GPIIb/IIIa inhibitors: From bench to bedside and back to bench again

Paul C. Armstrong; Karlheinz Peter
Atherothrombosis & Vascular Biology, Baker IDI Heart & Diabetes Institute, Melbourne, Australia

Summary
From the discovery of the platelet glycoprotein (GP) IIb/IIIa and identification of its central role in haemostasis, the integrin GPIIb/IIIa (αIIbβ3, CD41/CD61) was destined to be an anti-thrombotic target. The subsequent successful development of intravenous ligand-mimetic inhibitors occurred during a time of limited understanding of integrin physiology. Although efficient inhibitors of ligand binding, they also mimic ligand function. In the case of GPIIb/IIIa inhibitors, despite strongly inhibiting platelet aggregation, paradoxical fibrinogen binding and platelet activation can occur. The quick progression to development of small-molecule orally available inhibitors meant that this approach inherited many potential flaws, which together with a short half-life resulted in an increase in mortality and a halt to the numerous pharmaceutical development programs. Limited clinical benefits, together with the success of other anti-thrombotic drugs, in particular P2Y12 ADP receptor blockers, have also led to a restrictive use of intravenous GPIIb/IIIa inhibitors. However, with a greater understanding of this key platelet-specific integrin, GPIIb/IIIa remains a potentially attractive target and future drug developments will be better informed by the lessons learnt from taking the current inhibitors back to the bench. This overview will review the physiology behind the inherent problems of a ligand-based integrin inhibitor design and discuss novel promising approaches for GPIIb/IIIa inhibition.

Keywords
GP IIb/IIIa, platelet pharmacology, antiplatelet agents, thrombosis

Discovery of platelet glycoproteins
Glanzmann's thrombasthenia sufferers are characterised by a platelet-based bleeding disorder, first clinically described by Swiss physician Eduard Glanzmann in 1918. The underlying pathology of such a disease would remain ill-defined for a further 50 years until the optimisation of platelet washing techniques, protein radiolabelling, protease cleaving of surface receptors and SDS PAGE separation, allowed scientists such as Drs. Alan Nurden, David Phillips, James George and others to finally begin unravelling the complexities of platelet glycoproteins (GP), in particular IIb/IIIa (1). It was such discoveries that ultimately led us to the development of GPIIb/IIIa inhibitors. This vein of research continues in earnest as we continue to seek, and gain, insights into the relationship between glycoprotein structure and function, their suitability as therapeutic targets and, furthermore, the optimisation of therapeutic drug structure and application strategies.

GPIIb/IIIa structure and ligand binding
The GPIIb/IIIa complex numbers between 60,000 and 80,000 copies per platelet (2, 3). Also known as integrin αIIbβ3 or in the CD nomenclature CD41/CD61, it is a heterodimeric complex formed after synthesis of one IIb (α) and one IIIa (β) subunit (4). Each subunit is coded by individual genes, which are closely located, although not commonly regulated, on chromosome 17 (5). During megakaryopoiesis, the α subunit is formed after undergoing intracellular proteolysis resulting in a 105 kDa heavy chain and 25 kDa light chain. Notable structural features include a beta-propeller arrangement at the N-terminus and four divalent binding motifs at the base (6). In contrast, the beta-subunit is a 95 kDa polypeptide chain, with an A-domain at the N-terminus containing two-three divalent cation sites, a PSI (plexin/semaphoring/integrin) domain connecting to a protease resistant domain (7). The subunits utilise these divalent motifs to maintain a cation-dependant arrangement (8).

The principal ligand for GPIIb/IIIa is fibrinogen; however, fibronectin, vitronectin and von Willebrand factor (vWF) have also been demonstrated to have the ability to bind (9). From these ligands, two predominant peptide sequences have been found to encode recognition. The first is Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), found only in the carboxyl terminus of the fibrinogen gamma chain (10, 11). The second sequence, Arg-Gly-Asp (RGD) is found in the alpha chain with at least one copy contained in each known ligand. Of the amino acids in the recognised peptide sequences, the positively charged lysine (K) or arginine (R) residues
have been found to interact with the negatively charged aspartic acid 224 residue of the GPIIb beta-propeller. Likewise, the negatively charged aspartic acid (D) amino acid coordinates to the positively charged metal ion-dependent adhesion site (MIDAS) Mg\(^{2+}\) ion in \(\beta\)I domain of IIa (6, 12).

