a promising new class of oral antiplatelet agents for the treatment or prevention of atherothrombotic disease. This article will summarize the pathophysiology of atherothrombosis and the role of platelet activation pathways, the benefits and limitations of current standard-of-care therapy, thrombin–PAR-1 biology [including the rationale for thrombin receptor (PAR-1) inhibition for improved

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clinical outcomes], and pharmacological and clinical data for TRAs in clinical development.

Platelet activation and atherothrombosis

Platelets play a critical role in atherothrombosis, in that they mediate thrombosis, the major pathogenic event in disease progression.² Excessive platelet activation subsequent to plaque rupture or erosion overcomes regulatory haemostatic mechanisms and leads to generation of unwarranted levels of thrombin, initiating thrombosis and resulting in the formation of occlusive thrombi at sites of plaque disruption.² Platelet activation can be induced by the cooperative actions of multiple factors, including serotonin, epinephrine, thrombin, ADP, and TXA₂.^{1,3} Key among

the soluble agonists are ADP and TXA₂, which are released from adherent, activated platelets, and thrombin, which is produced locally by tissue factor (Figure 1).⁴ These agonists signal through G protein-coupled receptors (GPCRs) expressed by platelets and mediate paracrine and autocrine platelet activation.³ Platelet activation by these factors leads to platelet shape change,^{5,6} expression of proinflammatory molecules [P-selectin,⁷ soluble CD40 ligand (sCD40L)⁸] and other unidentified proteins,⁹ expression of platelet procoagulant activity,¹⁰ potentiation of aggregation by other prothrombotic factors such as collagen,¹¹ and, importantly, the conversion of the central platelet receptor GPIIb/IIIa into an active form.¹ A protective haemostatic plug develops into a vessel-occluding thrombus when exaggerated platelet activation occurs under pathological conditions. Consequently, agents targeting platelet activation pathways have become the mainstay of antithrombotic therapy.

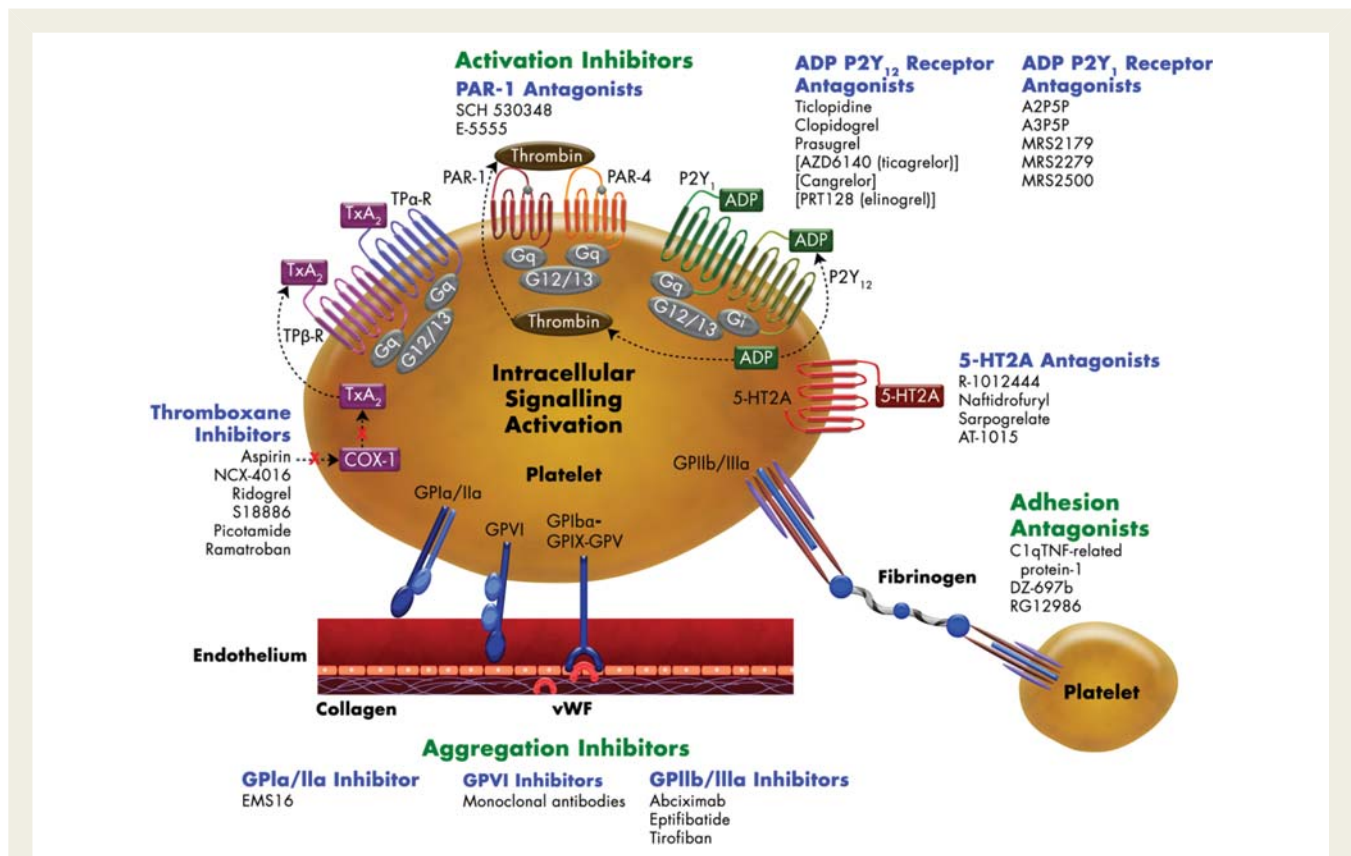


Figure 1 Sites of action of current and emerging antithrombotic drugs and antiplatelet agents. Adapted with permission. (Copyright © 2007 American Heart Association. All rights reserved.)⁴ Platelet adherence to the endothelium occurs at sites of vascular injury through the binding of GP receptors to exposed extracellular matrix proteins (collagen and vWF). Platelet activation occurs via complex intracellular signalling processes and causes the production and release of multiple agonists, including TXA₂ and ADP, and local production of thrombin. These factors bind to their respective G protein-coupled receptors, mediating paracrine and autocrine platelet activation. Further, they potentiate each other's actions (P2Y₁₂ signalling modulates thrombin generation). The major platelet integrin GPIIb/IIIa mediates the final common step of platelet activation by undergoing a conformational shape change and binding fibrinogen and vWF leading to platelet aggregation. The net result of these interactions is thrombus formation mediated by platelet/platelet interactions with fibrin. Current and emerging therapies inhibiting platelet receptors, integrins, and proteins involved in platelet activation include the thromboxane inhibitors, the ADP receptor antagonists, the GPIIb/IIIa inhibitors, and the novel PAR antagonists and adhesion antagonists. TP, thromboxane receptor; 5-HT_{2A}, 5-hydroxy tryptamine 2A receptor. Reversible-acting agents are indicated by brackets.

