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Platelet GPIb complex as a target for anti-thrombotic drug development

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Summary
Specific inhibition of platelet function is a major target of anti-thrombotic drug research. Platelet receptors are both accessible and specific but have multiple functions often linked to a wide range of ligands. GPIb complex is best known as a major platelet receptor for von Willebrand factor essential for platelet adhesion under high shear conditions found in arteries and in thrombosis. Recent animal studies have supported inhibition of GPIb as a good candidate for anti-thrombotic drug development with several classes of proteins showing important specific effects and the required discrimination between roles in haemostasis and thrombosis is important to protect against bleeding complications. These include antibodies, several classes of snake venom proteins, mutant thrombin molecules and peptides affecting subunit interactions. However, due to the nature of its receptor-ligand interactions involving large protein-protein interfaces, the possibility of developing classic pharmaceutical inhibitors for long term (and perhaps oral) treatment is still unclear, and additional information about structural interactions and signalling mechanisms is essential.

Keywords
Antiplatelet agents, atherothrombosis, GP Ib, platelet glycoproteins, platelet pharmacology

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Introduction

The main targets for anti-thrombotic drug development are platelets and coagulation factors (1, 2). Platelets have long been a goal, first of all because they are a major source of thromboxanes and their synthesis is inhibited by aspirin – the first anti-thrombotic drug (3). Since then it has become clear that platelet functions can be inhibited to reduce thrombotic tendencies, either by blocking surface receptors (4–8) or critical cytoplasmic enzymes, such as cyclooxygenase (9), or signalling proteins such as kinases (10) or phosphatases (11). Since many of these signalling proteins are common to a wide range of cells, including leukocytes (12, 13), important in immunology, the range of useful targets is very restricted. Because of this, and also due to their better pharmacological access, there is great interest in surface receptors, which are easier to aim at and in several cases are restricted to platelets. In the early days of receptor targeting there was much discussion about the best strategy (14, 15). According to current knowledge at that time it seemed safer to inhibit platelet aggregation rather than adhesion. Aggregation was seen as the result of all activation pathways and thought to collect together all agonist effects and therefore to be a safer approach. On the other hand, inhibition of adhesion, and early-stage haemostasis, was thought to more likely lead to bleeding problems. Since Bernard Soulier syndrome and Glanzmann's thrombasthenia, which affect the major adhesion and aggregation receptors, respectively, both lead to severe bleeding disorders, this was not completely logical. The choice was helped by some other factors, such as the differences in sequence between GPIbα from various species, making it difficult to test suitable human blocking antibodies (15) in small animal models. This problem was only accessible at a much later stage after development of large numbers of different monoclonal antibodies allowing the selection of some with a wider range of cross-species reactivity. This was less of a problem with GPIIb/IIIa, the first anti-human blocking antibody was also effective in small animal models (14), allowing

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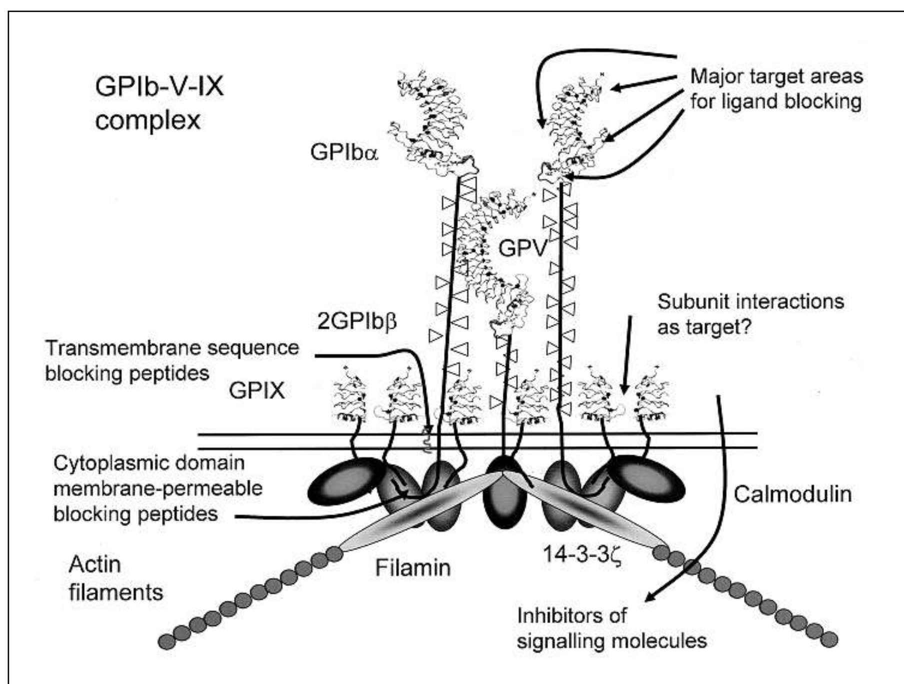