The integrin nature of GPIIb/IIa, through its assumption of conformational states, is also fundamental to facilitating the interaction with potential ligands. GPIIb/IIa exists in a resting conformational state, where the integrin is bent and the headpiece in a ‘closed’ form, meaning the RGD binding domain is concealed and thus it has only a low affinity for many physiological ligands. Upon appropriate stimulation, an inside-out signal, a conformational change occurs with the integrin transforming from a bent to an extended form with an ‘opening’ of the headpiece, exposing the extracellular RGD ligand binding domain (13) resulting in the integrin having a much higher affinity for its ligands. This is followed by the unclasping of the tail sections of both subunits, structurally repositioning the transmembrane domains (see ►Fig. 1A).

The intracellular pathways governing inside-out signalling are both numerous and complex. However, strong evidence exists demonstrating an important role for talin-1 and kindlin-3 translocation mediated by CalDAG-GEFI and Rap1 activation (reviewed in more detail by Ye et al. [14], Petrich [15] and Stefanini et al. [16]). In addition, it is believed that the conformational change of GPIIb/IIa may induce further signalling promoting actin polymerisation and cytoskeletal reorganisation in a process termed outside-in signalling (17). The exact mechanisms governing this process remain ill-defined, however a variety of molecules have been implicated including Src tyrosine kinases (18, 19), protein kinase C (20), phospholipases (21, 22) and phosphoinositide 3-kinase (23). More recently, Gong et al. have implicated the G-protein subunit \(G_{13}\) as being a key mediator (24).

Crucially, it is the above mentioned details concerning the conformational changes and ligand binding affinity that hold the key to our insights into utilising GPIIb/IIa as a therapeutic target (25–29).

Approved GPIIb/IIa inhibitors

There are currently three approved therapeutic inhibitors targeting GPIIb/IIa. The first to be used extensively in patients was the recombinant monoclonal antibody (mAb) abciximab (Reopro). Developed from the murine-raised 7E3 monoclonal antibody (mAb) by Dr. Barry Coller (30), the variable (Fab) antigen-specific region was utilised and the Fc region replaced and humanised using regions of human immunoglobulin to form a chimeric antibody (31), successfully reducing immunogenicity. Abciximab was approved by the US Food and Drug Administration (FDA) in 1994. A second inhibitor, eptifibatide (Integrillin), was approved in 1996 and is based on the disintegrin, barbourin, obtained from snake venom. Developed by Drs. David Phillips and Robert Scarborough with COR therapeutics/Portola pharmaceuticals; a Lys-Gly-Asp (KGD) sequence, analogous of the recognition sequences, is contained within a disulphide ring and acts as a competitive inhibitor for endogenous fibrinogen (32). In contrast, the third approved inhibitor tirofiban, is a small molecule non-peptide inhibitor developed by Merck. Using the RGD motif as a functional basis, an effective compound structure was established, which maintained the biochemical interactions important to the motif whilst removing the alpha-amino acids peptide bonds; a strategy which increased pharmacological survival time in vivo (33, 34).