Current standard-of-care dual oral antiplatelet therapy: benefits and limitations

Aspirin in combination with the thienopyridine clopidogrel is currently available as standard-of-care oral, dual antiplatelet therapy for reducing ischaemic events in patients with atherothrombotic disease. Aspirin is an irreversible cyclooxygenase-1 (COX-1) inhibitor that inhibits platelet activation by blocking TXA₂ production,¹² whereas clopidogrel prevents platelet activation by irreversibly inhibiting the major platelet ADP receptor P2Y₁₂ (Figure 1).^{4,5,13} Clinical trials have confirmed the therapeutic benefits of aspirin and clopidogrel in preventing thrombotic events in a broad range of patients with atherothrombotic diseases. The benefits of aspirin have been shown in acute coronary syndrome (ACS),^{14,15} percutaneous coronary intervention (PCI),¹⁶ and primary¹⁷ and secondary prevention of vascular events or death.¹⁴ The clinical efficacy of clopidogrel has been shown both as single antiplatelet therapy in high-risk patients with various manifestations of atherosclerotic disease,¹⁸ and in conjunction with aspirin in high-risk patients with ACS, including unstable angina, ST-segment elevation (STE) myocardial infarction (MI), and non-STE (NSTEMI) MI, or those undergoing PCI.^{19–22} The third-generation thienopyridine prasugrel, with a faster onset of action than clopidogrel, demonstrated superior efficacy to clopidogrel in the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel (TRITON-TIMI 38) in patients with ACS undergoing PCI.²³

Despite the benefit associated with these agents, significant clinical limitations are associated with use of aspirin and a P2Y₁₂ ADP receptor antagonist. The first among these is the risk of bleeding.^{19,24} A dose-dependent increase in bleeding has been shown with aspirin.²⁵ Although adjunctive therapy with clopidogrel in addition to aspirin reduces the risk of ischaemic events, this significantly increases the risk of TIMI major and minor bleeding and the need for transfusions.¹⁹ The dose of aspirin used in combination with clopidogrel contributes to the risk of bleeding complications.²⁵ In TRITON, prasugrel, a more potent P2Y₁₂ ADP receptor antagonist, was associated with a greater risk of major bleeding vs. clopidogrel (2.4 vs. 1.8%, $P = 0.03$), indicating that the bleeding risk increases proportionally with the degree of P2Y₁₂ inhibition.²³ Bleeding risk has been attributed to the fact that aspirin and P2Y₁₂ ADP receptor antagonists interfere with the TXA₂ and ADP platelet activation pathways that are crucial for normal haemostasis. Furthermore, P2Y₁₂ signalling plays an important role in the initiation of coagulation by modulating thrombin generation (Figure 1).^{26,27} More potent P2Y₁₂ inhibition by means of high loading and maintenance doses of clopidogrel is associated with reduced *ex vivo* thrombus cohesion and increased time to the *ex vivo* generation of the initial platelet-fibrin clot.^{28–30} The active metabolite of prasugrel has also been shown to reduce the kinetics of thrombin generation induced by ADP and to inhibit clot strength.³¹ This interplay between P2Y₁₂ signalling and thrombin generation^{26,27} may underlie the increased bleeding observed with the use of more potent P2Y₁₂ inhibitors.

Apart from bleeding risk, a considerable number of patients receiving dual antiplatelet therapy continue to experience recurrent thrombotic events. This residual risk may be due to the potential for continuing platelet activation and thrombosis via pathways independent of TXA₂ and ADP. Multiple pathways contribute to platelet activation, but aspirin and P2Y₁₂ ADP receptor antagonists do not inhibit pathways other than those stimulated by TXA₂ and ADP, respectively.¹ This potentially contributes to the increased incidence of thrombotic events in patients due to ongoing platelet activation via potent agonists such as thrombin, thereby increasing patient morbidity and mortality. New therapies that target pathways that are not affected by aspirin or P2Y₁₂ ADP receptor antagonists could provide complementary and more comprehensive inhibition of platelet activation when used in combination with the current standard-of-care therapies, and thereby contribute to greater inhibition of platelet-mediated thrombosis.

Finally, inadequate responsiveness to aspirin and clopidogrel has been shown in several studies to be associated with poor clinical outcomes (a full description of aspirin and clopidogrel resistance goes beyond the scope of this review and is described elsewhere).^{13,32,33} The variability in response is characterized by the inability of these agents to completely inhibit the COX-1 enzyme and TXA₂ generation (by aspirin) and the ADP P2Y₁₂ receptor (by clopidogrel), via mechanisms that are multifactorial and not fully elucidated. To date, phosphodiesterase inhibitors, such as cilostazol, have only partly been able to overcome this limitation.³⁴

Thus the challenge at present is to develop antiplatelet agents that inhibit thrombosis but leave haemostasis sufficiently intact to prevent bleeding. It is hoped that novel P2Y₁₂ receptor antagonists—prasugrel and ticagrelor—as well as antiplatelet therapies currently in clinical testing targeting novel pathways, such as the TRAs SCH 530348 and E-5555, will have superior clinical profiles to those of currently approved antiplatelet agents.^{35–37}

Thrombin–protease-activated receptor-1 biology and rationale for thrombin receptor (protease-activated receptor-1) inhibition