Figure 1: A recent model of the GPIb-V-IX complex showing inhibitor target sites. GPV reaches further out than in earlier models because the leucine-rich domains are now thought to fold like those in GPIbα giving a more open arc. Two molecules of GPIbβ linked covalently per GPIbα via disulphide bonds are shown, in line with recent findings. The fine detail of the binding sites for ligands other than the A1 domain of von Willebrand factor, thrombin and FXI are not yet known. Further connections to the cytoskeleton and to signaling pathways are known but have been omitted for the sake of clarity. General target areas for known or proposed inhibitors are indicated with arrows.

quick progress to in-vivo inhibitory studies. On the other hand, GPIIb/IIIa – belonging to the integrin family of receptors – was shown to have an RGD peptide loop binding pocket allowing the rapid development of small molecule inhibitors (16). The GPIb complex thus lagged behind in inhibitor development. While GPIIb/IIIa, i.e. aggregation, inhibitors have seen rapid development and great success in anti-platelet and anti-thrombotic treatment during surgery and in prevention of restenosis (17), the area of thrombosis prevention has been a different story. Although there is still much work to do in this area it now seems clear that while blocking GPIIb/IIIa following platelet activation certainly prevents platelet aggregation many other things, including signalling, secretion of granules, reorganisation of the cytoskeleton and membrane lipids as well as cleavage of receptors, continue to go on despite this. In addition, there are questions whether these inhibitors in fact caused platelet activation by inducing conformational changes in the receptor and if this might be avoidable. Thus, the release of granule contents, reorganisation of the cytoskeleton and membrane lipids are not necessarily prevented although signal transduction feedback via GPIIb/IIIa contributes to all of these. Activated but aggregation-inhibited platelets can still contribute to downstream effects (17). Because of this there has been much discussion and consideration of alternative strategies and new candidate targets. These have included a range of ADP or ATP receptors, P2Y12 (18), P2Y1 (19), and P2X1 (20), which all have a role in amplifying post activation signals. Thrombin receptors, PAR1 (21) and PAR4 (22) are also being actively investigated. The major adhesion receptors being considered include the collagen receptors α2β1 (7) and GPVI (8, 23) as well as the von Willebrand factor (VWF) receptor, GPIb (24). There has been considerable controversy about which of these receptors might be the most effective as an anti-thrombotic target. This is in large measure because of the different thrombosis models that have been used. In the simplest model, collagen/epinephrine is injected into a vessel in the small animal, and rapidly induces a blocking thrombus. Alternatively, rapid ligation of a vessel can be used to cause endothelial cell loss. This can be prevented by soluble collagen receptor or collagen blocking antibodies/inhibitors (25). More "physiological" models are based on laser damage to the endothelium with or without dye activation or $FeCl_3$-induced detachment of endothelial cells or the Folts model where endothelium damage is inflicted on an artery using a plastic ring or cuff resulting in cyclic flow with occlusion followed by embolism (26). More recent results suggest that the laser model does not necessarily expose subendothelium but causes *in situ* endothelial cell activation with expression of CD62P and large multimers of VWF (27). Despite some earlier claims, it was therefore not surprising that, in laser injury models, collagen and its receptors do not play major roles and thrombin was demonstrated to play a central role in thrombus formation (28). Nonne et al. (29) showed that thrombin had a greater role in more serious, laser-induced vascular injuries than in milder ones, based on inhibition with hirudin.