The first clinical data supporting the intravenous use of GPIIb/IIa inhibitors was established in early trials where abciximab (EPIC and EPILOG trials) and eptifibatide (IMPACT II and ESPRIT) were both found to achieve significant improvements in mortality in patients undergoing percutaneous coronary intervention (PCI) (35–38). Subsequent studies such as PRISM-PLUS (39) supported the use of tirofiban. These early studies also identified the importance of a bolus administration followed by an infusion. The relative risk reductions (RRR) achieved at 30-days with 35% and above were impressive and led to a widespread use of GPIIb/IIa inhibitors in cardiac catheter laboratories worldwide. However, the early clinical trials on GPIIb/IIa inhibitors were conducted before the introduction of the routine use of \(P_2Y_{12}\) ADP receptor blockers and also before the introduction of newer anti-coagulants such as bivalirudin. In more recent trials, pretreatment with the \(P_2Y_{12}\) blocker clopidogrel seems to offset the beneficial effects of GPIIb/IIa inhibitors in patients undergoing elective, low-risk PCI or in diabetic patients (40, 41). A very recent meta-analysis including trials on elective PCI concluded that the use of GPIIb/IIa blockers in combination with ADP receptor blockers did not improve mortality but are associated with a significant reduction of non-fatal myocardial infarction (49). Numerous clinical trials investigating GPIIb/IIa treatment in patients with unstable angina, non-ST-elevation as well as ST-elevation myocardial infarction, who are pretreated with \(P_2Y_{12}\) blocker, demonstrated no additional benefits of GPIIb/IIa inhibitors and thus resulted in recommendations that limit GPIIb/IIa blockers to high-risk cases only, such as high thrombus burden for example (42, 43). However, if patients did not receive timely pretreatment with an ADP receptor blocker or, as demonstrated in the recent 3T/2R study (44), reveal a low response to treatment with aspirin and/or clopidogrel then GPIIb/IIa inhibitors are recommended for patients undergoing PCI.

Two concerns, which also arose during these studies, were the occurrence of thrombocytopenia and the increased risk of major and minor bleeding complications. Thrombocytopenia is more frequently reported with abciximab than with eptifibatide and tirofiban (45, 46). Nevertheless, severe clinical problems arising from thrombocytopenia have not been seen to significantly influence outcome in the numerous clinical trials conducted with GPIIb/IIa inhibitors. However, bleeding complications are more and more recognised as major determinant of clinical outcome in PCI (47–49). This is particularly true for the currently clinically used GPIIb/IIa inhibitors. For example, although GPIIb/IIa inhibition was hoped to be pathophysiologically beneficial when given in combination with fibrinolytic drugs, as these have been shown to have a platelet-activating side effect, increased bleeding
complications have resulted in the recommendation not to combine fibrinolysis and GPIIb/IIIa inhibition (42, 49–51).

Overall in comparison to the widespread use of GPIIb/IIIa blockers directly following their approval, the use of these drugs nowadays has dropped substantially. Based on contradictory study outcomes and changing use, as well as availability of other new antithrombotic drugs in particular P2Y12 receptor blockers, the clinical role of GPIIb/IIIa inhibitors is poorly defined. In addition, it will keep changing and remain to be a matter of controversial discussion amongst cardiologists. Most cardiologists though agree that the specific but rather small group of patients without pretreatment with P2Y12 blockers as well as high-risk patients in regards to lesion pathology (e.g. large thrombus burden) will benefit from GPIIb/IIIa inhibitors.

Figure 1: Cartoon models of the conformational states of GPIIb/IIIa under physiological conditions or in presence of inhibitors. A) GPIIb/IIIa exists in a bent conformation in the resting state (stage 1) whereupon inside-out signalling induces a change to an extended conformation with increased ligand affinity (stage 2). Engagement of a ligand binding, such as fibrinogen, causes the IIb and IIIa subunits to unclasp and induce outside-in signalling (stage 3). B) Ligand (RGD)-mimetic therapeutics are the basis for the currently approved GPIIb/IIIa inhibitors. These are able to bind in a low affinity resting state but induce integrin extension causing a conformational change (also termed: priming). When these agents leave their interaction site and the drug plasma levels drop, fibrinogen or other ligands are able to bind. C) Future therapeutics include allosteric agents which hold the integrin in the bent resting conformation, GPIIb-specific structures, which do not intrinsically induce nor inhibit extension to stage 2, or activation-specific single-chain antibodies (scFv), which block ligand binding but only once stage 2 has been achieved. Figure based on Zhu et al. (12).
Failure of oral inhibitors