The serine protease thrombin is considered to be one of the most potent platelet activators and plays a seminal role in blood coagulation.³⁸ The potency of thrombin is supported by studies showing that among the pro-thrombotic activities of thrombin, platelet activation is observed most rapidly and requires the lowest biologically active thrombin concentration (~0.5 nM).³⁹ In contrast, *ex vivo* studies of platelet aggregation require micromolar concentrations of other platelet activators such as ADP.⁴⁰ Furthermore, thrombin is a more potent agonist than either thrombin receptor activating peptide (TRAP) or other activating peptides.⁴¹ Platelet responses to thrombin are mediated by surface GPCRs known as PARs or thrombin receptors. In humans, there are four known subtypes of PARs, which display wide tissue distribution (Table 1).^{42,43} PAR-1, PAR-3, and PAR-4 are activated by thrombin, whereas

Table 1 Properties of human protease-activated receptor family members and phenotype of mice lacking each receptor

	Primary activating protease ⁴³	Localization ⁴²	Phenotype of knockout mice ⁴³
PAR-1	Thrombin (50 pmol/L)	Platelets, endothelium, epithelium, fibroblasts, myocytes, neurons, astrocytes	Partial embryonic lethality, vascular matrix deposition after injury
PAR-2	Trypsin (1 nmol/L); Trypsin (1 nmol/L)	Endothelium, epithelium, fibroblasts, myocytes, neurons, astrocytes	Impaired leucocyte migration; impaired allergic inflammation of airway, joints, kidney
PAR-3	Thrombin (0.2 nmol/L)	Endothelium, myocytes, astrocytes	Delayed response to thrombin; no spontaneous bleeding; normal platelet counts; protection against thrombus formation/pulmonary embolism
PAR-4	Thrombin (5 nmol/L); Trypsin (1 nmol/L)	Platelets, endothelium, myocytes, astrocytes	Unresponsive to thrombin; no spontaneous bleeding; normal platelet counts; protection against thrombus formation/pulmonary embolism

PAR-2 is activated by trypsin and trypsin-like proteases and not by thrombin.³⁸ Thrombin-mediated platelet activation in humans is shown to occur through PAR-1 and PAR-4 receptors.³⁸ PAR-1 acts as the principal thrombin receptor on human platelets and mediates platelet activation by subnanomolar thrombin concentrations, whereas PAR-4 requires higher concentration of thrombin for activation (Table 1).^{38,43,44} Thrombin is also known to cleave PAR-3; however, the physiological relevance of this interaction is not known.⁴⁵ PAR-3 can dimerize with other PARs, which may contribute to regulation of PAR signalling.⁴⁵

A unique tethered ligand mechanism has been described for thrombin-mediated platelet activation via PAR.^{38,46} Thrombin's fibrinogen-binding exosite binds the hirudin-like extracellular amino (NH₂) terminal domain of PAR-1 on the platelet surface, cleaves the receptor between arginine 41 and serine 42, removes the amino terminal sequence, and generates a new protonated amino group at the NH₂ terminal of the tethered ligand—S⁴²FLLRN (serine–phenylalanine–leucine–leucine–arginine–asparagine). This newly exposed NH₂ terminus sequence acts as the tethered ligand and binds intramolecularly to the body of the receptor, activating it.⁴⁶ The hirudin-like sequence is important for receptor cleavage at low concentrations of thrombin and is present in PAR-1 and PAR-3.⁴⁷ PAR-4 lacks the hirudin-like sequence and thereby requires far greater levels of thrombin for activation.^{44,47} PAR-4 uses instead a negatively charged cluster of amino acid residues to facilitate slow disassociation from the positively charged thrombin molecule.⁴⁸ PAR-2 also lacks the hirudin-like sequence and cannot be cleaved by thrombin.^{38,47,49} Thrombin also binds to GPIIb α on the surface of human platelets, which has been proposed to act as a cofactor that localizes thrombin to the platelet surface to support thrombin cleavage of PARs.^{38,50}

Thrombin-mediated PAR-1 cleavage results in the activation of heterotrimeric G proteins of the G α 12/13, G α q, and G α i/z families that interconnect several intracellular signalling pathways to the various phenotypic effects of thrombin on platelets (Figure 2).^{6,38} These include TXA₂ production,⁵¹ ADP release,⁵² serotonin and epinephrine (adrenaline) release,^{53,54} activation/mobilization of P-selectin and CD40 ligand,^{55,56} integrin activation,⁵⁷ and platelet aggregation.⁵⁸ PAR-1 activation also stimulates platelet

procoagulant activity, leading to enhanced thrombin formation,¹⁰ and the generation of fibrin from fibrinogen.⁵⁹ PAR-4, like PAR-1, mediates its effects by activating G α q and G α 12/13 signalling⁶; however, unlike PAR-1, PAR-4 may not activate or require G α i signals.^{52,60} PAR-4 is activated, shut off, and internalized more slowly than PAR-1 and produces the majority of the integrated thrombin-triggered calcium signals of the two receptors.^{52,61} PAR-4 can also be activated by cathepsin G, a granzyme (serine esterase) released by neutrophils⁶²; however, the relevance of this function *in vivo* is not known. A counter-regulatory mechanism of PAR-1 and PAR-4 activity has been described in the control of platelet function, whereby PAR-1 activation causes release of vascular endothelial growth factor and PAR-4 activation causes release of endostatin.⁶³ The importance of these qualitative differences between PAR-1 and PAR-4 signalling for platelet function is not fully understood. The current hypothesis is that PAR-4 functions as an auxiliary receptor mediating thrombin signalling to distinct effectors or with different kinetics compared with PAR-1. PAR-4 may perhaps also interact directly or indirectly with PAR-1 or mediate platelet responses to proteinases other than thrombin.^{6,61} Indefinite signalling from PAR receptors is prevented by efficient receptor desensitization (via phosphorylation for PAR-1 but not for PAR-4⁶¹), receptor endocytosis, and delivery to lysosomes for degradation.^{38,64}

