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REVIEW

Future innovations in anti-platelet therapies

NE Barrett, L Holbrook, S Jones[1], WJ Kaiser, LA Moraes, R Rana, T Sage, RG Stanley, KL Tucker, B Wright and JM Gibbins

School of Biological Sciences, University of Reading, Whiteknights, Reading, Berkshire, UK

Platelets have long been recognized to be of central importance in haemostasis, but their participation in pathological conditions such as thrombosis, atherosclerosis and inflammation is now also well established. The platelet has therefore become a key target in therapies to combat cardiovascular disease. Anti-platelet therapies are used widely, but current approaches lack efficacy in a proportion of patients, and are associated with side effects including problem bleeding. In the last decade, substantial progress has been made in understanding the regulation of platelet function, including the characterization of new ligands, platelet-specific receptors and cell signalling pathways. It is anticipated this progress will impact positively on the future innovations towards more effective and safer anti-platelet agents. In this review, the mechanisms of platelet regulation and current anti-platelet therapies are introduced, and strong, and some more speculative, potential candidate target molecules for future anti-platelet drug development are discussed.
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Abbreviations: CCL, chemokine ligand; CCR, chemokine receptor; CVD, cardiovascular disease; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; ERp5, endoplasmic reticulum protein 5; FcR, Fc receptor; Gas6, growth arrest-specific 6; GP, glycoprotein; GPCR, G protein-coupled receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; MI, myocardial infarction; NK, neurokinin; PAR, protease-activated receptor; PDI, protein disulphide isomerase; PEAR1, platelet endothelial aggregation receptor 1; PECAM-1, platelet endothelial cell adhesion molecule-1; PGI_2, prostacyclin; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; RANTES, regulated upon activation and normal T-cell expressed and secreted; sCD40L, soluble CD40 ligand; sema, semaphorin; SHP-1/2, Src homology phosphatase-1/2; SLAM, signalling lymphocyte activation molecule; SP, substance P; TLT-1, TREM-like transcript-1; TRAP, thrombin receptor activating peptide; TXA_2, thromboxane A_2; vWF, von Willebrand factor

Introduction

Cardiovascular diseases (CVDs) are major causes of death in the developed world, and are becoming an increasing burden in a number of developing countries (Callow, 2006). In the UK, CVDs, predominantly myocardial infarction (MI), ischaemic stroke and peripheral vascular disease, contribute towards more than one in three deaths, affecting both men and women (British Heart Foundation, 2006). Improvements in prevention and treatment have reduced total mortality caused by CVD (in England this had decreased by 24% to 2005 in the under-75 age group over the preceding 10 years; British Heart Foundation, 2006), although the incidence continues to rise. With increasing levels of obesity, a strong independent risk factor for CVD, and an ageing population, the clinical burden of cardiovascular disorders is set to increase.

Thrombosis, the formation of a blood clot within the blood vessel resulting in occlusion of blood flow, is a major problem that triggers both MI and stroke. Ironically, this is the result of inappropriate triggering of a normal protective homeostatic mechanism, haemostasis, the function of which is to prevent excessive blood loss following injury. Platelets form a first line of defence, triggering haemostasis on encountering damaged tissue. The aetiologies of cardiovascular disorders are complex and controversial, but the underlying conditions such as atherosclerosis are commonly the precipitating factor. The rupturing of unstable lesions results in the release of prothrombotic factors, such as oxidized lipids, and the exposure of collagen, which trigger thrombosis. Furthermore, the presence of adhesive substrates within the lesion and rheological disturbances caused by the narrowed artery lumen may contribute to pathological platelet recruitment (Andrews and Berndt, 2004; Gibbins, 2004; Steinhubl and Moliterno, 2005; Davi and Patrono, 2007).

Correspondence: JM Gibbins, School of Biological Sciences, University of Reading, AMS Building, Whiteknights, Reading, Berkshire RG6 6AJ, UK.
E-mail: j.m.gibbins@reading.ac.uk
[1]Current address: National Heart and Lung Institute, Imperial College London, London, UK.
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The therapeutic targeting of platelets is recognized as effective in the prevention and treatment of CVD (Ferguson, 2006; Mayr and Jilma, 2006; Meadows and Bhatt, 2007). The morbidity and mortality figures, however, indicate that current anti-platelet strategies (and anti-coagulant therapy) are far from a panacea. Limited efficacy in some patients, drug resistance and side effects are limitations of current approaches.

The regulation of platelet function, and thereby haemostasis and thrombosis, is a precarious balance between activatory and inhibitory mechanisms that control platelet activation upon exposure to damaged tissues, yet enable platelets to remain quiescent in the undamaged circulation. Characterization of these mechanisms (agonists, receptors and signalling pathways) is essential for the understanding of platelet function in health and disease and for the development of more effective anti-platelet therapies.

The thrombus formation process

Platelets are anucleate cells derived from megakaryocytes within the bone marrow that possess the ability to respond explosively at sites of injury. Blood vessel injury results in the exposure of subendothelial extracellular matrix components, particularly collagens, which provide a surface on which platelets can adhere, and are capable of stimulating platelet activation. Activation results in platelet aggregation to form a thrombus, or haemostatic plug, to stem the flow of blood from the injury site.

The conversion of platelets from their circulating quiescent form to a thrombus may be characterized in three distinct phases (Figure 1): adhesion, activation and thrombus propagation.

Adhesion receptors

Initial slowing or rolling of platelets over exposed collagens is mediated by transient and indirect binding via the glycoprotein (GP) complex GPIb–V–IX on the platelet surface. Under arterial flow conditions, GPIb–V–IX binds to plasma von Willebrand factor (vWF), which also binds to exposed collagen. These interactions are superseded by more stable adhesion to collagen via integrin $\alpha_2\beta_1$ (Savage *et al.*, 1996). Stable adhesion enables collagen binding to GPVI, which is non-covalently associated with the Fc receptor (FcR) γ-chain. Clustering of the receptor complex upon collagen binding results in the stimulation of signalling pathways that result in shape change, secretion and aggregation (Gibbins *et al.*, 1996, 1997; Poole *et al.*, 1997; Tsuji *et al.*, 1997; Nieswandt and Watson, 2003; Gibbins, 2004). The FcR γ-chain is a component of several antibody receptors, which possesses within its cytoplasmic tail an immunoreceptor tyrosine-based activatory motif. This conserved sequence motif is present in a range of immunoreceptors and upon receptor clustering becomes tyrosine phosphorylated, facilitating the assembly of multiprotein signalling complexes that drive cell activation (Gibbins *et al.*, 1996, 1997; Poole *et al.*, 1997; Tsuji *et al.*, 1997). Progress in understanding the regulation of immune function has been helpful in characterizing the early signalling events that result from

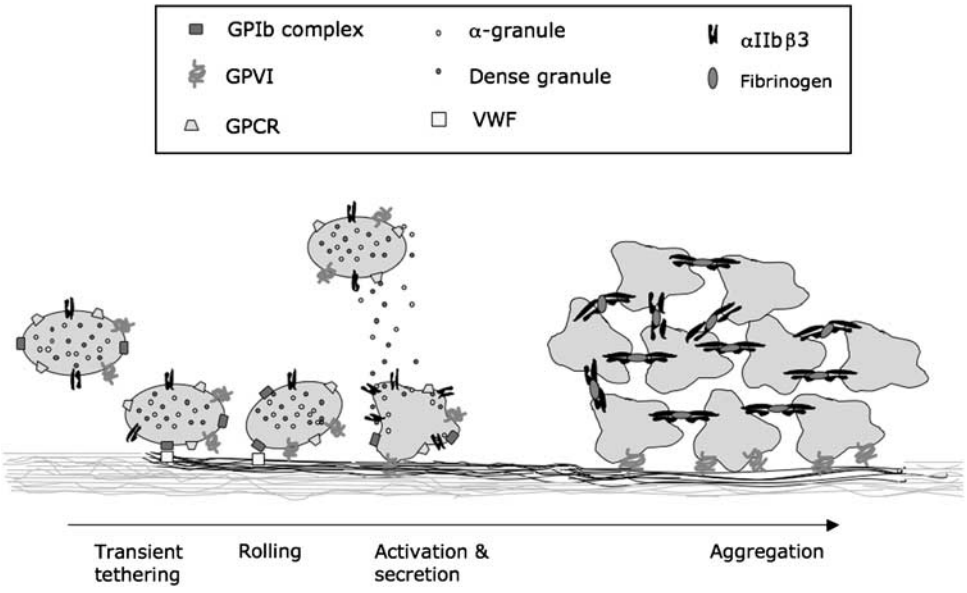

Figure 1 Platelet thrombus formation. Initial interaction of platelets with exposed collagens in the blood vessel wall under arterial shear conditions (left to right) is mediated indirectly through von Willebrand factor (vWF), which binds both GPIb and collagen. This transient interaction results in rolling along the collagen, effectively slowing down the platelet and enabling interaction of collagen with integrin $\alpha_2\beta_1$ and GPVI, supporting stable platelet adhesion and activation. Activation results in spreading, to cover the area of damage, the secretion of a range of pro-thrombotic factors from α-granules and dense granules, and the synthesis and release of thromboxane A_2. The binding of released factors to cognate G protein-coupled receptors (GPCRs) on the platelet surface leads to rapid thrombus propagation through positive feedback regulation. Platelet activation results in an increase in the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa), which through interaction with bivalent plasma fibrinogen results in stable platelet thrombus formation.

activation of GPVI. These pathways, which have been summarized in a number of reviews (Heemskerk *et al*., 2000; Leo and Schraven, 2000; Jackson *et al*., 2003; Nieswandt and Watson, 2003; Gibbins, 2004; Moroi and Jung, 2004; Watson *et al*., 2005), are dependent on the actions of a number of tyrosine and serine/threonine kinases, lipid kinases, phosphatases and adapter proteins, calcium mobilization and cytoskeletal reorganization, which in a highly coordinated manner orchestrate platelet spreading, secretion, integrin regulation, aggregation and thrombus formation (Gibbins, 2004; Watson *et al*., 2005).

