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Off-target drug effects on platelet function: Protecting an Achilles heel of drug development

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Abstract

The development of new drugs is often limited or even halted by their side effects on platelet number or function. This review introduces the signalling pathways and the role of various platelet receptors, such as GPIIb/IIIa, GPIb-IX-V, GPVI and P-selectin. The large scope of platelet function tests are described, including aggregometry, flow cytometry, VerifyNow, adhesion and *in vivo* thrombosis and haemostasis assays. Several important examples of drugs that have off-target effects influencing platelet function are discussed, including GPIIb/IIIa inhibitors, oligonucleotides, BH3 mimetics and Bruton tyrosine kinase inhibitors. Finally, challenges for future drug development with regards to platelet function are outlined, including the conclusion that no single assay can fully predict drug effects and thus a combination of platelet function tests is often required to assess platelet function in the context of newly developed therapeutics.

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Platelets are the second most abundant cell type in circulation, with nearly one trillion circulating at any time. They play a central role in mediating haemostasis and thrombosis [1]. This is underscored by the fact that a reduction in platelet numbers or impaired platelet function can result in significant bleeding complications, including fatal cerebral bleeding. Indeed, owing to the important role of platelets in mediating arterial thrombosis, pharmacological inhibitors of platelet function are one of the most commonly used therapeutics in the world. Platelets are small anucleate cells (2–5 μm in diameter) and have a short life span of approximately 7–10 days, and when these are aged, senescent platelets become cleared predominantly in the liver and spleen. The production of platelets (thrombopoiesis) occurs in the bone marrow, where their large nucleated progenitor cells, megakaryocytes, release platelets in a process largely controlled by the liver-derived growth factor thrombopoietin [2].

The role of platelets in thrombosis and haemostasis

To carry out their haemostatic function, platelets must have the ability to adhere and aggregate at sites of vascular injury. Accordingly, platelets express a large number of adhesion receptors on the cell surface to regulate platelet–extracellular matrix, platelet–platelet, and platelet–leucocyte interactions. Furthermore, adhesion receptors allow the transmission of extracellular signals by activating intracellular signalling pathways, which in turn can modulate platelet adhesion and activation responses. Platelet adhesion receptors can be grouped into different families, most prominent being the integrins (including GPIIb/IIIa [$\alpha_{IIb}\beta_3$]), the leucine-rich repeat family (including GPIb-IX-V), the immunoglobulin superfamily (including GPVI) and the C-type lectin receptor family (including P-selectin). Importantly, upon platelet activation, platelet GPIIb/IIIa undergoes a conformational change to adopt a high-affinity conformation towards its major ligand, fibrinogen [3]. P-selectin, which is stored in platelet α-granules, translocates to the plasma membrane [4]. The change in GPIIb/IIIa conformation and P-selectin expression can be measured with specific antibodies and serve as common markers of platelet activation. The importance of the platelet adhesion receptors in mediating haemostasis is underscored by the bleeding diathesis that results from congenital deficiency of integrin $\alpha_{IIb}\beta_3$ (Glanzmann thrombasthenia) and GPIb (Bernard–Soulier syndrome).

Platelet activation pathways

The molecular events leading to platelet activation have been extensively reviewed previously [5–7], and