The P2Y₁₂ and PAR-1 pathways are known to cross-react in mediating platelet activation.^{65,66} Potent platelet activation has been shown to require coactivation of G α q- and G α i-coupled receptors.^{67,68} Unlike ADP, which directly activates both G α i (via P2Y₁₂) and G α q (via P2Y₁) signalling,⁶⁷ thrombin cannot directly activate both signals and requires the secondary G α i signal from P2Y₁₂ and secreted ADP (thrombin's secondary response) to complement the G α q signals from PAR-1 and PAR-4.^{65,66} G α i signalling inhibits adenylyl cyclase, thereby decreasing the concentration of cyclic adenosine monophosphate, which is an inhibitor of platelet activation (Figure 2).^{38,66,69} Thrombin-dependent platelet aggregation is known to be mediated at least in part by secreted ADP acting on the G α i-linked ADP receptor in murine and human platelets.^{66,70–72} Building on these observations, the combined inhibition of thrombin and

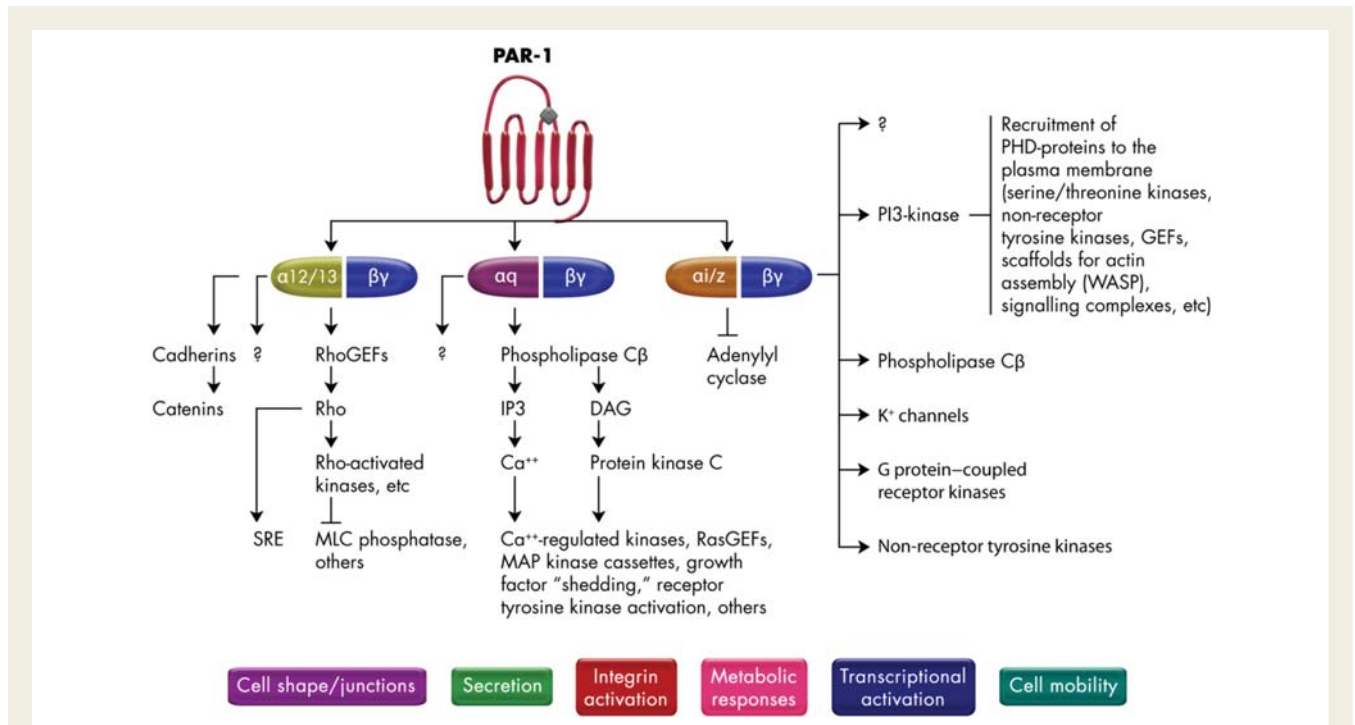


Figure 2 Intracellular signalling pathways and phenotypic effects mediated by PAR-1 activation by thrombin in platelets. Reproduced with permission. (Copyright © 2005 International Society on Thrombosis and Haemostasis. All rights reserved.)³⁸ Members of the $G_{\alpha 12/13}$, $G_{\alpha q}$, and $G_{\alpha i/z}$ families activate PAR-1 signalling pathways. First, the α -subunits of G_{12} and G_{13} bind RhoGEFs (which activate small G proteins) and drive Rho-mediated cytoskeletal responses leading to changes in platelet morphology. Second, $G_{\alpha q}$ activates phospholipase C β and triggers phosphoinositide hydrolysis, calcium mobilization, and protein kinase C activation. This then activates pathways leading to granule secretion, as PAR-1– $G_{\alpha q}$ -coupled ADP release is especially important for thrombin-mediated platelet activation. In addition, calcium-regulated kinases and phosphatases, GEFs, MAP kinases, and other proteins that mediate platelet aggregation are activated. $G_{\alpha i}$ promotes platelet responses by inhibiting adenylyl cyclase. $G\beta\gamma$ subunits can activate phosphoinositide-3 kinase, other lipid-modifying enzymes, protein kinases, MAP kinase cascades, and channels. Phosphoinositide-3 kinase modifies the inner leaflet of the plasma membrane to provide molecular docking sites. Finally, PAR-1 also promotes growth factor shedding and activation of receptor tyrosine kinases involved in cell growth and differentiation. GEF, guanine nucleotide exchange factors; MAP, mitogen-activated protein; IP3, inositol triphosphate 3; DAG, diacylglycerol; WASP, Wiskott–Aldrich syndrome protein; SRE, serum response element; MLC, myosin light chain.