A number of studies suggest that upon binding to the vWF–collagen complex, GPIb–V–IX also stimulates cell signalling that culminates in the intracellular mobilization of calcium and thereby contributes to platelet activation (Nesbitt *et al*., 2002; Goncalves *et al*., 2003; Schmugge *et al*., 2003; Ruggeri, 2007). The association of this receptor with the FcR γ-chain and indirectly with a number of components of the GPVI signalling pathway (Falati *et al*., 1999) indicates that these adhesion receptors may employ similar activatory mechanisms.

Receptors for secondary agonists

The secretion reaction upon platelet activation results in local high concentrations of a number of pro-thrombotic factors such as ADP and serotonin that act via cognate receptors on the platelet surface to reinforce stimulation and trigger positive feedback regulation (and thereby contribute to thrombus *propagation*). Platelets possess two receptors for ADP: $P2Y_1$ and $P2Y_{12}$. Both are G protein-coupled receptors (GPCRs): $P2Y_1$, which is essential for platelet activation, is coupled to Gq signalling; and $P2Y_{12}$, which synergizes via Gi coupling (Offermanns *et al*., 1997b; Hollopeter *et al*., 2001; Tolhurst *et al*., 2005; Gachet, 2006). During platelet activation, phospholipase A_2 is activated, resulting in the liberation of arachidonic acid from membranes, and via the actions of COX and thromboxane synthase, results in the generation of thromboxane A_2 (TXA_2) (Siess *et al*., 1983). Liberated TXA_2 binds to thromboxane-prostaglandin receptors, which through coupling to Gq also contribute to positive feedback activation (Armstrong, 1996; Halushka, 2000).

Individually, these secreted or released factors are weak agonists, but through synergism make important contributions to platelet activation, and are targeted by a number of current anti-platelet drugs. The lack of efficacy of these therapies in some patients is suggestive of additional uncharacterized primary or secondary agonists or pathways of activation.

Integrin receptors

A process known as inside-out signalling results in an increase in integrin affinity for respective ligands following platelet activation. It is this 'switch' mechanism that enables platelet–platelet adhesion and thrombus formation. This process, which is incompletely understood, is mediated through interactions with and between the cytoplasmic tails of the receptors, molecules on the external face of the membrane, and post-translational modifications such as phosphorylation (Jung and Moroi, 1998, 2000; Giancotti and Ruoslahti, 1999; Shattil, 1999; Ni and Freedman, 2003; Tadokoro *et al*., 2003; Shattil and Newman, 2004). Of particular importance for thrombus formation is integrin $\alpha_{IIb}\beta_3$, which through bivalent fibrinogen interactions and also by binding to vWF supports platelet aggregation. Integrin $\alpha_2\beta_1$ also contributes to thrombus formation through supporting platelet adhesion to collagen. There is extensive evidence that fibrinogen binding to its receptor results in the generation of 'outside-in' signalling, a second wave of signalling that further enhances thrombus stability. Although some enzymes such as focal adhesion kinase are uniquely involved in this process, curiously some proteins implicated in GPVI/GPIb–V–IX signaling, such as Syk and Src, also mediate outside-in signalling through integrin $\alpha_{IIb}\beta_3$ (Clark *et al*., 1994; Shattil, 1999; Obergfell *et al*., 2001; Gibbins, 2004; Watson *et al*., 2005).

Platelets and coagulation

Injury leads to the activation of the coagulation pathways, which result in the generation of thrombin in the vicinity of a platelet thrombus. The platelet thrombus provides a surface for the assembly of the prothrombinase complex and therefore thrombin is generated and fibrin produced within the developing platelet thrombus. Furthermore, through stimulation of the protease-activated receptors $(PAR)_1$ and PAR_4, present on the platelet surface, thrombin also acts as a powerful platelet activator (Kahn *et al*., 1998, 1999b; Leger *et al*., 2006).

Anti-thrombotic therapies

The therapeutic benefit of drugs that inhibit platelet function is well recognized. As described below, a number of beneficial therapeutic approaches are well established in the clinic for conditions, such as ischaemic stroke and MI for preventing reoccurrence in patients with a previous history of such conditions and during specific cardiovascular surgical procedures (Ischinger, 1998; Antithrombotic Trialists' Collaboration, 2002). Platelets perform a vital homeostatic function, the absence of which results in pathological bleeding. A balance is therefore required in the prevention of thrombosis while leaving haemostasis sufficiently intact to prevent haemorrhage. The challenge to produce the 'magic bullet' drug that targets only pathological platelet function may be unrealistic, but increased knowledge of the agonists, receptors and signalling mechanisms that control the function of these cells will enable more refined, platelet-specific and efficacious approaches to be developed that may be used in combination therapies.

To enable the focus for future development to be discussed, the mechanisms of action of anti-platelet drugs that are in current use are briefly summarized below. For more detailed discussion of the clinical aspects of the use of these approaches, the reader is directed to several recent excellent reviews in this area (Bhatt and Topol, 2003; Jackson and Schoenwaelder, 2003; Meadows and Bhatt, 2007).

Current anti-platelet therapies

Inhibition of thromboxane A_2 production

First generated in 1897, aspirin has been used as an anti-inflammatory, analgesic and anti-pyretic for many decades. In 1971, its mechanism of action to inhibit prostaglandin formation was elucidated (Ferreira *et al*., 1971; Smith and Willis, 1971; Vane, 1971; Vane and Botting, 2003). Taken orally, aspirin irreversibly acetylates and inactivates the enzyme COX, primarily COX-1, preventing the conversion of arachidonic acid to prostaglandins (see Figure 2), such as TXA_2 in platelets or prostacyclin (PGI_2) in endothelial cells. As the plasma half-life of aspirin is only 20 min and endothelial cells are able to generate new COX-1, the effect of aspirin on the endothelium is transient. Platelets, however, are unable to generate new COX-1, and the inhibition is irreversible (Awtry and Loscalzo, 2000).

The benefit of aspirin use in the prevention and treatment of various chronic or acute CVDs is widely recognized and supported by extensive clinical data (Lewis *et al*., 1983; Cairns *et al*., 1985; Theroux *et al*., 1988; Wallentin, 1990; Roux *et al*., 1992; Antithrombotic Trialists' Collaboration, 2002). Severe side effects, including gastric ulcers, renal failure and bleeding, are, however, experienced by some patients (Awtry and Loscalzo, 2000). The Antithrombotic Trialists' Collaboration (2002) compared several studies and concluded that 75–375 mg aspirin per day was at least as effective as higher doses (Antithrombotic Trialists' Collaboration, 2002). Aspirin is reported to be ineffective in 5–40% of patients in the prevention of thrombotic events and a phenomenon of aspirin resistance has been described (Gum *et al*., 2001).

ADP receptor antagonists

Clopidogrel, a thienopyridine, is an irreversible antagonist of the ADP receptor $P2Y_{12}$ (see Figure 2). Clopidogrel also has the advantage of being administered orally. When taken at 75 mg day^{-1}, it is able to reduce ADP-induced aggregation by 40–60% (Thebault *et al*., 1999) and is reported to be more effective than aspirin (325 mg day^{-1}) in preventing vascular death, MI or ischaemic stroke but with reduced gastric

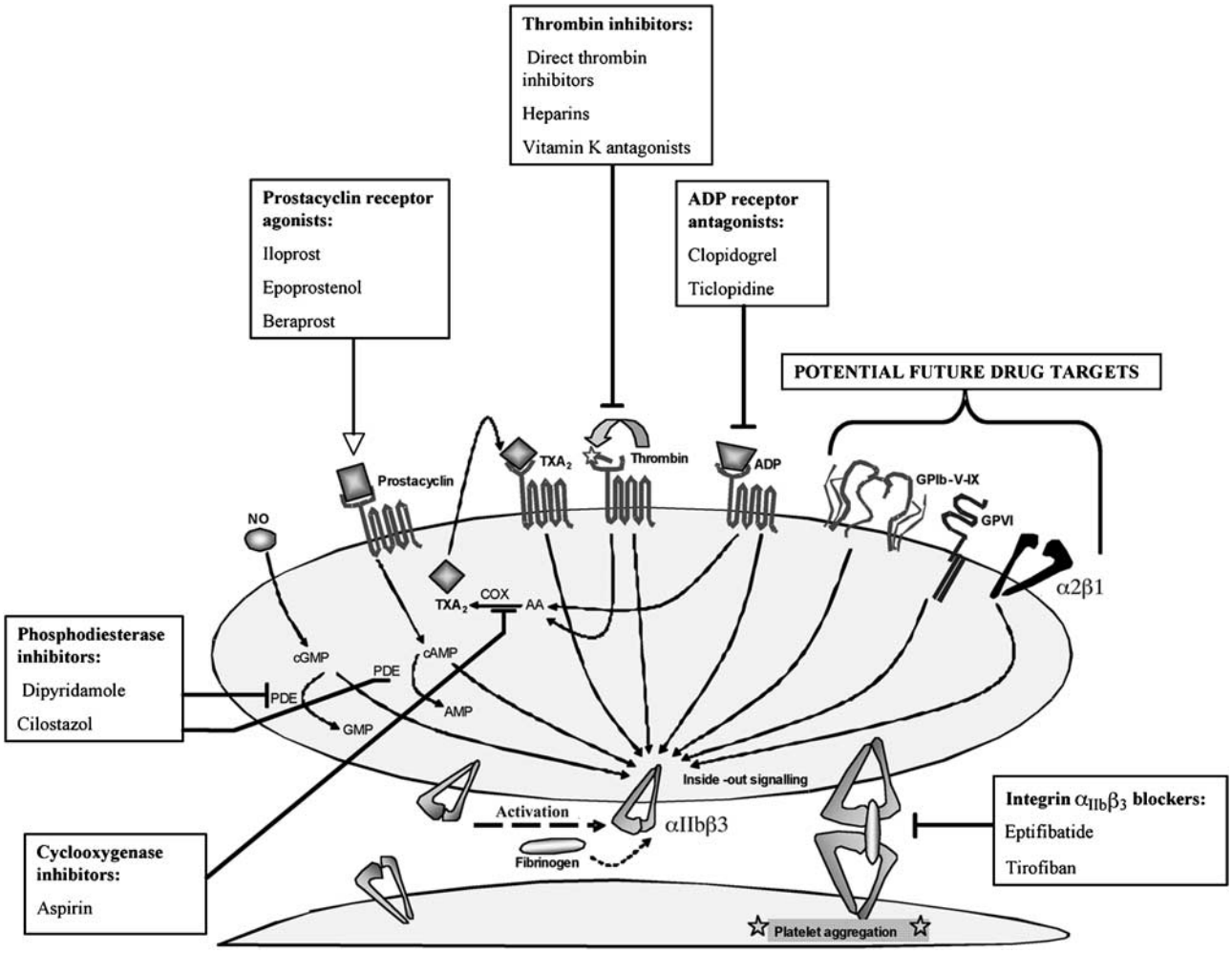

Figure 2 The molecular targets of anti-platelet drugs. Current anti-platelet drugs target key elements of the platelet regulatory machinery. This includes cyclooxygenase (COX) inhibitors for the inhibition of thromboxane A_2 (TXA_2) production, ADP receptor antagonists, fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$) blockers, prostacyclin receptor agonists, phosphodiesterase inhibitors and inhibitors of thrombin generation. Future potential targets for anti-platelet drug development include GPIb–V–IX, GPVI and integrin $\alpha_2\beta_1$. Adapted with permission from Jackson and Schoenwaelder (2003).