The approved and initially developed GPIIb/IIIa inhibitors could only be administered intravenously. Therefore, orally available structures were developed by several companies as a potential primary and secondary prevention therapy, potentially providing benefits to millions of patients (thus the term “superaspirin” was used for the proposed oral GPIIb/IIIa inhibitors). In total five major phase III trials (including over 42,000 patients) testing four different compounds were completed, reviewed in more detail by Cox (52). Two studies EXCITE and SYMPHONY, found no significant improvement over existing therapy (aspirin) and OPUS and BRAVO were terminated early due to excess mortality, including cardiovascular mortality (53–57). This negative clinical outcome essentially ended the prophylactic potential for GPIIb/IIIa inhibitors and led to the cancellation of all development programmes in this drug class with large financial loss to many of the pharmaceutical companies (52).

Ligand-mimetic GPIIb/IIIa inhibition: The wrong strategy for the right target?

Eighteen years on from the initial regulatory approval and the fast following commercial success, beginning with the failure of the oral GPIIb/IIIa inhibitor program and continuing with ongoing negative outcomes of large clinical trials, the enthusiasm that was once held for GPIIb/IIIa inhibitors has diminished (58). The unexpected finding of increased mortality caused by oral GPIIb/IIIa inhibitors and the limited benefits seen with intravenous GPIIb/IIIa inhibitors has not only questioned the specific GPIIb/IIIa inhibitors but also GPIIb/IIIa as a therapeutic target in general. However, there is increasing evidence that it is not the target as such but the targeting strategy that caused problems and based on a better understanding of the integrin physiology promising alternative targeting strategies are currently under development (58–60).

Integrin function was initially considered purely as mechano-transducer connecting the cell with ligands in the extracellular matrix or on the surface of other cells. Thus, the concept of using reagents that imitate ligands to inhibit this function was logical. Of the three approved agents, the R(K)GD-based reagents tirofiban and epifibatide directly imitate fibrinogen binding, whereas the mAb abciximab, binds close to the fibrinogen binding pocket (61), all three can be considered to function as a ligand mimetic. However, integrins also function as mechano-sensors and “detect” binding of ligands by conformational change that is then transferred as an outside-in signal into the cell (62). Du et al. demonstrated in 1991 that RGD peptides can cause a conformational change of GPIIb/IIIa (63). This response implied that integrins may be used by cells to sense their surroundings through ligand-specific responses; however, at this time the full implications were not known. Ligand-mimetic GPIIb/IIIa inhibitor induced conformational change and its link to paradoxical fibrinogen binding and potential platelet activation was first demonstrated by Peter et al. (29). Electron microscopy and crystallisation studies of GPIIb/IIIa occupied by ligand-mimetic inhibitors by Xiao et al. later provided proof of concept as well as a detailed structural model of how ligand-mimetic inhibitors induce a conformational change of GPIIb/IIIa and thereby induce outside-in signalling (7). This platelet activation is now known to induce the expression of CD40L, P-selectin, CD63, and phosphatidylserine as well as the release of dense granule content and other stored pro-inflammatory mediators (64–67). The release of such mediators may contribute to concurrent paracrine platelet activation or inflammatory stimuli.

One consequence of the induced conformational change of GPIIb/IIIa is the exposure of what have been termed ligand-induced binding sites (LIBS, see Fig. 1). A small number of patients have preformed circulating antibodies against these LIBS. When treated with intravenous ligand-mimetic GPIIb/IIIa inhibitors this can result in a rapid immunological reaction, causing thrombocytopenia (68–70).