P2Y $_{12}$ in human whole blood samples provided a synergistic inhibitory effect on thrombin-induced platelet activation and aggregation, via a mechanism that was proposed to involve concurrent inhibition of $G_{\alpha q}$ (via PAR-1) and $G_{\alpha i}$ (via P2Y $_{12}$) signals required for potent platelet activation.⁷³ P2Y $_{12}$ blockade was shown to be most effective at low (physiological) thrombin concentrations that are sufficient to cleave PAR-1 and release all of the ADP while leaving PAR-4 intact.^{52,69} Importantly, the synergistic inhibition was achieved with much lower dose combinations of thrombin and P2Y $_{12}$ inhibitors than single doses of the inhibitors required for the same effect.⁷³

In the vessel wall, PARs also mediate responses involved in contractility, proliferation, inflammation, and repair. Endothelial cells express all four PAR family members.⁴⁵ Preclinical studies have shown that activation of PAR-1 or PAR-2 induces vascular relaxation mediated by the production of nitric oxide.⁴⁵ PAR-1 agonists have also been shown to have the opposite effect on vascular tone, inducing contraction of vascular smooth muscle cells.⁷⁴ Proliferation of cultured vascular smooth muscle cells has also been observed in response to PAR-1, PAR-2, and PAR-4 activation.

PAR-2 also has proliferatory and angiogenic effects on endothelial cells. Other studies have suggested that PARs can promote inflammation by enhancing leucocyte adherence to endothelial cells and migration of leucocytes from the vasculature into inflamed tissue.⁴⁵ In atherogenic human arteries, expression of PAR-1 is enhanced in inflamed endothelium displaying macrophage influx and smooth muscle cell proliferation.⁶ PAR-2 and PAR-4 have also been implicated in inflammatory responses.⁴⁵ In contrast to the species difference in PAR expression on mouse vs. human platelets, PAR-1 is the major PAR family member present in endothelial cells in both mice and humans.³⁸ PAR-1 deficiency or blockade in mice reduces leucocyte infiltration and inflammation. PAR-1 antagonism has been shown to be protective in animal models of vascular injury.⁷⁵

Murine platelets utilize PAR-4 and PAR-3 receptors to respond to thrombin. PAR-4 is the principal thrombin receptor in mice.³⁸ PAR4 $^{-/-}$ mice are unresponsive to thrombin, display no spontaneous bleeding, and are protected from thrombosis (Table 1).⁶² Activation of PAR-3, a high affinity thrombin receptor, alone does not result in thrombin signalling. PAR3 $^{-/-}$ mice exhibit

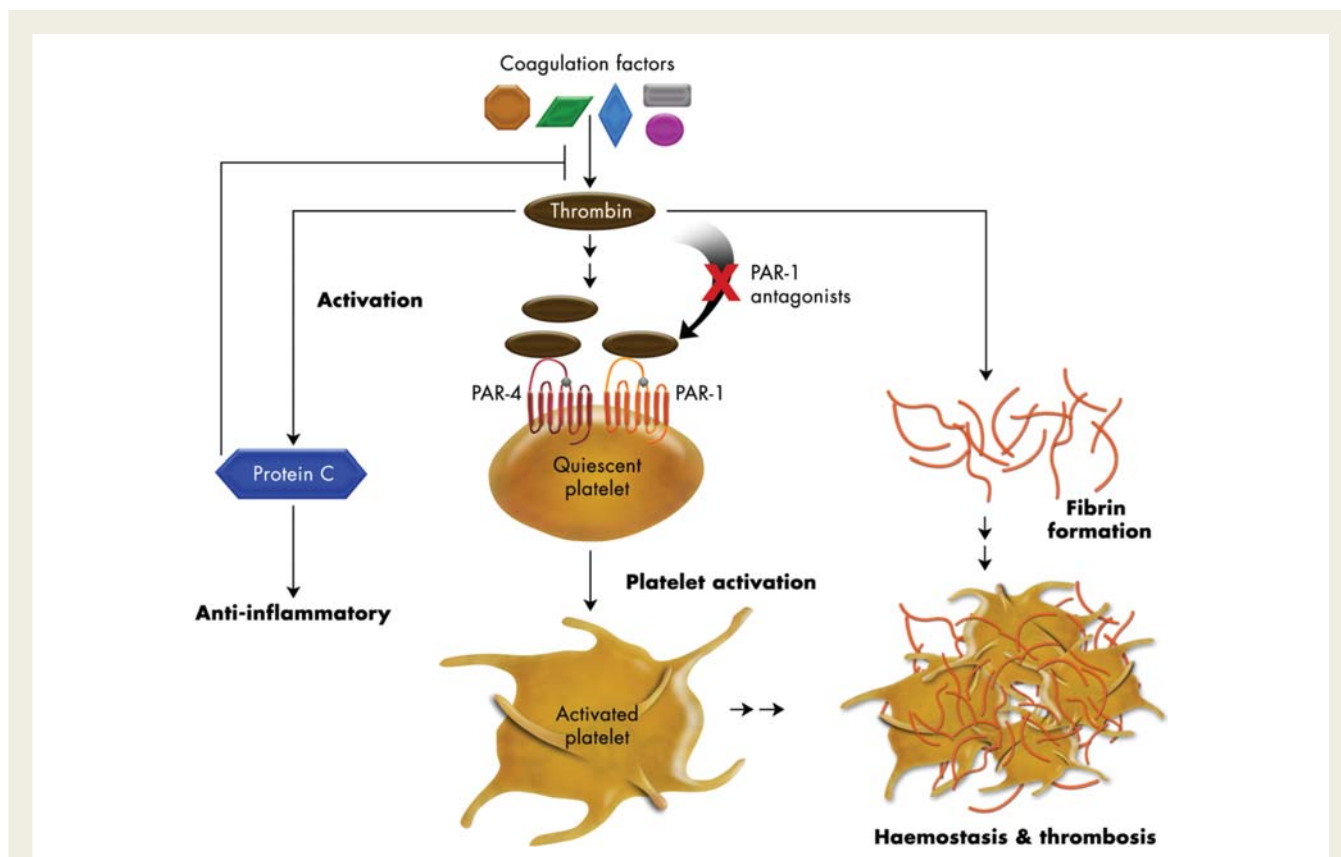


Figure 3 Role of thrombin-mediated PAR-1 signalling in haemostasis vs. thrombosis: rationale for PAR-1 antagonist.³⁸ In addition to activating PARs on platelets, thrombin facilitates fibrin formation and protein C activation. Available data in mice and non-human primate models indicate that PAR antagonists have the potential to block platelet activation by thrombin while sparing these other functions of thrombin, including coagulation, that are absolutely necessary for haemostasis. Murine studies suggest that fibrin(ogen) is relatively more important than thrombin-induced platelet activation for haemostasis, and this is supported by high bleeding risk associated with the use of anticoagulants in man. Thus, inhibition of thrombin-induced platelet activation might provide for a larger therapeutic index than can be achieved with coagulation inhibitors for the prevention or treatment of thrombosis.

a delayed response to thrombin.^{76,77} PAR-3 in murine platelets is proposed to serve as a cofactor, concentrating thrombin at the cell surface and promoting cleavage and activation of PAR-4 at low thrombin concentrations. Thus, in contrast to the human system, platelet activation in mice requires cooperative interaction between PAR-3 and PAR-4. The phenotypes of PAR knockout mice are indicated in Table 1.