irritation (CAPRIE Steering Committee, 1996). When used at higher doses, however, it increases bleeding time (Thebault *et al*., 1999). The overall effects of clopidogrel have been examined extensively in several clinical trials involving more than 80 000 patients (recently reviewed in detail by Meadows and Bhatt, 2007), demonstrating the ability of clopidogrel to reduce the incidence of vascular death, MI or ischaemic stroke. Occasional resistance to clopidogrel has been reported (Heptinstall *et al*., 2004), as have side effects such as cardiac events, gastrointestinal problems, neutropaenia and thrombotic thrombocytopaenic purpura (Bennett *et al*., 2000; Elmi *et al*., 2000). Such side effects are, however, reduced in comparison to ticlopidine, the forerunner thienopyridine to clopidogrel, and newer $P2Y_{12}$ antagonists in development aim to further reduce these limitations (Kam and Nethery, 2003).

Integrin $\alpha_{IIb}\beta_3$ blockers

Integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) supports fibrinogen-mediated platelet aggregation and thrombus formation (see Figures 1 and 2). As a culminating common component of platelet activation pathways, it has been a favoured target for anti-platelet therapies (Bhatt and Topol, 2003). The first such antagonist developed, abciximab, an anti-$\alpha_{IIb}\beta_3$ monoclonal $F(ab')_2$ fragment, is administered intravenously and is beneficial in preventing thrombosis in patients undergoing percutaneous coronary intervention (Popma and Satler, 1994; Genetta and Mauro, 1996; Bhatt and Topol, 2000; Casserly and Topol, 2002; Topol *et al*., 2002). The dose required for anti-thrombotic effect, however, is associated with a loss of haemostasis. Indeed, bleeding complications are a common feature of other $\alpha_{IIb}\beta_3$ receptor antagonists, such as epitifibatide and tirofiban (non-peptide and cyclic heptapeptide antagonists, respectively), which are also administered intravenously, and thus unsuitable for prophylaxis (Scarborough *et al*., 1999).

In contrast to abciximab, epitifibatide and tirofiban, orally available $\alpha_{IIb}\beta_3$ antagonists lack efficacy and cause bleeding and have therefore failed at the preclinical or clinical trial stages (Harrington *et al*., 2000; Chew, 2001; Chew *et al*., 2001).

Phosphodiesterase inhibitors

The elevation of the levels of cyclic nucleotides cAMP and cGMP in the platelet cytosol stimulates signalling pathways that inhibit platelet activation. Indeed the inhibitory effects of endothelium-derived nitric oxide and PGI_2 on platelet function are mediated through stimulation of cGMP- and cAMP-dependent signalling mechanisms, respectively. The inhibition of phosphodiesterase enzymes, which metabolize these second messengers, therefore suppresses platelet function (Feijge *et al*., 2004; Sim *et al*., 2004). The phosphodiesterase inhibitors cilostazol and dipyridamole are licensed for the treatment of stroke and intermittent claudication, for which clinical trials have demonstrated modest but consistent benefits (Reilly and Mohler, 2001). Cilostazol has been shown to decrease α-granule secretion (Inoue *et al*., 1999) and thereby reduce platelet–leukocyte interactions (Inoue *et al*., 1999; Ito *et al*., 2004) as well as acting as a vasodilator (Becker *et al*., 2005). In patients with peripheral arterial disease, cilostazol has not been associated with major adverse events or increased mortality although frequent but minor adverse effects have been noted (Reilly and Mohler, 2001).

Development of new anti-platelet drugs

The notion that the targeting of platelet function may be beneficial in the prevention of thrombosis is born out by several clinical trials and the wide use of anti-platelet therapies. A lack of efficacy in many patients, however, indicates that additional regulatory mechanisms (agonists, receptors and signalling pathways) are required to regulate platelet function and is suggestive of functional redundancy between pathways. In considering new approaches for the inhibition of platelets, two principal approaches may be used: (1) to develop more efficacious drugs that modulate the function of characterized and clinically proven platelet molecules (the 'best-in-class' approach) or (2) to target newly identified and characterized platelet molecules (the 'first-in-class' approach). Recent drug development in this area has favoured the former approach although considerable developments in the understanding of the platelet regulatory machinery in the last decade, and particularly the detailed characterization of platelet receptors, raise the possibility of new approaches. A number of commonly used anti-platelet drugs do not specifically target the platelet, which is likely to underlie the range of side effects observed. The characterization of platelet-specific receptors is likely to be of particular importance in overcoming these limitations.

Whatever the target, the challenge remains to inhibit thrombosis but to leave haemostasis sufficiently intact to prevent bleeding complications. Transgenic mice engineered to lack specific genes are ideal models in which to test the suitability of a specific protein as a drug target in platelets, and with the inability to readily genetically modify platelets *in vitro* this is the preferred approach. Such models are important to establish the role of specific proteins in the regulation of platelet function and have enabled the mapping of complex signalling pathways. To assess the potential of a specific protein as a drug target, it is important that platelet function is examined *in vivo*. A range of *in vivo* assays of thrombosis have therefore been developed and used in a range of species including primates, dogs, rabbits and rodents (Falati *et al*., 2004; Denis and Wagner, 2007; Eitzman, 2007; Westrick *et al*., 2007). The ability of a number of assays to be adapted for use in the mouse has enabled the detailed characterization of the physiological importance of specific proteins in both haemostasis and thrombosis, and the toleration of its absence in transgenic mouse models. In addition, bleeding assays may be used to assess the impact of the loss (or gain) of function of a specific protein on haemostasis. The ideal target will be important for thrombus formation, but have little impact on haemostasis. Care should, however, be taken in the interpretation of *in vivo* measurement of thrombosis, as different injury types or the extent of blood vessel injury may impact on the extent of the effects observed on thrombus formation. Recent studies

indicate that a range of injury models should be applied and the potential impact of different genetic backgrounds, for example in transgenic mouse studies, should not be overlooked (Zumbach *et al*., 2001; Nieswandt *et al*., 2005).

In the following sections, current 'strong candidates' as anti-thrombotic drug targets are discussed. The recent discovery of a number of new molecules and pathways that participate in the regulation of platelets raises the possibility of future new strategies in the development of drugs to prevent thrombosis ('New molecules with future promise'). This includes a number of proteins for which validation *in vivo* has yet to be performed, and therefore the potential for drug development remains to be established.

Strong candidates

Understanding of the molecular mechanisms that control the function of platelets, either on encountering tissue damage—triggering haemostasis—or unstable atherosclerotic lesions—triggering thrombosis, is likely to impact highly on innovations in anti-platelet therapies of the future. The importance of gene deletion in transgenic mouse models in elucidating these mechanisms is evident, but such models and *in vivo* analysis of platelet function (along with parallel techniques such as the infusion of receptor-blocking antibodies) also offer the potential to test the effects of the loss of function of specific proteins on both haemostasis and thrombosis. This strategy enables the promise of specific molecules to be assessed for potential as drug targets focusing on potential efficacy, that is, anti-thrombotic action in the absence of the protein or absence of protein function, but with minimal bleeding. This strategy is a good means to assess which of the currently characterized platelet molecules, be they ligands, receptors or intracellular signalling molecules, should provide the focus for current drug development in this area. The results of studies of platelet function in a wide range of transgenic mouse models are summarized in Table 1, where the impact of gene deletion on platelet function *in vitro*, thrombus formation *in vivo* and haemostasis is highlighted.

Cell surface adhesion/signalling receptors and their ligands

GPIb–vWF

The role of platelet-specific glycoprotein GPIb (a component of the GPIb–V–IX complex) is of critical importance in the initial entrapment of platelets at sites of collagen exposure, indirectly tethering platelets under high shear condition via plasma vWF. The importance of this protein in transient interactions that slow down and tether platelets, enabling cell signalling and activation, is evident in patients who lack this protein (Bernard–Soulier syndrome) or in GPIb-deficient mice, where a severe bleeding defect is observed (Lopez *et al*., 1998; Ware *et al*., 2000). Furthermore, the infusion of antibodies to vWF or GPIb inhibits thrombosis *in vivo*. Although defective haemostasis is apparent in the absence of GPIb function, this is not as severe as that observed on administration of integrin $\alpha_{IIb}\beta_3$ antagonists (Hodivala-Dilke *et al*., 1999; Ware *et al*., 2000). Indeed inhibition of platelet GPIb *in vivo* using antibodies results in the inhibition of thrombosis at doses where minimal effect on bleeding is observed (Wu *et al*., 2002). It is therefore possible that inhibition of GPIb may be more desirable than the inhibition of integrin $\alpha_{IIb}\beta_3$. The absence of the GPIb gene is, however, associated with abnormal thrombogenesis: giant platelets, thrombocytopaenia and reduced platelet responsiveness (Takahashi *et al*., 1999; Cauwenberghs *et al*., 2000). The effect of long-term use of anti-GPIb medication may therefore, through chronic effects on megakaryocyte function, lead to enhanced bleeding. A second approach to reduce GPIb-mediated thrombogenesis may be to target vWF. Indeed, antibodies against the A3 domain of vWF, through which interactions with GPIb are mediated, have been shown to inhibit arterial thrombus formation *in vivo* (Wu *et al*., 2002).