Treatment of arteries with $FeCl_3$ causes endothelial cells to detach from the subendothelium, exposing collagens, VWF and other subendothelial matrix proteins. In these latter models, inhibition of GPVI (30, 31) or α2β1 (31, 32) or GPIbα (33, 34) reduces thrombus size but has little effect on haemostasis, assessed as bleeding time. This is surprising but interesting and goes against intuition implying that 'dogma' about the physiological versus pathological roles of these adhesion receptors may be wrong. Thus, the inhibition of GPIbα (or GPIb complex by alternative methods) may be a good approach to inhibition of thrombosis.

The role of GPIb?

What is the role of GPIb in the overall process of haemostasis versus thrombosis, and why might it affect one more than the

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other? While it is an important platelet receptor it also has a number of key functions in coagulation and thus lies at an interface between both pathways. Figure 1 shows a model of the core structures of the GPIb complex with target sites for inhibitors indicated by arrows.

The major role of GPIb is generally thought to be as a receptor for VWF, acting under high shear to brake passing platelets to bring them into close contact with either exposed subendothelium or inflamed endothelium. Bernard-Soulier syndrome (BSS), where GPIb is either absent or defective (35), is characterised by major bleeding in response to trauma but otherwise, if patients are careful and have no other problems they can lead relatively normal lives. They may suffer severe nose bleeds and bruise easily but with appropriate therapy they can give birth or undergo major surgery without excessive bleeding. However, BSS platelets are not simply normal platelets lacking the GPIb complex but are much larger than normal. In addition, there are markedly fewer platelets than normal. BSS is thus characterised as thrombocytopenia, but despite this reduced count the total platelet mass may well be in the normal range (36). Expression of GPIb complex varies considerably in BSS patients from more or less complete absence in mutants where GPIbα is truncated or its folding is drastically affected (37), to about 7–10% expression in the common GPIX N45S mutant (38, 39), or even higher expression in certain other mutants (37). It is interesting that while these latter categories show some typical characteristics of BSS, such as large platelets and thrombocytopenia, this is variable and they rarely show major bleeding problems on trauma, which suggests that even a lower density of GPIb complex expression may be adequate for haemostasis under such conditions.

Table 1: Platelet GPIb ligands and their function.

Ligand	Function
A1 domain of von Willebrand factor	Critical in platelet binding to exposed sub-endothelium or activated endothelium
Thrombin	Binding to GPIb has a major role in platelet responses to low concentrations of thrombin (presentation to PAR-1?)
Protein C/activated protein C	Binding to GPIbα at the same time as thrombin may allow GPIbα to catalyse Protein C activation
Mac-1 (αMβ2)	Platelet binding to macrophages. Removal of "chilled" platelets from the circulation
P-selectin (CD62P)	Critical in platelet binding to activated endothelium or activated leukocytes
FXI, FXII	Critical roles in amplification of procoagulant cascade
High-molecular-weight kininogen	Supports GPIb interaction with Mac-1
Thrombospondin-1	Can replace von Willebrand factor in platelet binding to small vessel sub-endothelium at high shear
Collagen	Indirectly via A1 and A3 domains of von Willebrand factor
Soluble GPVI	May influence platelet responses to collagen

Thrombosis models in GPIbα$^{-/-}$ mice

GPIbα$^{-/-}$ mice have a similar phenotype to humans with Bernard-Soulier syndrome having large platelets and thrombocytopenia, enhanced bleeding after injury and much reduced thrombus size in thrombosis models (40, 41). It is not surprising that transgenic mice, with the extracellular domain of IL4Rα replacing that of GPIbα, also have a severe bleeding phenotype although the platelet size was reported to be normal and there is only mild thrombocytopenia (70%) (42). In thrombosis models, this IL4Rα/GPIbα chimera showed virtually no tethering platelets in damaged arterioles. The observation that platelets which lack the major ligand-binding (including VWF) domain, but with other regions, including cytoplasmic, cytoskeleton-linked domains intact, have a normal size and only a slight decrease in platelet number is intriguing from the standpoint of the role of the GPIb/VWF axis in regulating platelet production (36) from megakaryocytes, suggesting that it is the cytoskeleton-linkages of the GPIb complex that have the major role. This is reassuring in connection with the inhibition of GPIb, as it makes side effects on production from megakaryocytes affecting platelet size or count unlikely.