Rationale for thrombin receptor (protease-activated receptor-1) inhibition for treatment of atherothrombotic disease

In contrast to the ADP and the TXA₂ platelet activation pathways that are crucial for both routine haemostasis and pathological thrombosis, PAR-1-mediated platelet activation contributes to pathological thrombosis (formation of occlusive platelet-rich thrombus) but may not be required for protective haemostasis (formation of the initial platelet monolayer). Observations from

several pre-clinical studies indicate that thrombin-mediated cleavage of fibrinogen into fibrin is more important for haemostasis than thrombin-mediated platelet activation.³⁸ In contrast to the severe bleeding phenotype exhibited by mice deficient in fibrinogen, PAR-4-deficient mice (*Par-4*^{-/-}) exhibit normal platelet numbers and morphology, appear healthy, and do not show undue anaemia or spontaneous bleeding.^{62,78,79} *Par-4*^{-/-} mice have reduced and delayed platelet activation (measured by P-selectin expression) and diminished growth of thrombi, with no difference in kinetics or quantity of fibrin accumulation compared with wild-type mice.⁸⁰ Initial platelet accumulation at sites of injury was not different between wild-type and *Par-4*^{-/-} mice, and this early thrombus was proposed to support thrombin generation in sufficient amounts and allow normal fibrin deposition observed in this model.⁸⁰ Although a direct association between murine and human PAR function in platelets cannot be made because of species-dependent differences in expression, these findings suggest that platelet activation by thrombin is distinct and less important for haemostasis than fibrin generation. In addition, PAR-1 inhibition using the PAR-1 antagonist FR171113 has been shown to reduce arterial thrombosis in guinea pigs by reducing

PAR-1 agonist- or thrombin-induced platelet aggregation without prolonging bleeding times or influencing coagulation parameters.⁸¹ In contrast, the direct thrombin inhibitor argatroban inhibited thrombus formation but significantly prolonged bleeding and coagulation times in this study. Although extrapolation of these results to humans is highly speculative, these findings are suggestive of differentiation among antithrombotic agents based on the benefit/risk (i.e. bleeding) ratio. PAR-1 inhibition with FR171113 did not inhibit ADP-induced or collagen-induced platelet aggregation, suggesting that PAR-1 antagonism does not affect platelet activation pathways required for protective haemostasis. Inhibition of ADP signalling with AR-C69931MX (cangrelor) and, to a lesser extent, inhibition of TXA₂ signalling with indomethacin have been shown to inhibit platelet adhesion to immobilized collagen, demonstrating that inhibition of these pathways disrupts normal haemostasis.⁸² Of note, guinea pig platelets differ from human platelets in that they express PAR-1, -2, and -3.⁴² However, FR171113 has similar antithrombotic effects in human platelets, suggesting a similar role for PAR-1 signalling between humans and guinea pigs.^{81,83} As in human platelets, platelets from non-human primates express PAR-1 and PAR-4.⁸⁴ Treatment with a PAR-1 antagonist (RWJ-58259) or antibodies to PAR-1 in non-human primate models reduced vascular injury-induced thrombosis while sparing platelet-dependent coagulation functions such as the activated clotting time, activated partial thromboplastin time, and prothrombin times. Haematological parameters such as red blood cell count, haematocrit, white blood cell count, or platelet counts were not affected by PAR-1 inhibition.^{84,85} Importantly, thrombin signalling through the PAR-4 receptor remained intact in these studies, indicating a primary role for PAR-1 in mediating thrombin's prothrombotic effects in non-human primates. This suggests that inhibition of PAR-1 alone may be similarly sufficient in the prevention or treatment of thrombotic events in humans. These pre-clinical findings are also consistent with clinical observations showing substantial bleeding risk in patients treated with anticoagulants that interfere with the catalytic function of thrombin.⁸⁶ Inhibition of PAR-1 function rather than inhibition of

thrombin generation or activity (e.g. by factor Xa inhibitors) therefore provides a rational strategy for treatment of thrombotic disorders in humans. Platelets retain responsiveness to thrombin (albeit at much higher doses) via intact PAR-4 signals utilizing ADP/P2Y₁₂/G α i-independent mechanisms.^{52,60,66} Indeed, defective G α i signalling is known to be associated with chronic bleeding disorder in humans and is characterized by impaired platelet aggregation responses to ADP and epinephrine.^{71,87} These erroneous signalling events may underlie the increased bleeding observed with use of the more potent irreversible P2Y₁₂ inhibitor prasugrel when compared with clopidogrel.²³

Thrombin receptor antagonists in clinical development

Two TRAs are currently in clinical trials for the treatment and prevention of arterial thrombosis, SCH 530348 and E-5555 (Figure 4).^{88,89}

SCH 530348 (thrombin receptor antagonist)

SCH 530348 is a high-affinity, orally active, low-molecular weight, non-peptide, competitive PAR-1 TRA.⁸⁸ Pre-clinical functional assays have shown potent inhibition of thrombin and TRAP-induced platelet aggregation by SCH 530348.⁸⁸ In addition, SCH 530348 was inactive in functional assays with PAR-2, PAR-3, and PAR-4 and did not affect clotting parameters such as prothrombin time. A study in cynomolgus monkeys revealed no bleeding risk with the administration of SCH 530348 (1 mg/kg) alone, and no increase in bleeding time when used in combination with aspirin plus clopidogrel.⁹⁰ These results suggest that SCH 530348 is a potent and selective PAR-1 antagonist that does not impact bleeding and supported further clinical evaluation. Dose-ranging studies (daily doses of 0.5, 1.0, or 2.5 mg for 28 days or single doses of 5.0, 10, 20, or 40 mg) in healthy volunteers