It is the paradoxical fibrinogen binding to GPIIb/IIIa that is thought to be especially pertinent to the failure of the oral inhibitor class. As reversible small molecule ligand-mimetic inhibitors with relatively short half-lives (31) it would continually be binding and leaving the receptor, inducing GPIIb/IIIa to adopt a high-affinity conformation. During periods of high plasma drug level, sufficient drug is present to successfully compete with any physiological ligand. However, during the ‘troughs’ in the plasma drug levels, the GPIIb/IIIa would remain in a high affinity confirmation with the binding site exposed and more accessible to ligands (71–73). The repeated peaks and troughs of drug plasma levels and the paradoxical induction of fibrinogen binding are hypothesised, but not proven, to be contributing factors to the increased mortality associated with this drug class. Findings with other integrins indicate that ligand-mimetic inhibition might indeed have particular side effects at low concentrations (46). As an important example for this Reynolds et al. recently demonstrated that RGD-mimetic inhibitors cause paradoxical effects at low concentrations resulting in the induction instead of inhibition of tumour growth (74).

Another characteristic of the currently used GPIIb/IIIa inhibitors, although the relevance is difficult to determine, is the lack of specificity for the target integrin GPIIb/IIIa. Abciximab binds to the integrins αvβ3 (75) as well as αMβ2 (Mac-1, CD11b/CD18) (76), whereas tirofiban and epifibatide possess various levels of cross-reactivities to integrin receptors that share RGD containing ligands, such as fibronectin (77).

The three approved GPIIb/IIIa inhibitors differ in their pharmacology, particularly in their binding affinity towards GPIIb/IIIa with abciximab demonstrating the strongest affinity and longest half-life (78). The question arises whether these drugs also differ in their characteristic to mimic ligand binding to GPIIb/IIIa and thus their ability to cause paradoxical fibrinogen binding and potentially platelet activation. Using LIBS exposure as a measure of conformational change of GPIIb/IIIa, abciximab induced less binding of an anti-LIBS antibody to GPIIb/IIIa than epifibatide and tirofiban (79). Whether this results in less paradoxical side effects with the use of abciximab compared to epifibatide or tirofiban has not been investigated yet. In the few trials, in which GPIIb/IIIa block-

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ers were compared to each other, abciximab seems to provide greater benefits compared to small molecule GPIIb/IIIa inhibitors (80–82). However, several factors besides differences in induced conformational change may account for this, such as differences in receptor affinity and differences in the percentage of GPIIb/IIIa receptors blocked by the three competing GPIIb/IIIa inhibitors.

Future novel strategies targeting GPIIb/IIIa

Whilst the currently approved inhibitors have not been as successful as originally hoped or intended, GPIIb/IIIa remains to be a promising target for future developments of anti-platelet drugs. Recent work by Drs. Robert Blue and Barry Coller has identified a novel type of inhibitor, RUC-1, which effectively inhibits fibrinogen binding to GPIIb/IIIa and thus agonist-induced platelet aggregation (83, 84). However, in contrast to the ligand-mimetic based antagonists discussed above, this novel compound only binds to the GPIIb subunit but not to GPIIIa (Fig. 1C). This represents a novel and attractive strategy of inhibiting GPIIb/IIIa, without the intrinsic property of inducing a conformational change of GPIIb/IIIa (12). By avoiding such a change it is anticipated that associated thrombocytopenia, induction of fibrinogen binding and paradoxical platelet activation would be prevented. Whether or not the current molecule structure is to prove clinically successful, it offers an informative basis for the rational design of future anti-GPIIb/IIIa drugs but also anti-integrin drugs in general.

Another potential alternative approach aiming to avoid a conformational change of the non-activated GPIIb/IIIa, is to block the activated form of the integrin receptor GPIIb/IIIa only (Fig. 1C). Using phage display technology, Schwarz et al. (71, 85) generated human single-chain antibodies that only bind to the activated form of GPIIb/IIIa. These single-chain antibodies, which are small recombinant antibody fragments consisting of the variable regions of the antibody heavy and light chain fused together to a single molecule, have attracted major interest from the pharmaceutical industry based on their expected low immunogenicity especially when human antibody libraries are used (71, 85, 86). These anti-GPIIb/IIIa single-chain antibodies were systematically tested in vitro and in vivo and it was shown that they do not cause a conformational change of GPIIb/IIIa and thus do not induce LIPS epitope expression, which makes the development of thrombocytopenia less likely. Furthermore, they do not induce paradoxical fibrinogen binding to GPIIIa or paradoxical platelet activation (85). Their selective binding and blocking of activated platelets can explain the unique and promising in vivo finding of strong anti-thrombotic properties without bleeding time prolongation (85). Whereas platelet adhesion was still intact, platelet aggregation and therefore thrombus formation was effectively inhibited (85). Furthermore, this GPIIb/IIIa inhibition approach is highly flexible as additional effector molecules can be fused to these activation-specific single chain antibodies allowing not only blocking of GPIIb/IIIa but also targeting and thus enrichment for example of fibrinolytics or anticoagulants at the activated platelet and thus clot (87).