therefore, we will only précis the key elements here. The inciting event leading to platelet thrombus formation is endothelial injury or the rupture of an atherosclerotic plaque. Both lead to the exposure of a number of subendothelial matrix proteins including collagen, von Willebrand Factor (vWF), laminin and fibronectin. Each of these matrix proteins engages with their cognate platelet receptor to facilitate platelet adhesion and activation. These interactions of GPIb-vWF integrin $\alpha_{IIb}\beta_3$ with fibrinogen and GPVI with collagen triggers intracellular signalling pathways that ultimately result in an increase of intracellular calcium flux and activation of integrin $\alpha_{IIb}\beta_3$ such that it adopts a high-affinity conformation for its major adhesive ligand, fibrinogen [8,9]. Fibrinogen serves as a linker that cross-links adjacent activated platelets and therefore allows platelets to aggregate. The process of platelet aggregation can be measured *ex vivo* by light transmission aggregometry. Moreover, these platelet—receptor interactions lead to the generation of soluble agonists and platelet degranulation, resulting in the synthesis and release of thromboxane A2 and adenosine diphosphate (ADP), respectively. The soluble liberated agonists act via their respective G-protein—coupled receptor in an autocrine and paracrine fashion to amplify platelet activation and stabilize the nascent platelet thrombus, while granule-derived vWF and fibrinogen act as an adhesive surface to mediate further platelet recruitment [5—7]. An important G-protein—coupled receptor is the P2Y12 receptor, which serves as one of the receptors for ADP. ADP activation of the P2Y12 receptor triggers downstream signalling events, which ultimately leads to sustained integrin activation [10]. Indeed, therapeutic inhibition of the P2Y12 receptor remains a common approach to inhibit platelet function, and the effect of these drugs can be measured using point-of-care testing assays [11]. As can be appreciated, any dysregulation of megakaryopoiesis leading to thrombocytopaenia or effect on platelet function may have significant effects on haemostasis. The following section will discuss some recent important examples of new drugs developed that have displayed important 'off-target' effects on platelet function.

Examples of drugs that revealed side effects on platelet function

GPIIb/IIIa inhibitors

Inhibition of the platelet GPIIb/IIIa (αIIbβ3, CD41/CD61) allows blockade of platelet aggregation and therefore is an effective antithrombotic strategy [12]. The initial enthusiasm for this strategy, especially oral approaches of GPIIb/IIIa inhibition, came to an abrupt halt when it became clear that this strategy was associated with increased mortality compared with placebo [13]. Although being effective inhibitors of ligand binding, all developed and clinically tested GPIIb/IIIa inhibitors also mimic ligand function. As a prototypical integrin, GPIIb/IIIa is also a sensor of ligand engagement, and thus, ligand binding causes outside-in signalling. Therefore, in the case of ligand-mimetic inhibitors, this can result in paradoxical platelet activation resulting in platelet degranulation as described previously [14]. Furthermore, after dissociation of the GPIIb/IIIa inhibitor, the receptor in its activated state can bind the natural ligand fibrinogen [15]. Overall, ligand-mimetic GPIIb/IIIa inhibitors have the potential to paradoxically activate platelets and to cause thrombocytopaenia, the opposite of what they are intended for [3,14,16,17]. Given intravenously for a short period in acute coronary syndromes, the benefits of acute inhibition of platelet aggregation seem to supersede these side effects. However, this was not the case for long-term oral therapy. The latter has caused one of the biggest development failures in the pharmaceutical industry and is a strong example that systematic testing of these drugs in the aforementioned assays might have revealed the problems inherent to the strategy of ligand-mimetic integrin inhibition [3,18].

Biotherapeutics

The use of biotherapeutics, particularly monoclonal antibodies (mAbs), has emerged as a significant advance in clinical medicine with biotherapeutics now widely used in the treatment of cancer and cardiovascular and autoimmune diseases [19]. In fact, mAbs are currently attracting the major share of research and development investment in the pharmaceutical industry. Importantly, platelets have the largest circulating pool of the activating IgG receptor, FcγRIIa, which is expressed on their surface [20]. Therefore, thrombocytopaenia is a well-described side effect of a number of biotherapeutic agents, especially of Fc-containing antibody formats as a consequence of their ability to bind and cross-link the FcγRIIa receptor, or the presence of antidrug antibodies [21]. The potential for mAbs to cause unintended thrombocytopaenia and significant adverse events is exemplified by the mAbs developed to target CD40 ligand (CD40L) for the treatment of inflammatory diseases such as systemic lupus erythematosus and transplant rejection [22,23]. Indeed, the development of CD40L-targeted therapeutics was halted after this approach resulted in thrombocytopaenia and thrombotic events in humans and nonhuman primates [24,25]. Subsequently, it was demonstrated that CD40L is also expressed on activated platelets where anti-CD40L mAbs can inhibit the disaggregation of ADP-activated platelets and can bind to CD40L on the surface of activated platelets to form higher order immune complexes. This mechanism is similar to that observed in heparin-induced thrombocytopaenia [26,27] where anti-CD40L mAbs binds to CD40L expressed on the surface-activated platelets, resulting in the formation of higher order immune complexes that cause potent platelet activation via the cross-linking and activation of platelet FcγRIIa receptors. These represent the likely