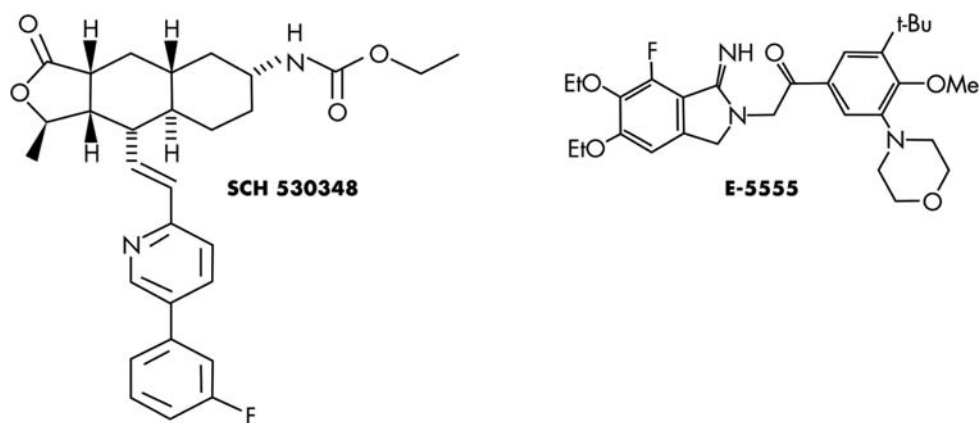


Figure 4 PAR-1 antagonists in clinical development. Reproduced with permission. (Copyright © 2006 and 2008. The American Chemical Society. All rights reserved.)^{88,89} Chemical structure of PAR-1 antagonist SCH 530348 and presumed structure of E-5555.

indicated that SCH 530348 stimulated dose-dependent inhibition of platelet aggregation induced by 15 μ M TRAP.⁹¹ SCH 530348 was rapidly absorbed and slowly eliminated, with a mean terminal-phase half-life ($t_{1/2}$) of 165–311 h. Recovery of platelet function to $\geq 50\%$ of baseline was dose-dependent and occurred at 4 weeks after treatment discontinuation.⁹² SCH 530348 was safe and well tolerated after single oral doses up to 40 mg and multiple doses up to 5 mg daily in healthy volunteers.⁹³ There were no significant changes in the results of laboratory tests, including tests of liver and kidney function, coagulation parameters, and vital signs or ECGs including specific changes in the QT interval, or any significant effect on Ivy bleeding times with SCH 530348 vs. placebo groups.

The Phase 2 Thrombin Receptor Antagonist-Percutaneous Coronary Intervention (TRA-PCI) trial evaluated the safety and efficacy of SCH 530348 used in combination with standard oral antiplatelet therapy (aspirin and clopidogrel) and an antithrombin agent (heparin or bivalirudin) over a 60-day treatment duration in a total of 1030 patients undergoing non-urgent PCI or coronary angiography with planned PCI. Patients were randomized to receive one of three oral loading doses of SCH 530348 (10, 20, or 40 mg) or placebo in addition to aspirin plus clopidogrel. Patients who underwent PCI ($n = 573$) were randomized to receive one of three oral daily maintenance doses of SCH 530348 (0.5, 1, or 2.5 mg) or placebo.⁹⁴ There was no significant difference in the primary endpoint of rate of TIMI major bleeding (e.g. any intracranial haemorrhage or overt sign of bleeding requiring intervention and associated with a decrease in haemoglobin concentration of >5 g/dL) and minor bleeding (e.g. any overt sign of bleeding requiring intervention that did not meet the requirements for TIMI major bleeding and associated with a decrease in haemoglobin concentration of 3 to ≤ 5 g/dL) between patients who underwent PCI at the end of the 60-day treatment (3% in the collective SCH 530348 treatment arms vs. 3% with standard care therapy). TIMI major plus minor bleeding was observed in 2, 3, and 4% of patients receiving SCH 530348 10, 20, and 40 mg loading dose, respectively. Non-coronary artery bypass graft TIMI major plus minor bleeding was observed in less than 1, 3, and 4% of patients receiving 10, 20, and 40 mg, respectively (vs. 3% with placebo). No significant trends were observed. TIMI major plus minor bleeding was observed in 2, 4, and 3% of patients receiving SCH 530348 0.5, 1.0, and 2.5 mg/d maintenance dose, respectively. The combination of SCH 530348 40 mg oral loading dose plus a 2.5 mg/d maintenance dose (the highest dose combination tested) was associated with no cases of TIMI major bleeding vs. no cases with placebo and two cases (3%) of TIMI minor bleeding vs. one case (2%) with placebo.

The incidence of death, major adverse cardiovascular events (MACE), or stroke was not significantly different between the SCH 530348 and placebo groups (6% in the collective SCH 530348 groups vs. 9% with placebo). There was a non-significant trend for dose-dependent reduction in the secondary outcome of non-fatal MI in the SCH 530348 groups vs. the placebo group (5, 4, and 3% with SCH 530348 10, 20, and 40 mg loading dose vs. 7% with placebo).⁹⁴ SCH 530348 also demonstrated dose-dependent inhibition of TRAP-induced platelet aggregation.⁹⁴ The 40 mg loading dose was the most effective; it inhibited

TRAP-induced platelet aggregation in almost 30% of patients as early as 0.5 h after administration and by 2 h this proportion had more than tripled. Levels of inhibition were sustained at 30 and 60 days with 1.0 and 2.5 mg maintenance doses. Furthermore, a substudy of TRA-PCI evaluated the effect of SCH 530348 on platelet aggregation induced by various agonists. SCH 530348 did not have any measurable effects on platelet aggregation induced by ADP, arachidonic acid, or collagen, supporting the specificity of SCH 530348 for PAR-1.⁹⁵