GPVI and integrin $\alpha_2\beta_1$–collagen

Targeting GPIb or vWF would indirectly interfere with platelet interactions with collagen. As stable platelet adhesion to collagen is mediated by integrin $\alpha_2\beta_1$, and platelet activation is stimulated through collagen binding to GPVI, the consequences of inhibition of either of these has gained considerable attention. Furthermore, human studies involving patients lacking either receptor, or equivalent mouse models, reveal reduced platelet responsiveness to collagen, with only mild deficiencies in haemostasis (Nieuwenhuis *et al*., 1985; Moroi *et al*., 1989; Holtkotter *et al*., 2002; Kato *et al*., 2003). Although GPVI-deficient mice were reported to be protected from experimentally induced thrombosis (Nieswandt *et al*., 2001b), a more recent study in which a range of *in vivo* models of thrombosis were employed indicates that the protective effects are evident only in the absence of thrombin (Mangin *et al*., 2006). This suggests that the inhibition of thrombosis through administration of molecules that block GPVI may be ineffective unless co-administered with anti-coagulants that block either the formation or activity of thrombin.

An alternative means to inhibit collagen–GPVI interactions that has been explored is to target collagen itself. This has been approached using recombinant dimeric extracellular domain of GPVI (PR-15, Trigen, London, UK). As with all recombinant therapeutics, use would be limited to an intravenous route of administration, and the low affinity of GPVI–collagen interactions may limit efficacy, which has yet to be explored in humans.

Platelet G protein-coupled receptors

The most widely employed anti-platelet drugs target the effects of secondary mediators of platelet function, many of which act via GPCRs. These include inhibition or antagonism of ADP binding to the ADP receptor, $P2Y_{12}$, and inhibition of TXA_2 formation/action. More effective means to modulate such mechanisms, either individually or in combination, have been considered.

New $P2Y_{12}$ antagonists have been developed such as prasugrel (an oral thienopyridine) and the reversible non-thienopyridine drugs AZD6140 and cangrelor to try to overcome variability of response, the length of administra-

Table 1 Effect of deletion of genes for platelet regulatory proteins on haemostasis and thrombosis in transgenic mice

		Effect of gene deletion on platelet function/thrombosis		*Effect of gene deletion on haemostasis*	*Reference*
		In vivo	In vitro		
Cell adhesion receptors					
GPIb–V Platelets and megakaryocytes	GPIbα	Platelet adhesion and thrombus formation absent in $FeCl_3$ thrombosis model (IL4Rα/GPIbα transgenic mouse)	Aggregation unchanged to U46619 (TXA_2 analogue) or thrombin	Severe bleeding. Reduced platelet count. Giant platelets	Ware *et al*., 2000; Bergmeier *et al*., 2006
	GPIbβ			Severe bleeding. Reduced platelet count. Giant platelets. Increased α-granule size	Kato *et al*., 2004
	GPV		Aggregation increased to thrombin	Unchanged bleeding time	Ramakrishnan *et al*., 1999
		Increased time to occlusion and decreased platelet-endothelial detachment time in $FeCl_3$ thrombosis model	Aggregation decreased to collagen, unchanged to ADP and U46619. Decreased adhesion to a collagen surface under flow		Moog *et al*., 2001
			Aggregation unchanged to thrombin		Kahn *et al*., 1999a
		Accelerated platelet adhesion and thrombus formation in $FeCl_3$ thrombosis model. Decreased occlusion time	Flow: no change in adhesion or thrombus volume on a collagen surface		Ni *et al*., 2001
GPVI/FcR γ-chain Platelets and megakaryocytes		Occlusive injury and cyclic flow reductions unchanged in stenosis, electrolytic and severe laser injury models. Reduced size of thrombi with weak laser injury	Thrombus formation and platelet spreading absent when flowed over a collagen surface. Aggregation to collagen and convulxin decreased. Normal to phorbol 12-myristate 13-acetate	Unchanged bleeding time	Kato *et al*., 2003; Mangin *et al*., 2006
Integrin $\alpha_2\beta_1$ Widely expressed	α_2 Receptor for laminin, collagen, collagen C-propeptides, fibronectin and E-cadherin	Delayed occlusion. Possible unstable thrombi. No change in platelet count with collagen + adrenaline embolization model	Non-adherent to collagen. Aggregation to collagen with prolonged lag phase and decreased rate	Unchanged bleeding time	Chen *et al*., 2002; Holtkotter *et al*., 2002; He *et al*., 2003
	β_1 Receptor for collagen and any ligand containing the sequence RGD		Aggregation to fibrillar collagen delayed; abolished to soluble collagen. Normal to collagen, collagen-related peptide, thrombin and ADP	Unchanged bleeding time	Nieswandt *et al*., 2001a
Integrin $\alpha_{IIb}\beta_3$ Platelets and megakaryocytes	α_{IIb}		No aggregation to ADP or collagen. Reduced binding to fibrinogen surface	Severe bleeding. Failure to undergo clot retraction	Tronik-Le Roux *et al*., 2000
	β_3	Reduced haemorrhage to subcutaneous lipopolysaccharide with reduced number of thrombi. No occlusion to $FeCl_3$. Platelet counts not reduced with i.v. ADP; partial with collagen + adrenaline; minimal with tissue factor	Shape change but not aggregation to ADP	Prolonged bleeding time. No clot retraction to thrombin—fibrinogen absent from clots	Hodivala-Dilke *et al*., 1999; Smyth *et al*., 2001

Table 1 *Continued*

		Effect of gene deletion on platelet function/thrombosis		*Effect of gene deletion on haemostasis*	*Reference*
		In vivo	In vitro		
Integrin $\alpha_v\beta_3$ (vitronectin) Platelets and megakaryocytes		Reduced time to occlusion. No change in the amount of $FeCl_3$-induced thrombus formation	Aggregation increased to thrombin, no change to ADP	Reduced prothrombin time	Fay *et al.*, 1999
PECAM-1 (CD31) Platelets, endothelial cells and some leukocytes		Both unchanged and increased rates of thrombus formation and occlusion reported. Increased thrombus size. More stable thrombi	Increased aggregation to collagen. Increased thrombus size. Enhanced spreading on vWF. Hyperaggregation to vWF	Prolonged and unchanged bleeding times reported	Mahooti *et al.*, 2000; Patil *et al.*, 2001; Vollmar *et al.*, 2001; Rathore *et al.*, 2003; Falati *et al.*, 2006
Other receptors/ligands					
$P2Y_1$ Widely expressed		Resistance to collagen + adrenaline-induced thromboembolism. Resistance to thromboplastin-induced thromboembolism. Reduced number of occlusion sites; no reduction in platelet count	No shape change or aggregation to ADP. Extended lag-time to collagen	Prolonged bleeding time	Fabre *et al.*, 1999; Leon *et al.*, 1999, 2001
$P2Y_{12}$ Platelets, brain, lung, appendix, pituitary and adrenal gland		Resistance to $FeCl_3$-induced injury. Delayed thrombus formation, increased occlusion time. Smaller thrombi	Aggregation: shape change but not aggregation to ADP. Impaired to low-dose collagen, TRAP, thrombin and epinephrine. Normal aggregation to U46619 Flow: reduced adhesion to vWF. Loosely packed thrombi over collagen Flow cytometry: reduced fibrinogen binding	Severe bleeding. No change to prothrombin time or activated partial thromboplasmin time	Foster *et al.*, 2001; Andre *et al.*, 2003
PAR_3 Platelets, endothelial cells, myocytes and neurons		Reduced thrombus formation in $FeCl_3$ thrombosis model. Resistance to thromboplastin-induced pulmonary embolism. Reduced thrombus size	Aggregation delayed and reduced to thrombin	Both prolonged and unchanged bleeding times reported	Kahn *et al.*, 1998; Weiss *et al.*, 2002
PAR_4 Platelets, endothelial cells, myocytes and neurons		Resistance to thromboplastin-induced pulmonary embolism		Prolonged bleeding time	Kahn *et al.*, 1998; Weiss *et al.*, 2002; Hamilton *et al.*, 2004
Thromboxane-prostaglandin receptor Widely expressed		Resistance to U45519- and arachidonic acid-induced thromboembolism	Aggregation: none to U46619. Increased lag-time to collagen. Unchanged to ADP	Prolonged bleeding time	Thomas *et al.*, 1998
Tissue factor Subendothelial tissue, platelets and leukocytes		Reduced platelet accumulation in developing thrombi. Very small thrombi lacking TF and fibrin		Unchanged prothrombin time and activated partial thromboplasmin time	Parry *et al.*, 1998; Chou *et al.*, 2004
Gas6 receptors Widely expressed. Tyro3 is abundant in the brain	Mer	Inhibition of $FeCl_3$-induced thrombosis. Resistance to thromboplastin-induced pulmonary embolism. Delayed occlusion	Aggregation to collagen, U46619, PAR_4 analogue and thrombin all decreased. Unchanged to ADP	Unchanged bleeding time, prothrombin time and activated partial thromboplasmin time	Chen *et al.*, 2004a
	Mer, Sky/ Tyro3 or Axl	Reduced thrombosis. Reduced thromboplastin-induced pulmonary thromboembolism	Aggregation: abolished to low-dose ADP, collagen or U46619. Normal to thrombin and PAR_4-activating peptide. Delayed binding to fibrinogen surface. Impaired secretion	Unchanged bleeding time. Increased rebleeding time. Impaired clot retraction to thrombin	Angelillo-Scherrer *et al.*, 2005a