mechanisms of the thrombocytopaenia and thrombotic events observed with the use of anti-CD40L mAbs. Because the FcγRIIa receptor is not expressed on rodent platelets, none of these adverse effects of CD40L mAbs were observed in rodent models, exemplifying the need to include different species, including human platelets, for toxicology testing. Alternatively, an FcγRIIa transgenic mouse model can be used for toxicology studies to address this question. In the case of anti-CD40L antibodies, this mouse model in fact recapitulated the thromboembolic side effects seen in humans [27].

Thrombocytopaenia, as a consequence of antidrug antibodies, is a well-described complication of Abciximab, a Fab fragment of a chimeric mAb that binds and inhibits GPIIb/IIIa. Thrombocytopaenia occurs in up to 5% of patients and typically occurs after the first dose [21,28]. As such, the cause of thrombocytopaenia secondary to Abciximab is thought to be due to pre-existing or newly formed antibodies that recognize and bind the murine sequence of the Fab, leading to the immune clearance of platelets [29]. In addition, as discussed previously (see section GPIIb/IIIa inhibitors), Abciximab can induce paradoxical platelet activation by inducing outside-in signalling. The binding of Abciximab to the GPIIa/IIIa receptor can also lead to the exposure of neoantigens on GPIIb/IIIa, which then can lead to antibody-mediated immune thrombocytopaenia [30].

Oligonucleotides

Nucleotide-based therapeutics such as antisense oligonucleotides, aptamers, immunoreceptor-activating nucleotides or anti-microRNAs are an emerging class of drugs that hold promise for the treatment of a range of diseases. Indeed, this class of drugs had Food and Drug Administration approval for the treatment of Duchenne muscular dystrophy [31], spinal muscular atrophy [32], cytomegalovirus [33] and familial hypercholesterolaemia [34]. One of the common structural modifications of nucleotide-based drugs is the introduction of a phosphorothioate backbone to protect the drug from the rapid degradation by plasma nucleases [35]. Interestingly, we recently reported that this common structural modification is associated with significant effects on platelet function [36]. Indeed, the modified phosphorothioate backbone induces potent platelet activation via binding to the platelet GPVI and potentially the Clec-2 receptor [36–39].

BH3 mimetics

The targeting of the Bcl-2 family of prosurvival proteins has emerged as a promising new approach for the treatment of haematological malignancies such as chronic lymphocytic leukaemia (CLL) [40,41]. However, the development of early generations of Bcl-2 inhibitors, such as navitoclax, was halted after it emerged that thrombocytopaenia was a major dose-limiting toxicity [42]. In contrast to traditional chemotherapeutic agents, the thrombocytopaenia was not due to bone marrow suppression but a result of shortening platelet life span by inducing platelet apoptosis as a consequence of Bcl-xl inhibition [43]. This has spurred the development of a newer, more specific inhibitor Bcl-2 inhibitor (venetoclax) that does not bind Bcl-xl and has gained Food and Drug Administration approval for the treatment of CLL.

Bruton tyrosine kinase inhibitors

The first in class Bruton tyrosine kinase inhibitor, ibrutinib, has proven effective for the treatment of CLL [44]. However, its use has been associated with an increased risk of major bleeding [45]. It is now apparent that ibrutinib also inhibits Tec and other Src family kinases important for platelet functional responses downstream of GPIb, GPVI and GPIIb/IIIa [45,46].

