The Development of an Assay to Evaluate the Efficacy of a Novel GPIb Antagonist, and the Functional Characterisation of the Drug

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The Development of an Assay to Evaluate the Efficacy of a Novel GPIb Antagonist, and the Functional Characterisation of the Drug

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Dr. D Cox

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Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MSc by research is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of my work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged with the text.

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List of Abbreviations

GP	glycoprotein
ADP	adenosine di-phosphate
vWf	von Willebrand factor
MI	myocardial infarction
LRR	leucine rich repeat
PRP	platelet-rich plasma
PPP	platelet-poor plasma
COX	cyclooxygenase

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Summary

Thrombosis is central to the pathogenesis of many life-threatening diseases, including stroke and myocardial infarction. Current treatments for thrombosis, particularly anti-platelet agents that target GPIIb/IIIa, can cause serious bleeding complications. Unlike GPIIb/IIIa, GPIb initiates thrombus formation at the high shear stress that is prominent in atherosclerotic vessels. Therefore, this target could prevent pathological thrombosis while leaving normal haemostasis at low shear rates relatively unaffected. Anfibatide is a novel GPIb antagonist isolated from snake venom in China. Anfibatide will enter phase Ib-IIa trials in early 2015 and therefore, it is necessary to develop an assay to measure its efficacy in patients. We have developed a flow cytometry based receptor occupancy assay. In this assay, total GPIb receptor numbers are measured using an anti-GPIb monoclonal antibody, and the percentage of GPIb receptor occupancy by anfibatide is measured. Receptor occupancy by anfibatide correlated with dose-dependent inhibition of ristocetin-induced aggregation in whole blood by anfibatide. Functional studies were carried out using anfibatide. Anfibatide did not inhibit binding of two anti-GPIIb/IIIa antibodies. Anfibatide partially inhibited the anti-GPIb antibodies VM16d, and SZ 2, indicating that it binds to GPIbα between the thrombin-binding site and the sulfated tyrosine motif. Anfibatide did not cause platelet activation or loss of single platelets *in vitro*. Anfibatide had no effect on aggregation induced by low dose or high dose thrombin, ADP or arachidonic acid. Anfibatide inhibited *Streptococcus sanguinis* 133-79 induced aggregation by 50% in half of healthy donors, but had no effect on *Staphylococcus aureus* Newman aggregation. The receptor occupancy assay is optimised and available for use in Phase Ib-IIa clinical trials, and in future clinical use of anfibatide.

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Chapter 1: Introduction

Platelets are anucleate fragments from megakaryocytes. They are the smallest and most abundant cellular component of blood, ranging from 3 x 10^8/ml to 4 x 10^8/ml. The GPIb-IX-V complex is present on the platelet membrane and interacts with von Willebrand factor to initiate thrombus formation at high shear stress. This interaction is central in pathological thrombus formation, which can lead to stroke and myocardial infarction. Thrombosis is treated with anti-platelet agents, usually targetting the GPIIb/IIIa receptor. This receptor causes thrombus formation at low shear stress, and its inhibition leads to bleeding complications. Since GPIb inhibition would not affect haemostasis at low shear stress in the periphery, it is an enticing target for anti-thrombotic treatment (1-6).

Glycoprotein Ib-IX-V Complex

The glycoprotein (GP) Ib-IX-V complex is a receptor present on the platelet membrane with a central role in primary haemostasis under high shear conditions. GPIb binds to von Willebrand factor (vWF), which is exposed on the subendothelium following vascular damage, leading to thrombus formation (1-6). GPIb bound to the endothelium may mediate endothelial cell migration during wound repair (7). GPIb-IX-V has also been implicated in regulating platelet size (2).