Table 1 *Continued*

	Effect of gene deletion on platelet function/thrombosis		*Effect of gene deletion on haemostasis*	*Reference*
	In vivo	In vitro		
Gas6 Widely expressed	Reduced thrombus size. No sign of pulmonary embolization with collagen and adrenaline model	Aggregation decreased to low-dose ADP, collagen and U46619. Abnormal to thrombin—loosely packed aggregates, incomplete degranulation	Unchanged bleeding time	Angelillo-Scherrer *et al*., 2001
Plasma fibronectin Widely expressed	No change in adhesion. Delayed $FeCl_3$-induced thrombus formation and occlusion. Unstable thrombi. Increased infarct size with a stroke model (transient cerebral ischaemia)	Aggregation unchanged to collagen	Unchanged bleeding time and activated partial thromboplasmin time	Sakai *et al*., 2001; Ni *et al*., 2003
SLAM (CD150)	Delayed $FeCl_3$-induced thrombus formation and occlusion in mice. Increase in embolization of small thrombi	Aggregation reduced to TRAP and collagen. Normal to ADP	Unchanged bleeding time	Nanda *et al*., 2005a
CD40L (CD154) Activated CD4 + T-lymphocytes and platelets	Unchanged $FeCl_3$-induced thrombus formation time. Frequent rupture and embolization of large thrombi. Delayed occlusion	Aggregation to ADP, collagen or thrombin unchanged. Decreased aggregation to collagen at high shear	Unchanged bleeding time and plasma clotting time	Andre *et al*., 2002
NK_1 receptor		Reduced thrombus size when flowed over collagen. Aggregation response to thrombin slightly diminished	Moderately increased	Graham *et al*., 2004; Jones *et al*., 2007
SP CNS and peripheral nervous system, some leukocytes and platelets		Reduced thrombus volume when flowed over collagen		Jones *et al*., 2007
Intracellular signalling molecule				
PI3Kγ	Unable to form stable thrombi in $FeCl_3$-induced thrombosis model. Reduced platelet accumulation, thromboembolism and occlusion in i.v. ADP-induced thrombosis model	Aggregation and fibrinogen binding impaired to ADP but normal to collagen and thrombin. Disaggregation to low-dose ADP. Slow spreading on immobilized fibrinogen	Unchanged bleeding time	Hirsch *et al*., 2001; Lian *et al*., 2005
Akt1		Aggregation: reduced and delayed to thrombin and collagen + fibrinogen. None to collagen. Reduced to phorbol 12-myristate 13-acetate. Delayed to ADP Fibrinogen binding: decreased to thrombin and PMA, normal to ADP, increased to LY294002. Delayed spreading on collagen	Prolonged bleeding time	Chen *et al*., 2004b
Akt2	Resistant to thrombosis after $FeCl_3$ -induced injury	Aggregation: none to low-dose PAR_4 agonist or U46619. Unstable aggregates to ADP. Granule secretion and binding to fibrinogen inhibited with low-dose PAR_4 agonist	Unchanged bleeding time	Woulfe *et al*., 2004
Syk		Decreased secretion and aggregation to collagen but not thrombin	Unchanged bleeding time	Poole *et al*., 1997

Table 1 *Continued*

	Effect of gene deletion on platelet function/thrombosis		*Effect of gene deletion on haemostasis*	*Reference*
	In vivo	In vitro		
Rap1b	Highly delayed thrombus formation and no occlusion in $FeCl_3$-induced injury model	Aggregation reduced to ADP and epinephrine. Aggregation to collagen and convulxin only present at high concentrations. Reduced spreading on fibrinogen	Prolonged bleeding time	Chrzanowska-Wodnicka *et al.*, 2005
CalDAG-GEFI	Thrombus formation absent in $FeCl_3$-induced injury			Bergmeier *et al.*, 2007

Abbreviations: CD40L, CD40 ligand; FcR, Fc receptor; Gas6, growth arrest-specific 6; NK_1, neurokinin receptor 1; PAR, protease-activated receptor; SLAM, signalling lymphocyte activation molecule; SP, substance P; TRAP, thrombin receptor agonist peptide; TXA_2, thromboxane A_2; vWF, von Willebrand factor.

tion to see benefit, and the non-reversibility of clopidogrel. Indeed prasugrel displays 10-fold higher potency than clopidogrel in animal studies (Niitsu *et al.*, 2005), potent effects are observed in humans and phase 3 trials with patients undergoing percutaneous coronary intervention have been recently completed (Jakubowski *et al.*, 2007). Clinical trials are also underway with AZD6140 and cangrelor, again for percutaneous coronary intervention (recently reviewed by Meadows and Bhatt, 2007). Future developments may also include exploring the simultaneous blockade of $P2Y_1$ and $P2Y_{12}$ receptors although transgenic mouse studies indicate that excessive bleeding may be encountered (Fabre *et al.*, 1999; Leon *et al.*, 1999).

The use of aspirin to inhibit TXA_2 synthesis is also associated with a reduction in PGI_2 synthesis by endothelial cells. An alternative approach is to inhibit TXA_2 binding to thromboxane receptors (thromboxane-prostaglandin α/β). Attempts to inhibit TXA_2 synthesis and thromboxane-prostaglandin receptor antagonism in combination therapies, however, have failed to show benefit over aspirin (RAPT Investigators, 1994).

The PARs (PAR_1 and PAR_4) mediate the activation of platelets on exposure to thrombin. The antagonism of these receptors as anti-platelet agents has gained considerable attention. Of these GPCRs, PAR_1 has higher affinity for thrombin and is considered the principal effector, with PAR_4 synergizing at later stages in the activation process (Kahn *et al.*, 1999b). Several peptide and non-peptide antagonists have been developed, which display synergism when administrated together (Wu and Teng, 2006). Clinical studies are underway for two PAR_1 antagonists, E5555 and SCH 530348, in a coronary artery disease and percutaneous coronary intervention context. Furthermore, the use of direct thrombin inhibitors in development for the inhibition of coagulation may also be of benefit for the reduction of platelet stimulation.

Intracellular targets

Phosphoinositide 3-kinase–p110β

A considerable number of studies have revealed the critical importance of specific signalling proteins in the regulation of platelet function. Although this raises the possibility of targeting such molecules to inhibit thrombus formation, their absence or inhibition is frequently associated with excessive bleeding (Offermanns *et al.*, 1997a; Mangin *et al.*, 2003). In many cases, the wide expression profile of many cell signalling molecules limits the utility of their selective inhibitors. Notably, however, one isoform of phosphoinositide 3-kinase (PI3K) appears to be important for thrombus formation.

PI3Ks are classified into three classes based on structure, substrate and action (Vanhaesebroeck *et al.*, 1997; Anderson and Jackson, 2003). Class I PI3Ks are further subdivided into four isoforms based upon the p110 catalytic subunit (α, β, γ and δ), all of which are present in platelets (Vanhaesebroeck *et al.*, 1997; Zhang *et al.*, 2002; Watanabe *et al.*, 2003). PI3Ks operate downstream of the major platelet receptors to produce the second messenger phosphoinositide 3,4,5-trisphosphate (among others) with the different isoforms operating in different platelet receptor pathways. p110α, p110β and p110δ are recognized to be regulated by tyrosine kinases (Jackson *et al.*, 1994; Kasirer-Friede *et al.*, 2004), and p110γ is involved in GPCR signalling (Kauffenstein *et al.*, 2001; Foster *et al.*, 2003; Resendiz *et al.*, 2003).

Knockout mouse models exist for all four p110 isoforms. Deficiency of either of the ubiquitously expressed p110α and p110β subunits is embryonic lethal, whereas p110γ- and p110δ-deficient mice are viable (Jackson *et al.*, 2005). The use of general PI3K inhibitors has established a role for members of this family in adhesion of platelets under flow conditions and consequent activatory signalling (Goncalves *et al.*, 2003; Jackson *et al.*, 2005). Such adhesion and calcium signalling is unaffected in p110γ- and p110δ-deficient blood, ruling out the involvement of these isoforms (or indicate redundancy) (Jackson *et al.*, 2005). In the absence of viable mice to investigate the roles of p110α and p110β in haemostasis and thrombosis, selective inhibitors have been developed. The most promising of these, the p110β inhibitor TGX-221, is able to prevent the formation of stable $\alpha_{IIb}\beta_3$-dependent adhesion *in vitro*, inhibit aggregation induced by threshold doses of collagen, thrombin and TXA_2, abolish occlusive thrombus formation and yet cause no prolonged bleeding in rats and rabbits (Jackson *et al.*, 2005). This study defines p110β as a novel anti-thrombotic target, but acknowledges that given the ubiquitous expression of this

enzyme, the challenge will be to minimize the effects in other cell types.

New molecules with future promise

In recent years, a number of new platelet agonists, receptors and cell signalling molecules have been discovered, characterized and shown to be of importance for the regulation of platelet function. Although the potential value of many of these molecules for drug development has yet to be tested fully using *in vivo* models, they may offer the possibility of new approaches in the future. The following section, which is not exhaustive, summarizes some recent discoveries of particular note in this regard.