In addition such a genetic fusion for example of a molecular switch, allowed the development of a unique temperature steerable GPIIb/IIIa inhibitor that can be turned on by hypothermia and off by rewarming to body temperature. This would allow the use of a GPIIb/IIIa blocker as effective anti-thrombotic protection during hypothermic extracorporeal circulation with immediate recovery of platelet function on rewarming at the end of surgery (88).

Allosteric inhibition has been shown to be possible for the integrins LFA-1 (αLβ2) (89) and Mac-1 (αMβ2) using small molecules (90). This potential approach would involve holding GPIIb/IIIa in the bent conformation (Fig. 1C). One proposed approach is to target residues 95–105 on the β3 subunit, as this region has been identified as being a fulcrum to the conformational hinge and as such offers a potential allosteric inhibition site (91).

Each of these approaches is so far experimental in nature only and is yet to reach clinical testing. It is also an open question whether after the massive financial losses of the pharmaceutical industry in the development of oral ligand-mimetic GPIIb/IIIa blockers and the fading financial benefits of intravenous GPIIb/IIIa blockers there will be enough financial support available that is necessary for the clinical testing of new GPIIb/IIIa blockers. Nevertheless, it is hoped that this review might provide the basis for a better understanding of the problems of the current clinically used GPIIb/IIIa blocking strategy and might help to promote the development of newer and better approaches to inhibit thrombus formation by targeting GPIIb/IIIa.

Conclusion

GPIIb/IIIa plays a critical role in the regulation of platelet aggregation and clinical thrombosis and was, and still is, therefore a rational target in the search for improved anti-thrombotic therapeutics. However significant limitations associated with the currently approved inhibitors, coupled with the failure of the oral GPIIb/IIIa inhibitors, has led to reduced expectations and questioning of the suitability of GPIIb/IIIa as a good target for anti-platelet/anti-thrombotic therapy. The currently clinically used intravenous GPIIb/IIIa inhibitors have helped to establish the benefits of anti-platelet therapy in PCI. However, the emergence of P2Y12 ADP receptor blockers as routine pretreatment of patients undergoing elective PCI or PCI whilst suffering from acute coronary syndrome has offset many of the benefits initially seen with intravenous GPIIb/IIIa inhibitors. Nevertheless, in patients without timely or effective P2Y12 blocker treatment and in high risk patients (e.g. high thrombus burden) and despite a potentially problematic inhibition strategy the use of the currently clinically available GPIIb/IIIa inhibitors provide some proven benefits. In addition, it is in part due to these limitations and lack of success that has directed research to further investigate and gain greater understanding of the mechanisms of GPIIb/IIIa inhibition. The progress in this research area, as outlined above, infers that we may now be able to consider the current inhibitors as ‘first-generation’ and with this increased knowledge as well as additional “bench work” we can im-

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prove future drug design appropriately and identify more suitable strategies for maximising the protection offered by GPIIb/IIIa inhibition. The "second generation" of GPIIb/IIIa inhibitors remain to be clinically tested. Since bleeding complications have been the reason for major clinical limitations of the currently clinically used GPIIb/IIIa, the activation-specific anti-GPIIb/IIIa inhibitors seem to be particularly attractive for further drug development.

Conflicts of interest
None declared.

References

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