Comparison of the IL4Rα/GPIbα chimera with VWF$^{-/-}$ animals showed far less difference from normal in the latter but an ~80% reduction in tethering platelets. Thrombus formation was, however, fairly stable, often leading to occlusion. It was concluded that GPIb plays a more critical role in haemostasis and thrombosis than VWF (33). Thus, there must be alternative, back-up mechanisms, which enable platelet tethering in the absence of VWF but nevertheless requiring GPIb.

Over the past few decades an increasing number of ligands for GPIb have been identified (43) (Table 1). It is now well established that GPIb, as well as the protease activated receptors (PAR1 and 4 in humans), is an important receptor for thrombin but its mode of action remains obscure (44). Platelets can bind to and be activated on thrombin-coated surfaces by a GPIb-dependent mechanism (45, 46). Other candidate ligands include P-selectin (47), which is expressed on the surface of activated platelets, and endothelial cells or thrombospondin-1, which was shown to support GPIbα-dependent platelet adhesion *in vitro* under high-shear conditions (48). GPIbα has several other ligands and plays an important role in binding components of the coagulation cascade and catalyzing their activation.

GPIb complex function can be inhibited by means of various approaches. Although these are not the same as pharmaceutical type, small molecule inhibitors give some indication regarding what might be expected in such studies with human platelets.

Inhibitors of GPIb

Some of the first studies were conducted with a recombinant peptide from VWF (residues 504–728), cross-linked internally by a single disulphide bond, and termed VCL peptide (49). This is now identified as the A1 domain of VWF, in the "activated" GPIb-binding conformation due to the lack of the constraining,

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highly O-glycosylated, adjacent regions. VCL was shown to inhibit platelet adhesion to VWF under both static and shear conditions and to prevent thrombus formation *in vivo* (50).

Another approach has been to use specific proteases to cut GPIbα without affecting other platelet receptors. Two classes of proteases have been used; on the one hand a bacterial O-sialylglycopeptide endopeptidase (51) that cleaves GPIbα to liberate a 45 kDa fragment containing the ligand binding sites, and on the other hand, snake venom metalloproteases, in particular mocarhagin, which cleaves GPIbα in the anionic peptide region to liberate a slightly smaller fragment (52). Other snake venom metalloproteases have been shown to cleave GPIbα but they also affect other proteins such as VWF (53). These are useful tools, especially for human platelet studies but are difficult to apply in practice because the degree of completion of cleavage varies, depending on the donor and the platelet preparation.

A second approach involved the use of antibodies, which dates back to the early 1980s and established some early functional properties and structure/functional relationships of the GPIb complex (15). As previously mentioned, a major problem has been the lack of species cross-reactivity to enable a comparison between human and animal models due to sequence differences in critical regions of the molecule. Nevertheless, it had been possible to establish critical epitope locations (54–56) even before X-ray crystallographic structures of the 45 kDa outer domain of GPIbα (57), and its complexes with the A1 domain of VWF (58, 59) or thrombin (60, 61), were obtained.

When anti-GPIbα antibodies are injected into animals, a strong, transient thrombocytopenia is often observed (34). This seems to require the presence of a divalent antibody because Fab fragments do not have this effect, which suggests that platelets are activated and agglutinate or aggregate in response to this treatment. Weak GPIb expression on endothelial cells, which was indicated in several studies (62), remains another possible explanation, with these antibodies inducing platelet binding to the endothelium.

Antibodies to GPIbα, or at least their Fab fragments, have proven to be effective in the prevention of thrombosis in non-human primates without causing undue bleeding problems (34), and there is considerable interest in the development of "nanobodies" from camels or llamas to block the GPIb/VWF axis. However, a major problem that arises when applying to acute thrombotic situations is that, for many possible applications they will need to undergo head-to-head clinical testing with the best treatments currently available, including αIIbβ3 inhibition. This involves very large and hence expensive clinical trials. However, for market niches outside present applications of anti-platelet therapy the situation is probably more promising. Recently, Kleinschnitz et al. (63) showed that anti-GPIbα antibody (Fab fragments) was more effective than either depleting GPVI or an anti-αIIbβ3 antibody (Fab fragments) in a mouse, middle cerebral, artery occlusion model without increasing bleeding complications. Improved treatments are still urgently needed in stroke. Likewise, the recent observation that replacement of the extracellular domain of GPIbα on platelets by IL4R reduced lung metastasic foci in a B16F10.1 melanoma model in congenic C57BL/6J mice compared to the normal GPIbα mice (64) suggests other important applications for anti-GPIbα antibodies.