Cell surface adhesion/signalling receptors and their ligands

Eph kinases and ephrins

A growing body of evidence indicates that signalling between platelets in the later phases of thrombus formation is important for thrombus stability and effective haemostasis (Brass *et al*., 2006). Molecules implicated in this include members of Eph kinases and ephrin families of cell surface receptors, more recognized for their roles in axonal steering and vasculogenesis. The Eph kinases comprise a large family of receptor tyrosine kinases, and the ephrins are either glycosylphosphatidyl inositol-linked to the plasma membrane (A ephrins) or are transmembrane proteins (B ephrins). Generally, A Eph kinases bind to A ephrins, and B Eph kinases bind to B ephrins, although a degree of promiscuity of interaction is observed (Gale *et al*., 1996). Ligand binding results in receptor clustering and stimulation of signalling, which for both the Eph kinase and the ephrin ligand may be tyrosine phosphorylation-dependent (Hock *et al*., 1998; Lin *et al*., 1999). Platelets have been reported to possess EphA4, EphB1 and ephrinB1 proteins (Prevost *et al*., 2002). Clustering EphA4 and ephrinB1 using oligomeric recombinant binding partners results in adhesion and spreading on fibrinogen and α-granule secretion. Adhesion requires PI3K and protein kinase C activity, but does not require ephrinB1 phosphorylation (Prevost *et al*., 2004). Furthermore, ephrinB1 clustering results in activation of Rap1B, a signalling molecule implicated in the upregulation of integrin $\alpha_{IIb}\beta_3$ affinity. Aggregation of platelets is associated with the interaction of EphA4 with intracellular kinases and the cell adhesion molecule L1, which may be important for the stabilization of platelet–platelet interactions, as the inhibition of ligation between platelets through the addition of excess monomeric recombinant Eph kinases or ephrins results in only reversible aggregation in response to ADP (Prevost *et al*., 2002). More recent insights into the mechanisms of action stem from the observation that EphA4 is physically associated with integrin $\alpha_{IIb}\beta_3$ on the platelet surface and, notably, at points of platelet–platelet interaction (Prevost *et al*., 2005). Outside-in signalling through the integrin drives clot retraction that is believed to be important for thrombus stability. Inhibition of EphA4 and EphB1 ligand interactions results in diminished clot retraction, which is associated with reduced tyrosine phosphorylation of β_3 integrin and binding to myosin. In whole blood, such treatment is reported to result in a moderate reduction in thrombus volume formed on perfusion at arterial shear rates over collagen (Prevost *et al*., 2005).

Collectively, these data implicate Eph kinases and ephrins in the regulation of the thrombus stability through sustained platelet–platelet signalling following thrombus formation. The physiological significance of this has yet to be established.

Semaphorins

Members of the membrane-bound and secreted family of ligands known as semaphorins have been recently implicated in the control of platelet function. These ligands, which are defined by the presence of a semaphorin domain at the C-terminus, bind to receptor complexes that comprise members of the neurophilin and plexin families and are recognized for their roles in axonal steering (Tamagnone and Comoglio, 2000; Goshima *et al*., 2002; Antipenko *et al*., 2003). The class 3 semaphorin (sema) 3A is a disulphide bonded homodimer that has been reported to be secreted by endothelial cells, in which it is implicated, through regulation of integrin function, adhesion and spreading, in the control of angiogenesis (Tamagnone and Comoglio, 2000; Serini *et al*., 2003). Platelets have been reported to express the sema3A receptors neurophilin-1 and plexin A. Incubation of platelets with sema3A results in reduced upregulation of integrin $\alpha_{IIb}\beta_3$ affinity and aggregation in response to a wide range of platelet agonists including thrombin, convulxin, ADP, U46619 and the calcium ionophore A23187, and inhibition of adhesion and spreading on fibrinogen (Kashiwagi *et al*., 2005). Sema3A signalling in platelets does not affect calcium mobilization in stimulated platelets or intracellular cyclic nucleotide levels, although filamentous actin formation and associated cytoskeletal signalling is reduced. The mechanism of sema3A inhibition of platelet function is therefore believed to be regulated by impairment of Rac-1-dependent actin rearrangement.

A more complex dual role has been identified for the class 4 semaphorin sema4D, which is expressed on platelets along with its receptors plexin-B1 and CD72 (Conrotto *et al*., 2005; Zhu *et al*., 2007). This protein in soluble form is recognized to stimulate angiogenic responses in endothelial cells and the inhibition of monocyte influx (Delaire *et al*., 2001; Basile *et al*., 2004; Conrotto *et al*., 2005). sema4D is an integral membrane protein of platelets, and its levels at the cell surface are increased during activation, whereupon it is cleaved from the cell surface by the metalloprotease ADAM17 (Zhu *et al*., 2007). Mouse platelets deficient in sema4D display defective aggregation in response to collagen (although responses to ADP and PAR_4 agonist are normal) and delayed arterial occlusion using *in vivo* models of thrombosis (Zhu *et al*., 2007). Therefore, it is possible that sema4D possesses dual roles in the context of tissue injury: (1) the promotion of platelet thrombus formation through binding to receptors on adjacent platelets, and (2) following cleavage, contribution to tissue repair through its effects on endothelial cells.

Gas6 and its receptors Sky, Axl and Mer

The growth arrest-specific 6 (*Gas6*) gene was originally identified in fibroblasts, and codes for a vitamin K-dependent secreted protein related to the anti-coagulant protein, protein S. Gas6 protein is expressed in a range of tissues such as endothelial cells, vascular smooth muscle and bone marrow, and is implicated in the control of a number of functions including cell adhesion, growth and survival. The effects of Gas6 are mediated through three receptor tyrosine kinases, Axl, Sky and Mer, which have been shown to be expressed on platelets (Gould *et al*., 2005). Gas6-deficient transgenic mice are protected against fatal collagen/adrenaline-induced thromboembolism, although haemostasis appears unaffected (Angelillo-Scherrer *et al*., 2001). Similarly, the deletion of any one of the Gas6 receptors or intravenous administration of soluble extracellular domain of Axl results in protection from experimental thromboembolism (Chen *et al*., 2004a; Angelillo-Scherrer *et al*., 2005). The incubation of platelets with anti-Sky or Mer antibodies has been shown to result in decreased platelet aggregation and secretion in response to ADP and PAR_1 agonists, where a stimulatory anti-Axl antibody resulted in potentiated responses (Gould *et al*., 2005). Furthermore, administration of Sky blocking antibodies *in vivo* resulted in reduced thrombus weight, but tail bleeding assays of haemostasis were unaffected (Gould *et al*., 2005). Transgenic mice lacking individual Gas6 receptors bind normal levels of fibrinogen, indicating that Gas6 signalling is not required for initial upregulation of integrin $\alpha_{IIb}\beta_3$ affinity, but may be necessary for subsequent outside-in signalling by this integrin that is necessary for thrombus stabilization (Angelillo-Scherrer *et al*., 2005). Consistent with this model are the observations that Gas6-deficient mice appear to have reduced β_3 tyrosine phosphorylation, delayed spreading and clot retraction, along with a tendency to re-bleed. This is similar to the phenotype in mice in which integrin $\alpha_{IIb}\beta_3$ cytoplasmic tail tyrosine residues were mutated to phenylalanine (Law *et al*., 1999; Angelillo-Scherrer *et al*., 2005).

The presence of Gas6 in platelets and its release upon activation has been reported (Ishimoto and Nakano, 2000; Angelillo-Scherrer *et al*., 2001), and this has led to the proposition that this molecule performs an autocrine function in platelets; however, the absence of Gas6 in platelets has also been reported (Balogh *et al*., 2005). Nonetheless, these data indicate an important role for this soluble protein in the regulation of the stabilization of platelet thrombi following activation, through modulation of outside-in integrin signalling.

Tachykinins

A potential role for tachykinins in the regulation of platelet function was first suggested in 1981, when it was demonstrated that a tachykinin family member, substance P (SP), could stimulate platelet shape change in rabbit platelets (Gudat *et al*., 1981). Tachykinins are a family of peptides characterized by the C-terminal motif F-X-G-L-M-NH_2, where X represents a hydrophobic or aromatic amino acid. The mammalian tachykinins include SP, neurokinin (NK) A encoded by the TAC1 gene, NKB encoded by the TAC3 gene and a number of predicted peptides encoded by the recently identified TAC4 gene (Page *et al*., 2003). SP, NKA and NKB mediate their responses through preferentially binding G protein-coupled NK receptors, NK_1, NK_2 and NK_3 respectively. Binding studies using a C-terminal amino-acid sequence peptide, common to the predicted products of the TAC4 gene, termed endokinin A/B or hemokinin-1, demonstrated high affinity for the NK_1 receptor (Zhang *et al*., 2000; Page *et al*., 2003).

A handful of studies have reported effects of SP on platelets *in vitro* (Damonneville *et al*., 1990; Savi *et al*., 1992) and *in vivo* (Ohlen *et al*., 1989). It is only recently, however, that the responses evoked in platelets by SP have been characterized (Graham *et al*., 2004). SP and endokinin A/B can mediate a variety of functional responses in platelets including shape change, secretion and aggregation (Graham *et al*., 2004). The study of platelets from NK_1-deficient mice confirmed these responses to be largely mediated through the NK_1 receptor (Graham *et al*., 2004; Jones *et al*., 2007). Unlike TAC1 and TAC3, the TAC4 gene is predominantly expressed in peripheral non-neuronal cells (Kurtz *et al*., 2002; Page *et al*., 2003), and it has therefore been suggested that the products of this gene may be the endogenous agonists of peripheral NK_1 receptors (Page *et al*., 2003) such as those found on platelets. mRNA transcripts from both TAC1 and TAC4 genes have been demonstrated in the megakaryocytic cell line HEL (Page *et al*., 2003), and TAC1 also in platelets. Furthermore, SP, endokinin A and endokinin B have each been detected in platelets and SP-like immunoreactivity is secreted upon platelet activation. These data suggest that SP and possibly TAC4 products may act as secondary platelet agonists in a similar manner to ADP. In support of this, inhibiting NK_1 signalling abrogates collagen-mediated platelet aggregation and thrombus formation *in vitro*, and mice injected with an NK_1 receptor antagonist are protected from collagen-induced thromboembolism (Graham *et al*., 2004; Jones *et al*., 2007).

CD36 and oxidized choline glycerophospholipids

CD36 has long been recognized to be present on platelets, and its ability to bind to collagens led, in the early 1990s, to the incorrect proposition that this was the activatory collagen receptor, later discovered to be GPVI (Tandon *et al*., 1989; Yamamoto *et al*., 1992; Saelman *et al*., 1994; Gibbins *et al*., 1997; Tsuji *et al*., 1997). CD36 is best recognized, however, as a scavenger receptor for oxidized low-density lipoprotein on macrophages. Platelet CD36 has been recently identified as a missing link in the association between dyslipidaemia and a prothrombotic phenotype (Podrez *et al*., 2007). Dyslipidaemia was shown to result in elevated plasma levels of structurally defined oxidized choline glycerophospholipids ($oxPC_{CD36}$) that serve as ligands for CD36. Although the intracellular signalling mechanisms that underlie this have yet to be established, $oxPC_{CD36}$ binding to CD36 results in platelet activation in mouse and human blood. Decreased thrombus formation time in hyperlipidaemic mice (ApoE deficient) fed on a western diet was reversed in mice also deficient in CD36. Importantly, $oxPC_{CD36}$ is not only revealed to platelets upon injury, but is

also present in plasma. It has therefore been proposed that CD36 serves to prime platelets for subsequent activation, thereby contributing to increased propensity to thrombosis in dyslipidaemic individuals. The same group has also reported that oxidized high-density lipoprotein (Podrez *et al*., 2007) inhibits platelet activation, and that this is dependent on another scavenger receptor expressed on platelets, SR-B1 (Valiyaveettil *et al*., 2008).