Platelet function tests

A broad scope of *in vitro* and *in vivo* platelet function assays, testing various of the previously described signalling pathways, is available with the ones most relevant for assessing potential drug side effects listed in Table 1. As a caveat, it is important to stress that there is not one test that can fully examine platelet function in its entirety, and thus, typically new drugs need to be evaluated with a combination of tests to fully characterize and examine for any unintended effects of a new drug on platelet function.

Platelet counts

Clearly, the most convenient and widely available marker to measure is the platelet count to assess for thrombocytopaenia as a result of any bone marrow suppression or effect on platelet life span. However, it is important to note that multiple mechanisms may lead to thrombocytopaenia. That is, thrombocytopaenia can be due to direct myelosuppression, immune-mediated effects where a drug may be associated with drug dependent antibodies that lead to premature sequestration or platelet destruction in the reticuloendothelial system. In addition, as discussed, drugs may interfere with platelet cell death pathways leading to a shortened life span and thus induce thrombocytopaenia. However, while a normal platelet count of 150–450 $\times$ 10^9/L is well defined, there is a lack of data regarding what platelet counts confer an increased risk of bleeding. In this context, it appears that aside from the absolute platelet count, other clinical factors such as age, comorbid conditions and the underlying disease process are important factors influencing the risk of haemorrhage [47,48]. While studies in mice suggest that bleeding times are largely unaffected with reductions in platelet counts of up to 97.5% [49], how this translates into humans remains uncertain. However, it is important to note that there are significant species differences in

Table 1 Summary of available laboratory tests to examine platelet function.

Test	Benefits	Limitations
Flow cytometry assessment of platelet activation		
P-selectin surface expression Activated GPIIB/IIIa expression	• Sensitive marker of platelet activation • Not restricted to a specific pathway of platelet activation — multiple agonists can be used to test multiple pathways	• Lack of standardized protocols for clinical practice • Time- and labour-intensive
VASP phosphorylation	• Standardized assay • Defined thresholds associated with ischaemic and bleeding events on treatment with P2Y12 inhibitor	• Expensive assay • Time- and labour-intensive • Specific to the P2Y12 receptor activity — does not evaluate other pathways
Measuring platelet aggregation to determine platelet function		
Light transmittance aggregometry	• Historical gold standard • Well validated with clinical outcomes • Not restricted to a specific pathway of platelet activation	• Requires sample preparation with risk of platelet activation, large sample volume and varying protocols with varying reproducibility
Impedence aggregometry	• Whole-blood assay reduces intrinsic activation and preparation time • Validated against light transmittance aggregometry with improved reproducibility	• Large sample volume, expensive
VerifyNow	• Rapid point-of-care test • Standardized • No sample preparation	• Expensive assay • Specific to ADP-related platelet activation using P2Y12 cartridge or thromboxane A2 activity using Aspirin cartridge — does not test other pathways
Shear-related clot formation to determine platelet function		
Shear-based plaatelet adhesion assays	• Provides information on platelet adhesion/thrombus formation in whole blood under physiological shear rates • Can test different platelet receptor–matrix protein interactions • Can identify defects not identified with non–shear-based assays	• Can require large blood volumes • Time- and labour-intensive

platelet counts, which is important when interpreting the potential effects of therapeutics on the platelet count. For example, mice typically have significantly higher platelet counts than humans in contrast to nonhuman primates, which generally display platelet counts similar to those of humans.

Flow cytometry

Flow cytometry can be performed to examine platelet receptor expression and, importantly, to investigate GPIIb/IIIa activation and P-selectin expression as markers of platelet activation [50]. Therefore, flow cytometry can resolve whether a novel drug induces platelet activation and can also discern whether platelet activation is inhibited or amplified, and the potential signalling pathways involved, in the presence of physiological agonists. In addition, measuring phosphatidylserine exposure on platelets (via Annexin V staining) can be used as a marker of platelet 'cell death' and can therefore provide important clues as to how a particular drug may induce thrombocytopaenia. A particular advantage of flow cytometry is the ability to examine multiple markers with small volumes of whole blood [51,52]. However, a potential limitation of flow cytometry for toxicology studies is the fact that antibodies that recognize GPIIb/IIIa are often species-specific, and thus, the repertoire of activation-specific platelet antibodies may not be available for species commonly used for toxicology studies such as those performed in dogs and monkeys.