The safety and tolerability of SCH 530348 was also confirmed in a recent Phase 2 clinical trial in Japanese patients with NSTEMI ACS ($n = 117$).⁹⁶ Addition of SCH 530348 (either 20 or 40 mg loading dose, followed by 1 or 2.5 mg maintenance dose) for 60 days to standard of care (aspirin, ticlopidine, and heparin) was not associated with an increase in the occurrence of the key safety endpoint of TIMI major and minor bleeding or non-TIMI bleeding vs. patients receiving standard of care plus placebo, confirming the previous findings in elective PCI. Patients undergoing PCI (primary cohort) treated with SCH 530348 plus standard of care ($n = 71$) experienced a significant reduction in periprocedural MI (defined as a more than 3-fold elevation in creatine kinase-myocardial band or troponin I, with $\geq 50\%$ increase from baseline) compared with the 21 patients receiving standard of care alone (16.9 vs. 42.9%, respectively; 61% relative reduction, $P = 0.013$). There were no deaths or any other MACE.⁹⁶ The data from these Phase 2 trials demonstrate the potential clinical benefit of SCH 530348 when incorporated into the standard-of-care therapy for patients with vascular atherothrombotic disease undergoing PCI.

Taken together, the results of these initial studies indicate that SCH 530348 has the potential to reduce recurrent ischaemic events without exposing patients to increased bleeding risk. These findings provide a rationale for evaluation of SCH 530348 used in combination with current standard-of-care dual antiplatelet therapy in Phase 3 trials. Two Phase 3 trials for SCH 530348 are currently ongoing. The Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischaemic Events (TRA-2P-TIMI 50, NCT00526474) is a multinational, double-blind, randomized, placebo-controlled trial that is evaluating the efficacy of SCH 530348 plus standard of care, which includes aspirin and/or clopidogrel therapy, in the secondary prevention of ischaemic events in patients with prior MI, stroke, or PAD and is recruiting $\sim 20\,000$ patients. Patients are randomized to receive a 2.5 mg maintenance dose of SCH 530348 or placebo as secondary prevention. The primary endpoint of the trial is the composite of cardiovascular death, MI, urgent coronary revascularization, or stroke. The key secondary endpoint is cardiovascular death, MI, or stroke. Patients are being followed for a minimum of 1 year. The Phase 3 Thrombin Receptor Antagonist Clinical Event Reduction in Acute Coronary Syndrome (TRA-CER, NCT00527943) trial is a multinational, randomized, double-blind, placebo-controlled study evaluating the primary prevention of ischaemic events in $\sim 10\,000$ patients with NSTEMI ACS over ≥ 1 year of follow-up with a loading dose of SCH 530348 40 mg and a maintenance dose of SCH 530348 2.5 mg in addition to aspirin and clopidogrel. The primary endpoint is the composite of cardiovascular death, MI, stroke, rehospitalization for ACS, and urgent revascularization; the secondary endpoint is the composite of cardiovascular death, MI, and stroke.

E-5555 (thrombin receptor antagonist)

E-5555 is a novel, orally active, potent PAR-1 antagonist that has demonstrated antiplatelet effects without increasing bleeding times in pre-clinical studies (Figure 4).⁸⁹ Upon incubation with human platelet-rich plasma from healthy volunteers, E-5555 was shown to inhibit thrombin- and TRAP-mediated sCD40L release (IC₅₀: 47 and 38 nM, respectively) without affecting ADP-induced sCD40L release.⁹⁷ E-5555 also inhibited thrombin-induced release of interleukin-6 and P-selectin expression in human coronary artery smooth muscle cells and human coronary artery endothelial cells.⁹⁷ Interestingly, E-5555 was also shown to prevent vascular spasm at the site of intracranial bleeding in rabbits.⁹⁸ The *in vitro* effects of E-5555 on platelet function in blood samples from healthy subjects ($n = 10$), patients with coronary artery disease (CAD) treated with aspirin ($n = 10$), and patients with CAD treated with aspirin plus clopidogrel ($n = 10$) were recently reported.⁹⁹ Thrombin receptor activating peptide-induced platelet aggregation was inhibited almost completely at all tested doses of E-5555 (20 and 50 ng/mL and 100 mg/mL).⁹⁹ No dose-dependent effect was observed at the doses used in the study. The safety and tolerability of a once-daily oral dose of E-5555 50, 100, or 200 mg and its effects on inhibition of platelet aggregation, endovascular inflammatory processes, and the incidence of MACE is being evaluated over 24 weeks in patients with CAD aged 45–80 years and over 12 weeks in patients with NSTEMI ACS aged 45–80 years in two randomized, double-blind, placebo-controlled Phase 2 clinical trials called Lessons From Antagonizing the Cellular Effects of Thrombin (LANCELOT) 201 and 202 (NCT00312052 and NCT00548587). The enrolment for each study is anticipated to be about 600 patients.

Conclusion

Platelet activation is crucial for normal haemostasis but may also lead to the formation of occlusive platelet-rich thrombi, responsible for the manifestations of atherothrombotic disease. Consequently, antiplatelet agents such as aspirin and clopidogrel have become the cornerstone of treatment for patients with atherothrombotic complications. Although aspirin alone or in combination with clopidogrel has demonstrated significant clinical benefits, the residual morbidity and mortality associated with ischaemic events remains substantial in patients receiving such therapy, and these agents are associated with bleeding complications, emphasizing the need for antiplatelet therapies that afford more comprehensive platelet inhibition without increasing bleeding risk. The PAR-1-mediated platelet activation pathway via thrombin is a key contributor to platelet-mediated thrombosis, but it does not play a critical role in haemostasis. Thus PAR-1 antagonists may provide more comprehensive antithrombotic effects when used in combination with the current standard of care, with the potential to incrementally reduce atherothrombotic complications without increased bleeding risk. Results from Phase 2 trials of the PAR-1 antagonist SCH 530348 have provided promising data in support of this approach, in particular for patients undergoing PCI. The PAR-1 antagonist E-5555 is currently under Phase 2 clinical investigation and will provide further insights on

the safety and tolerability of PAR-1 blockade, as well as the potential to reduce ischaemic events when used in conjunction with standard antiplatelet treatment regimens. Ongoing Phase 3 clinical trials with SCH 530348 being performed in a very large cohort of patients with various manifestations of atherosclerotic disease will better define the overall net clinical benefit associated with inhibition of this important signalling pathway.

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