CD84 and signalling lymphocyte activation molecule

A proteomics screen has led to the identification of two homophilic adhesion receptors in platelets, CD84 and CD150/signalling lymphocyte activation molecule (SLAM) (Nanda *et al*., 2005a). In T cells, homophilic interaction of SLAM induces its tyrosine phosphorylation (Chan *et al*., 2003; Engel *et al*., 2003). Both CD84 and SLAM are tyrosine phosphorylated upon platelet aggregation (presumably as a result of homophilic interactions) (Nanda *et al*., 2005a) and thus signalling generated through these may serve to enhance platelet activation subsequent to platelet–platelet contact. Consistent with this, SLAM-deficient mice display delayed thrombus formation without affecting tail bleeding times (Nanda *et al*., 2005a), indicating a role in aggregate stability. Although SLAM signalling in platelets has yet to be characterized, this is believed not to be identical to SLAM signalling in lymphocytes (Nanda and Phillips, 2006).

Platelet endothelial aggregation receptor 1

Proteomic and microarray analysis of platelet proteins that become phosphorylated upon platelet aggregation have converged to identify platelet endothelial aggregation receptor 1 (PEAR1), an epidermal growth factor repeat-containing transmembrane receptor, on platelets and endothelial cells (Nanda *et al*., 2005b). PEAR1 becomes tyrosine and serine phosphorylated upon aggregation independently of platelet activation (Nanda *et al*., 2005b). It is postulated that platelet–platelet contact enables an unidentified surface ligand to bind PEAR1, inducing clustering and phosphorylation of its cytoplasmic tail (Nanda and Phillips, 2006). PEAR1 was found to associate with the adaptor protein ShcB upon thrombin receptor agonist peptide (TRAP)-induced platelet aggregation (Nanda *et al*., 2005b). Localization of ShcB to the plasma membrane may enable it to enhance activation of Ras signalling, and thus PEAR1 may contribute to platelet regulation, amplifying activation after initial agonist-induced aggregate formation (Nanda and Phillips, 2006).

Chemokines and their receptors

In addition to a role in haemostasis, platelets contribute to non-adaptive immunity and inflammation, by secreting chemokines that attract leukocytes to sites of injury and inflammation (Rottman, 1999; Gear and Camerini, 2003; Le *et al*., 2004; von Hundelshausen and Weber, 2007). Platelets store chemokines (CXC chemokine ligands (CXCLs) and chemokine ligands (CCLs)) in α-granules that are secreted upon activation, and express chemokine receptors (CXCR4, CCR1, CCR3 and CCR4) on their surface (Kameyoshi *et al*., 1992; Clemetson *et al*., 2000; Mause *et al*., 2005; von Hundelshausen and Weber, 2007). Platelet-derived chemokines include connective tissue-activating protein III, the connective tissue-activating protein III precursors or truncation products, platelet factor 4 (CXCL4), macrophage inflammatory peptide-1 (CCL3), monocyte chemotactic protein-3 (CCL7), growth-regulated oncogene-α (CXCL1), ENA-78 (CXCL5), interleukin-8 (CXCL8) and regulated upon activation and normal T-cell expressed and secreted (RANTES/CCL5) (Gear and Camerini, 2003; Mause *et al*., 2005; von Hundelshausen and Weber, 2007). Platelet chemokine receptors have been demonstrated to be functional due to the ability of their ligands to activate platelets. Chemokines, including RANTES, monocyte chemotactic protein-1, MIP-1α, macrophage-derived chemokine, thymus and activation-regulated chemokine, eotaxin and stromal derived factor-1, have been shown to induce intracellular calcium mobilization, serotonin secretion, aggregation under low and arterial shear conditions and platelet adhesion to collagen and fibrinogen under high shear (Clemetson *et al*., 2000; Abi-Younes *et al*., 2001; Gear *et al*., 2001). They also increase tyrosine phosphorylation of the FcR γ-chain, Syk and PLCγ2, and these effects are potentiated by ADP and diminished by TRAP (Clemetson *et al*., 2000; Kowalska *et al*., 2000; Gear *et al*., 2001). The signalling and functional responses provoked by some chemokines (stromal derived factor-1, macrophage-derived chemokine, thymus and activation-regulated chemokine) are dependent on the presence of ADP and decreased by ADP scavengers and receptor antagonists (Clemetson *et al*., 2000; Kowalska *et al*., 2000; Suttitanamongkol and Gear, 2001). Interestingly, some chemokine responses have been shown to be enhanced or inhibited by other chemokines. Stromal derived factor-1 potentiates aggregation caused by macrophage-derived chemokine and thymus and activation-regulated chemokine, and RANTES non-competitively inhibits platelet activation caused by stromal derived factor-1 (Abi-Younes *et al*., 2001; Shenkman *et al*., 2004). Complex regulatory mechanisms exist, therefore, linking platelet activation and inflammation. A complete understanding of associations between responses caused by platelet-derived cytokines and endogenous inflammatory mediators may enable the development of anti-platelet drugs that repair or prevent inflammatory damage.

CD40 and CD40 ligand

Platelets are one of the main producers of soluble CD40 ligand (sCD40L) and CD40 is expressed on their surface (Cipollone *et al*., 2002; Inwald *et al*., 2003). The CD40L–CD40 interaction is a well-characterized mediator of inflammatory and immune responses (Lederman *et al*., 1992; Grewal and Flavell, 1998; Laman *et al*., 1998; van Kooten and Banchereau, 2000). Platelets expose CD40L on their surface within seconds of activation with various agonists including thrombin, ADP, collagen and adrenaline and *in vivo* during thrombus formation (Henn *et al*., 1998; Andre *et al*., 2002). This is then shed from the platelet surface as sCD40L, which has been shown to regulate endothelial and immune cell function by upregulating adhesion receptors

(E-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule 1) on human umbilical vein endothelial cells and binding T cells following the release of RANTES triggered by thrombin stimulation (Henn *et al*., 1998; Danese *et al*., 2004). CD40L has been shown, via a KGD (amino-acid sequence) integrin-binding motif, to bind to integrin $\alpha_{IIb}\beta_3$, thereby stabilizing platelet thrombi (Andre *et al*., 2002). Furthermore, normal thrombotic activity in CD40L-deficient mice is not restored by recombinant sCD40L lacking the KGD integrin-recognition sequence, and $\alpha_{IIb}\beta_3$ antagonists inhibit sCD40L cleavage from activated platelets (Andre *et al*., 2002; May *et al*., 2002; Nannizzi-Alaimo *et al*., 2003; Furman *et al*., 2004).

sCD40L has been demonstrated at significantly higher levels in the plasma of patients with unstable angina compared to those with stable angina or healthy individuals, and also in patients with untreated autoimmune thrombocytopaenic purpura compared to treated patients (Nagahama *et al*., 2002; Aukrust *et al*., 2007). Furthermore, high sCD40L levels have been linked to the progression of atherosclerosis due to increased expression of chemokines including RANTES and monocyte chemotactic protein-1, which may allow CD40-positive cells, such as platelets and monocytes, to adhere to the endothelium and contribute to the weakening of plaques (Cipollone *et al*., 2002; Nagahama *et al*., 2002; Danese *et al*., 2004; Chakrabarti *et al*., 2007). The perturbation of sCD40L–CD40 interactions has been investigated with knockout mouse models ($CD40^{-/-}$/$ApoE^{-/-}$; LDL-$R^{-/-}$) and anti-CD40L antibodies to develop therapeutic strategies for atherosclerosis (Mach *et al*., 1998; Lutgens *et al*., 1999, 2000). These studies reported a reduction in atherosclerotic lesions and a stable plaque phenotype in $CD40^{-/-}$/$ApoE^{-/-}$ mice and LDL-$R^{-/-}$ or wild-type mice treated with anti-CD40L antibodies. Platelets are therefore mediators of CD40L–CD40 signalling, at the interface between inflammatory responses, atherosclerosis, haemostasis and thrombosis, highlighting another potentially beneficial therapeutic route.

Inhibitory receptors

The involvement of immunoreceptor-like signalling in the activation of platelets (for example, GPVI) has been recognized for some time. Increasing evidence suggests a role for inhibitory immunoreceptor-like signalling by receptors that possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Three ITIM-containing receptors have been identified in platelets: platelet endothelial cell adhesion molecule (PECAM)-1, G6b and TREM-like transcript-1 (TLT-1) (Newman *et al*., 1990; Barrow *et al*., 2004; Newland *et al*., 2007).

Homophilic ligand interactions are believed to underlie the functions of PECAM-1 (Albelda *et al*., 1990; Buckley *et al*., 1996). It has been reported, however, to have a number of heterophilic binding partners including CD38, CD177, $\alpha_v\beta_3$ and glycosaminoglycans, the roles of which are unknown (DeLisser *et al*., 1993; Piali *et al*., 1995; Deaglio *et al*., 1998; Sachs *et al*., 2007). PECAM-1 signalling induced by antibody crosslinking or soluble recombinant PECAM-1 results in inhibition of platelet function (Cicmil *et al*., 2000, 2002; Jones *et al*., 2001), although the signalling mechanisms through which PECAM-1 mediates this effect remain to be established. PECAM-1-deficient mouse platelets display enhanced aggregation, dense-granule secretion and adhesion to collagen (Patil *et al*., 2001), indicating a negative regulatory role for this receptor in platelets. In an *in vivo* model of thrombosis, thrombus formation has also been shown to be enhanced in PECAM-1-deficient mice (Falati *et al*., 2006). As an adhesion receptor, cell–cell contact is necessary for function and therefore its role is likely to be distinct from potent soluble inhibitory molecules such as NO and PGI_2 that are released from the endothelium. The more subtle effect of this ITIM receptor, and others described below, suggests a role in the fine-tuning of platelet reactivity and potentially within a developing thrombus.