Platelet aggregometry

Platelet aggregation involves the addition of soluble agonists to platelet-rich plasma. This induces platelets to aggregate together which results in changes in the optical transmission of light, which is measured as a readout of platelet aggregation [53]. Light transmission aggregometry (LTA) developed by Born in the 1960s is still used as a standard test for platelet function and is the historical gold standard [54,55]. The development of this test was a major milestone in the understanding of platelet function. Its elegance is based on the broad coverage of platelet function using platelet aggregation as the final and common endpoint of various platelet activation pathways. Similar to flow cytometry analysis of platelet activation, platelet aggregation assays allow

the assessment of a range of soluble agonists. Thus, if a defect in platelet aggregation is found as a consequence of a novel therapeutic, this can infer the signalling pathway that may be affected. However, there are no normal values for platelet aggregation studies, and there remains no standardization regarding the panel (and concentrations) of agonists used in platelet aggregation studies [56]. Typically, ADP, thrombin, collagen and arachidonic acid are used to activate platelets because these platelet agonists have cognate receptors expressed on the platelet surface that trigger specific downstream signalling cascades. Therefore, any inhibition observed with any of these agonists can provide insights into the signalling receptor and/or pathway inhibited by a particular therapeutic. An important consideration, however, is the difference in platelet receptor expression between human and rodent platelets. For example, human platelets express the FcγRIIa receptor and the protease-activated receptor-1 (PAR-1) receptor, neither of which is expressed on mouse platelets. As discussed, the lack of FcγRIIa on rodent platelets is particularly important when assessing for any off-target effects of biotherapeutics, especially mAbs. Further limitations include reduced sensitivity and specificity of LTA to detect mild platelet function defects, limited reproducibility and spontaneous platelet activation during sample preparation [57–61]. The efficacy of clopidogrel's antiplatelet effects has been measured extensively by aggregometry (using ADP as an agonist) and poor inhibition correlated to increased adverse cardiovascular outcomes [62–66]. However, one major advantage of platelet aggregometry for toxicology studies is that humans and nonhuman primates have similar platelet counts, repertoire of platelet receptor expression, display similar responses to platelet agonists such as ADP, thrombin and collagen and, unlike flow cytometry, does not rely on the availability of species-specific antibodies [67].

Impendence aggregometry, a newer technique, has been shown to correlate well with LTA [68,69] while having improved reproducibility and sensitivity [70]. This method assesses platelet reactivity by measuring increase in electrical resistance, which occurs with increasing aggregation of platelets (stimulated by an agonist) on devices' platinum electrodes. The Multiplate analyzer (Dynabyte, Munich, Germany) is a standardized, point-of-care assay utilizing this methodology. This assay also uses whole blood, reducing preparation time and the risk of intrinsic activation through centrifugation. It is a standardized assay which has been used extensively in clinical trials to evaluate the efficacy of antiplatelet agents [68,71–73].

VerifyNow assay

The VerifyNow assay (Accumetrics, US) is a whole-blood, point-of-care test developed to monitor responses to antiplatelet agents. This assay uses fibrinogen-coated beads that agglutinate in the presence of activated platelets in response to soluble agonist stimulation. Simplicity of technique, reproducibility of results and correlation with established tests of platelet function are advantages of this test [74,75]. However, the one-use cartridges are expensive, and this assay does not provide any further information than traditional platelet aggregation and, similarly, does not have any reference ranges. In addition, using the cartridges (aspirin or PRU) available, the assay is relatively specific to platelet activation via ADP and Thromboxane A2 pathways.

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay

This is a flow cytometric assay that measures the degree of VASP phosphorylation as a marker of the degree of P2Y12 receptor inhibition. Therefore, this assay only has utility to monitor the effect of P2Y12 inhibitors because other platelet inhibitors do not influence the degree of VASP phosphorylation [76].