Another important class of GPIb binding and, mostly, inhibiting molecules, are the snake venom C-type lectins or C-type-like lectins (65, 66). These are very widespread and examples have been found in nearly all Viperidae and Crotalidae venoms analyzed but also in some Elipidae venoms. Most of these inhibitory molecules belong to the simple heterodimeric class consisting of α- and β-subunits linked by swapping loops and a disulphide bridge, such as echicetin (67, 68). They form a large concave surface that is thought to bind to the outer domains of GPIbα blocking access to the A1 domain of VWF and in some cases (not all were tested) also preventing thrombin binding (69). So far there are no crystallographic studies of such complexes but this interpretation appears to be reasonable based on other recent findings. Thus, the structures of both the botrocetin/45 kDa outer domain of GPIbα/A1 domain of VWF complex (70), and the bitiscetin/45 kDa outer domain of GPIbα/A1 domain of VWF complex (71), have been determined. Both botrocetin and bitiscetin are simple heterodimeric class snake venom C-type lectins. They were originally thought to activate platelets by binding to the A1 domain of VWF and changing its conformation to the GPIbα binding state. Crystallographic studies of the molecular trimer show that botrocetin and bitiscetin bind to both the 45 kDa outer domain of GPIbα and A1 domain of VWF, and hold them together, thus enhancing their interaction. Therefore, molecules of this class with GPIb as target can either inhibit or activate platelets depending on their mode of action. There is a further category of snake venom C-type lectins that belong to the tetrameric, heterodimeric class where four heterodimers are held together by additional disulphide bridges linking the N-terminal and C-terminal regions of each in order to form a cyclic structure with four binding sites facing outwards that can activate platelets via GPIb. This structure allows binding of four GPIbα molecules on the same or adjacent platelets. Several examples of this class are known with various effects on platelets. These include flavocetin (72), mucrocetin (73) and mucetin (74). While flavocetin simply induces platelet agglutination with no evidence of activation as defined by αIIbβ3 activation or granule release, mucrocetin is able to form larger agglutinates, and mucetin is able to induce classic aggregation involving αIIbβ3. All three, flavocetin, mucrocetin and mucetin, have very similar sequences with only a few amino acid differences which makes it difficult to establish these functional differences. Minor differences in overall hydrophobicity might affect their ability to form larger clusters known to influence platelet activation. A somewhat similar phenomenon is known for the tetrameric, heterodimeric class molecules convulxin (75), stejnuluxin (76) and ophioluxin (77) that activate platelets via GPVI binding, where ability to form higher clusters seems to regulate ability to activate platelets. Recombinant convulxin, despite having the necessary basic structure and the ability to bind GPVI, is nevertheless a very poor platelet activator (78).

In early studies of GPIb binding snake venom C-type lectins, at a time when their structure was still poorly understood, attempts were made to isolate peptides or sub-domains that still retained a substantial part of the overall inhibitory power of the intact molecule. While these experiments were largely unsuccessful, in rare cases peptides were obtained that retained some inhibitory capacity, although in general higher concentrations were necessary.

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The number of GPIbα molecules present per platelet remains controversial despite the large number of studies that have been conducted. Early work based on monoclonal antibodies revealed numbers in the 20–25,000 range (15), whereas studies with monovalent snake venom C-type lectins indicated numbers in the 40–50,000 range (67). More recent flow cytometry results have tended to support these higher numbers and the simplest explanation for the disparity is likely to be that the divalent nature of the monoclonal antibodies was not considered in the earlier studies. These differences are relevant when inhibitory studies are analysed.