G6b is an ITIM-containing receptor recently reported to be present in platelets (Macaulay *et al*., 2007; Newland *et al*., 2007; Senis *et al*., 2007). G6b splice variants (G6b-A and G6b-B) share extracellular domains that bind heparin, contain a single Ig-like domain and have a single transmembrane domain, but differ in the cytoplasmic tail (de Vet *et al*., 2001, 2005). Like PECAM-1, G6b-B contains two intracellular ITIM motifs that associate with Src homology phosphatase (SHP)-1 when phosphorylated upon platelet stimulation with either collagen-related peptide or thrombin (Senis *et al*., 2007) and SHP-2 in K562 or COS-7 cells (de Vet *et al*., 2001). Crosslinking with antisera raised against G6b inhibits collagen-related peptide- or ADP-induced platelet aggregation in a calcium-independent manner (Newland *et al*., 2007) suggesting a different signalling mechanism to PECAM-1. A G6b-deficient mouse has yet to be developed, and in the absence of a known ligand the physiological role of this receptor is unknown.

The ITIM-containing receptor TLT-1 is found exclusively on platelets and megakaryocytes (Washington *et al*., 2002, 2004). TLT-1 is in platelet α-granules and its exposure on the platelet surface is upregulated by stimulation with TRAP/thrombin (Barrow *et al*., 2004; Washington *et al*., 2004; Giomarelli *et al*., 2007). TLT-1 also exists as a soluble fragment lacking the cytoplasmic tail (20–25 kDa) (Barrow *et al*., 2004) that is thought to be secreted from platelets upon activation (Gattis *et al*., 2006). Although the ligand and function of TLT-1 are unknown, upon phosphorylation it has been shown to bind SHP-1 when expressed in HEK293T cells (Washington *et al*., 2002) and bind SHP-2 in platelets (Barrow *et al*., 2004). The ability to interact with SHP-1 and SHP-2 suggests an inhibitory role in platelet regulation, although TLT-1 has been shown to have a co-stimulatory role in immunoglobulin receptor-induced calcium release when expressed in RBL (rat basophilic leukaemia) cells (Barrow *et al*., 2004). Conversely, anti-TLT-1 antibody fragments were able to inhibit thrombin- and U46619- but not collagen-, ADP- or phorbol ester-induced aggregation (Giomarelli *et al*., 2007); however, the underlying mechanisms have not yet been characterized.

Recent characterization of transgenic mice lacking the cell adhesion molecules endothelial cell-selective adhesion molecule (ESAM) and junctional adhesion molecule-A (JAM-A) revealed enhanced platelet thrombus formation which is indicative of inhibitory roles for these proteins (Naik *et al*., 2007; Stalker *et al*., 2007). Although these are not ITIM-

containing receptors, these reports suggest multiple potential routes to inhibit thrombus formation through cell adhesion receptors.

Extracellular thiol isomerases

Platelet activation leads to a conformational change in integrin $\alpha_{IIb}\beta_3$ from a low-affinity state to a high-affinity, fibrinogen binding state (see Figure 1) (Shattil *et al.*, 1998). Studies using mutant $\alpha_{IIb}\beta_3$ (Ruiz *et al.*, 2001) and reducing agents (Yan and Smith, 2001) have shown that this switching involves remodelling of disulphide bonds within the extracellular portion of the receptor (Yan and Smith, 2000). Thiol isomerase enzymes on the platelet surface are believed to be key to this process (Ferrari and Soling, 1999; Sevier and Kaiser, 2002; Essex, 2004; Essex and Li, 2006; Jordan and Gibbins, 2006). Furthermore, the integrin $\alpha_{IIb}\beta_3$ has been shown to possess intrinsic thiol isomerase activity in the β_3 subunit, which may contribute to the modulation of receptor affinity (O'Neill *et al.*, 2000; Walsh *et al.*, 2004). Thiol isomerases are ubiquitously expressed with restricted localization to the endoplasmic reticulum where they are required for correct folding of nascent proteins. Two thiol isomerases, protein disulphide isomerase (PDI) (Chen *et al.*, 1992) and endoplasmic reticulum protein 5 (ERp5) (Jordan *et al.*, 2005), have been characterized in platelets where localization is not restricted to endoplasmic reticulum. Low levels of these enzymes are present on the resting cell surface and their exposure is increased dramatically upon activation (Chen *et al.*, 1995; Essex *et al.*, 1995; Jordan *et al.*, 2005). Platelets secrete PDI and ERp5 and the enzymes re-associate with the cell surface where they are believed to play a role in receptor remodelling by thiol–disulphide exchange. Although the exact mechanism of release and binding kinetics of these proteins are poorly understood, platelet responses to physiological agonists are inhibited by anti-PDI and anti-ERp5 antibodies, indicating a role for these proteins in normal platelet function (Essex and Li, 1999; Lahav *et al.*, 2000, 2002; Jordan *et al.*, 2005).

Tissue factor, an important coagulatory protein, is expressed in a cryptic form, which is inactive. Cleavage of an allosteric disulphide bond between Cys186 and Cys209 transforms tissue factor to a pro-coagulant state, enabling it to participate in the enzymic-based coagulation cascade (Chen and Hogg, 2006). Ahamed *et al.* (2006) suggest that the regulation of tissue factor de-encryption is mediated by PDI, with PDI suppressing coagulant activity. Other studies, however, suggest that PDI chaperone activity alone (and not thiol isomerase activity) is required for the effects of PDI on coagulation. Either way, these studies suggest novel roles for platelet-released thiol isomerases in the regulation of haemostasis.

Intracellular targets

As described, the absence or presence of function of cell signalling molecules of importance for platelet regulation results frequently in bleeding, and wide expression profiles raise issues of cell specificity. A new group of intracellular targets within the platelet have been recently described, however, and surprisingly these are members of the intracellular receptor families, which have been characterized extensively in other cell systems in the context of gene regulation.

Intracellular hormone receptors

Although platelets are anucleate, recent reports demonstrate that they express intracellular receptors such as the glucocorticoid receptor (Moraes *et al.*, 2005), retinoic X receptor (Moraes *et al.*, 2007) and the peroxisome proliferator-activated receptor (PPAR) isoforms PPARγ (Akbiyik *et al.*, 2004) and PPARβ/δ (Ali *et al.*, 2006). Furthermore, platelet intracellular receptors have been shown to possess the ability, in a non-genomic fashion, to regulate platelet function. Retinoic X receptor ligands inhibit platelet activation stimulated by ADP or the TXA_2 mimetic U46619. This is believed to be mediated through suppression of Gq signalling, resulting in inhibition of intracellular calcium mobilization (Moraes *et al.*, 2007).

PPARs are ligand-activated transcription factors that act as heterodimers with retinoic X receptors, to recognize PPAR response elements, located in the promoter of target genes. Alternative signalling mechanisms of action include interaction with nuclear factor-κB and consequent inhibition of nuclear factor-κB-mediated gene regulation (Berger *et al.*, 2005). PPARγ can be activated by a number of ligands, including docosahexaenoic acid, linoleic acid, the anti-diabetic glitazones (for example, rosiglitazone, used as insulin sensitizers), a number of lipids and eicosanoids, such as 5,8,11,14-eicosatetraynoic acid and the prostanoids PGA_1, PGA_2, PGD_2 and 15d-PGJ_2. PPARγ ligands have been reported to inhibit platelet aggregation in response to ADP. Furthermore, human studies have shown the glitazones to reduce significantly markers for platelet reactivity such as P-selectin expression and sCD40L release (Marx *et al.*, 2003; Berger *et al.*, 2005; Irons *et al.*, 2006). Although no mechanism has been proposed for this, the ability of PPARγ to interact with retinoic X receptor may suggest common modes of action. It is pertinent to note that the treatment of diabetes with PPARγ agonists is associated with a reduced risk of cardiovascular complications (Irons *et al.*, 2006), although no formal link between this and the inhibition of platelet function has been reported. Recent clinical trials, however, have indicated that administration of PPARγ agonists is associated with an increased incidence of congestive heart failure and MI (Lago *et al.*, 2007; Lipscombe *et al.*, 2007). Agonists for PPARβ/δ, which is a putative intracellular receptor for PGI_2, have also been reported to inhibit platelet function (Ali *et al.*, 2006). These studies raise the curious potential that drugs targeted to these intracellular receptors may enable the dual treatment of metabolic diseases and CVDs, including the prevention of thrombosis.

Concluding remarks

With a strong clinical need to improve the efficacy and safety of current anti-platelet therapies in various clinical scenarios, a small number of characterized platelet receptors, which include proven anti-platelet drug targets, are currently the

focus of drug discovery and clinical trials for the prevention and treatment of thrombosis. These include ADP receptor ($P2Y_{12}$) antagonists and molecules that inhibit activation of PARs for thrombin. Owing to the array of regulatory pathways employed by platelets to enable activation and inhibition of function, it is likely that the current preference for combination therapies, frequently with aspirin, is likely to continue. A more detailed appreciation of the platelet regulatory systems is, however, likely to result in the development of more refined, safer and more efficacious approaches to prevent thrombosis. In recent years, considerable progress has been made by platelet cell biologists that in the future will impact on drug discovery. These include the identification and characterization of new platelet agonists, cell adhesion receptors, GPCRs, intracellular receptors and the complex signalling pathways that these control. The application of technologies such as proteomics to the study of platelet biology is beginning to yield the identities of new platelet proteins and fill gaps in existing signalling pathways. Indeed this has also resulted in the identification of functionally active stimulatory and inhibitory platelet orphan receptors. Although the translation of effects observed in animals to the prevention of human disease cannot be guaranteed, current advances in the understanding of platelet biology provide a number of intriguing directions for the future.

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Conflict of interest

The authors state no conflict of interest.

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