Adhesion assays

The adhesive function of platelets can be examined using static adhesion assays or flow-based adhesion assays. Static adhesion assays involve the real-time monitoring of washed platelets adhere and activate (spread) on immobilized matrix proteins such as fibrinogen or vWF [77]. In contrast, flow-based adhesion assays use whole blood perfused through microcapillaries or microfluidic devices with immobilized matrix proteins such as fibrinogen, vWF or collagen at physiological, or pathophysiological, shear rates [78–80]. These assays can be used to examine for specific defects in platelet signalling (such as integrin outside-in signalling) or defects in platelet adhesive function under shear, which may not be detected using assays such as flow cytometry or platelet aggregation.

In vivo assays of thrombosis and haemostasis

A common approach to assess a novel drug or drug candidate's effect on platelet function is the use of *in vivo* mouse models of thrombosis and haemostasis. *In vivo* models of thrombosis are based on the principle that exposure of a blood vessel to chemical injury (ferric chloride) or photoactivation (laser injury) can induce endothelial injury and subsequent thrombus formation [81–83]. Thrombus formation is then monitored either using an ultrasound probe (to monitor blood flow) or by intravital microscopy (to visualize thrombus growth and size over time). Similarly, haemostasis is often assessed in murine models using tail bleeding time. This involves the transection of the anaesthetized mouse tail (3 mm from the tip) with the time to bleeding cessation, volume of blood loss or the amount of haemoglobin measured [84]. An alternative method is the template bleeding time that involves an incision, as opposed to transection, of the tail vein with

the same outcomes measured. Although commonly used, these models suffer from poor reproducibility and ability to predict bleeding [84]. This has been exemplified by a number of therapeutics, which have not shown any effect on tail bleeding time, yet have been associated with bleeding in clinical trials. A jugular vein puncture assay and saphenous vein injury model have recently been developed as other tests of haemostasis [85,86]. These models rely on the direct puncture or laser injury to rupture the endothelium of a large vessel, with the outcome being the time to bleeding cessation. Whether these assays will prove to be better models of haemostasis than the tail vein injury models remains to be tested. Another important consideration when interpreting rodent models of thrombosis is that rodents have important differences in their vascular anatomy compared with humans. In contrast, nonhuman primates are relatively homologous to humans with regards to their haematological profile and vascular anatomy in addition to being phylogenetically similar, thus providing a more translatable and reliable immunological and pharmacokinetic analysis of new therapeutics [87]. However, the use of photochemical or mechanical disruption of a vessel to incite thrombus formation differs significantly to the pathophysiology of thrombosis in humans (typically atherosclerotic plaque rupture), which remains a limitation of *in vivo* thrombosis studies irrespective of the species. Commonly used models of haemostasis used in nonhuman primates include the liver, renal and mesenteric bleeding times [88]. These models appear to be better predictors of haemostasis than those used in mouse models; however, they are costly and require specific expertise because they involve a midline abdominal incision to an anaesthetized nonhuman primate. Bleeding time after an incision to the liver or kidney or puncture to a mesenteric artery will be measured. Therefore, the *in vivo* assessment of haemostasis for new drug development is of central importance but remains unresolved, given no assay has been demonstrated to predict bleeding in patients. This remains a challenge because, even in patients, the skin bleeding time lacks sensitivity or the ability to predict procedural bleeding or reliably diagnose patients with platelet function disorders [89,90].

Summary and perspectives

The processes regulating platelet production, platelet life span and platelet activation are complex and are governed by distinct signalling pathways. Moreover, the *in vivo* haemostatic state requires the rapid and coordinated response from platelets, the coagulation system and the endothelium. These complexities make assessing new drugs for undesired off-target effects on platelets a challenge because no individual assay can adequately assess all the platelet functional responses. Nevertheless, the combined application of targeted platelet assays, as discussed, can be used to detect changes in platelet number or function, particularly for those drugs whose therapeutic target may overlap with key platelet signalling pathways.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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* of special interest
** of outstanding interest

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