Thrombin mutants form another interesting category of GPIb inhibiting molecules. Two major mutants have been described, the S195A mutant (79) where the active site serine has been inactivated, and the W215A/E217A (80) mutant which affects fibrinogen binding and activation. The S195A mutant can still bind to GPIbα but is unable to cleave PAR1 or PAR4. The W215A/E217A mutant can also still bind to GPIbα and can cleave PAR1 but at a 1,000-fold reduced level compared to wild type thrombin. On the other hand, it can still activate protein C at levels comparable to the wild type in the presence of thrombomodulin, shifting the overall balance to down regulation of thrombin generation. Surprisingly, while the S195A mutant had no effect on platelet rolling on a VWF coated surface or binding to a collagen-coated surface, the W215A/E217A mutant inhibited both. There had been some debate regarding whether both thrombin and the VWF A1 domain can bind simultaneously to GPIbα, and whether binding of one could affect binding by the other (81). These data support other studies showing that both can bind and that binding of one may enhance the binding of the other. On the other hand, the W215A/E217A mutant might not be able to bind simultaneously and the collapse of the 215–217 strand might well alter the binding of this mutant to GPIbα so as to prevent the A1 domain binding simultaneously. Since two molecules of thrombin can bind simultaneously to each GPIbα, the conformations of each may be drastically altered by the mutations.

Recent studies suggest that GPIb can bind to both protein C and activated protein C (82) and that, via concurrent thrombin binding, it might play a catalytic role in this conversion. Thus, while the key role of GPIbα in thrombin generation is now well known, it may also play a key role in down-regulation.

The W215A/E217A mutant neither supports platelet adhesion nor does it induce platelet signalling, which again suggests that its anti-thrombotic effects are due to prevention or alteration of key catalytic events involving GPIbα or blockage of GPIbα interaction with VWF.

Inhibition by aurin tricarboxylic acid

Aurin tricarboxylic acid has been known to inhibit platelet GPIb-VWF interactions for a long time (83) and has been used as a standard method to compare results of such inhibition. It was shown in early experiments that it binds to VWF but not to platelets but it may have additional effects. Since aurin tricarboxylic acid is a highly negatively charged molecule it is likely that it prevents interactions with GPIbα by blocking positively charged residues (lysines?) on VWF that interact with the anionic peptide in GPIbα. After the effects were first described later studies fractionated the commercial aurin tricarboxylic acid preparations (200 to >6000 Da) by size exclusion chromatography and showed that the >2,500 Da fraction was the most potent (84). This could be interpreted as being due to enhanced binding between the larger molecules and the larger (and more effective) VWF multimers.

Inhibition by transmembrane peptides

Recent studies have shown that receptors involving multi-subunit complexes each with transmembrane sectors can be influenced by a synthetic peptide with one of the transmembrane sequence (GPIIb/IIIa) (85). Studies indicate that the association of the subunits within the complex is regulated to a large degree by the transmembrane sectors. This conclusion was drawn based on the observation that mutations in transmembrane domains could lead to defective complex formation in disorders such as Glanzmann's thrombasthenia and BSS. Thus, peptides made to complement transmembrane domains of αIIbβ3 or αvβ3 can be used to manipulate, inhibit, or activate, these receptors. Similar techniques should be applicable to the GPIb complex.

In addition (86), the preparation of membrane penetrating peptides made up of a 9 arginine domain, linked to cytoplasmic domain sequences of GPIbα corresponding to the filamin-binding site were shown to decrease platelet agglutination/aggregation in response to VWF, while those that corresponded to the 14–3–3ζ-binding site had no effect. These results support studies in CHO cells that express GPIb complex but lack filamin (87), or with a modified GPIbα molecule lacking the 14–3–3ζ-binding site (88).

Inhibition of GPIb/VWF interactions by other peptides and/or small molecules

Although GPIb interaction with its ligands generally seems to involve large protein-protein interactions, at least for those crystal structures of complexes that have so far been determined, there have been some reports of successful inhibition using small molecules, in particular, cyclic peptides. Recently, Smith et al. (89) reported that screening a library of nine amino acid, cysteine-constrained peptides expressed in phage, for binding to GPIbα, yielded three peptides with binding constants of 0.74, 31 and 106 nM measured by surface plasmon resonance. They inhibited VWF binding to GPIbα and delayed closure time in the PFA-100 platelet function analyzer, which has a high VWF/GPIbα interaction dependence. These results clearly demonstrate that small molecules can interfere with GPIbα function. What remains unclear is whether they only block interactions with VWF, or whether they also affect interactions with other GPIb ligands.

Conclusions

GPIbα as the main ligand binding molecule of the GPIb complex presents highly interesting, but quite difficult, challenges as a target for anti-thrombotic molecules. Nevertheless, over the last few years a lot of new information has surfaced and research has shown that it is not impossible to find suitable molecules. The next few years will show if this promise can be held.

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