The GPIb-IX-V complex is part of the leucine rich repeat (LRR) family (3). The adhesion receptor contains four membrane spanning polypeptides (GPIbα, GPIbβ, GPIX and GPV) in the ratio 2:2:2:1 on the platelet plasma membrane (5). GPIbα (610 residues) is disulfide linked to GPIbβ (181 residues), and is noncovalently complexed with GPIX (160 residues) and GPV (544 residues) (2). A stable complex between GPIbα, GPIbβ, and GPIX is required for normal transport of each gene product though the membrane systems of maturing megakaryocytes, as well as for expression of the complex on the platelet surface (8). GPV has a high affinity binding site for thrombin (9). The LRRs on GPIX, GPIbβ, and GPV may be vital in controlling subunit structure (8). GPIb-IX-V is formed in the endoplasmic reticulum and transported to the Golgi apparatus for subsequent modification, including O-glycosylation of GPIbα (8,10). GPIb-V-IX is extensively palmitoylated (11). A GPIb-V-IX subset is constitutively associated with lipid rafts in unstimulated platelets, and the number of receptors present can triple following stimulation with vWF (11, 12). Lipid raft disruption impairs some GPIb-IX-V dependent reactions, including vWF induced aggregation, tyrosine phosphorylation and adhesion to vWF (11).

GPIb (CD42b)

The largest subunit in the GPIb-IX-V complex is GPIbα, which has an apparent molecular mass of 135 kDa (2, 6). It is a major sialoglycoprotein with multiple N- and O- linked oligosaccharides (10). The N-terminal domain (1-282 residues) of GPIbα contains binding sites for vWF (amino acids 26-200), Mac-1 (on neutrophils), P-selectin (on activated platelets and endothelial cells), α-thrombin (a potent platelet agonist *in vivo*), clotting factors XI/XIIa, and high-molecular-weight kininogen (2, 6, 13). Glycocalicin, a soluble fragment of GPIb, is produced by proteolytic cleavage, for example by endogenous platelet calpain, of the external part of GPIbα. Glycocalicin circulates freely in normal plasma (10, 14). GPIb interaction with its ligands generally involves large protein-protein interactions (15). GPIb usually binds thrombin when the agonist is present at low doses, due to the high affinity of the binding site (4, 13, 16). Heparin blocks thrombin induced platelet activation through GPIb (4). The N- terminal domain of GPIbα is enclosed by two conserved disulphide loop structures, and contains eight LRRs (12). Asparagine residues in these LRRs are required for correct conformation and function of the ligand binding regions (17). LRRs 2, 3, and 4 are critical for vWF binding and platelet adhesion. Residues 283-302 are mostly negatively charged, and include aspartic and glutamic acids, and three sulfated tyrosines (Tyr^{276}, Tyr^{278}, Tyr^{279}). Mutation of the three sulfated tyrosine residues strongly reduces vWF levels, and thrombin binding (12). It is suggested that this sulfated region is necessary for GPIbα to bind vWF under shear flow (9). A long and highly glycosylated mucin-like macroglycopeptide domain with a molecular mass of 118 kDa (residues 303-485) connects these to a single transmembrane region (residues 486-514) (12, 18). The macroglycopeptide may position the ligand-binding region above other molecules present on the platelet surface (9). The cytoplasmic tail of GPIbα consists of 96 amino acids (residues 515-610) and is necessary for normal processing and function of GPIb-V-IX (12). Filamin-1 binds to residues 557-579, and adaptor protein 14.3.3ζ binds to residues 605-610 (19).

Some reports estimate 25,000 GPIbα receptors per platelet (5, 6, 13), but this remains controversial. Yamamoto *et al* reported approximately 28,000 GPIb molecules per platelet using a monoclonal antibody that binds to a part of glycocalicin containing overlapped binding sites for vWF and thrombin (20). Other monoclonal antibody studies indicate that there are 20,000 to 25,000 receptors per platelet, compared to 40,000 to 50,000 receptors indicated in studies using monovalent snake venom C-type lectins. Recent flow cytometry studies generally support higher receptor numbers, suggesting that the divalent nature of the monoclonal antibodies was not considered in the earlier studies (15).

Von Willebrand Factor

Von Willebrand factor (vWF) is a multimeric glycoprotein bound to collagen in the subendothelial matrix or plasma (21). It circulates in the plasma tightly bound to the glycoprotein factor VIII (FVIII). FVIII accelerates activation of factor X by activated factor IX in the coagulation cascade. VWF has an important role in production, conformation, and stabilisation of FVIII (12). Mature vWF is 250 kDa, with 2,050 amino acids, and is characterised by A, B, C, and D structural domains. It is rapidly secreted as a range of multimers (over 2,000 kDa) from endothelial Weibel-Palade bodies and platelet α-granules following endothelial or platelet activation by pro-thrombotic or inflammatory signals (2, 12, 22, 23). Patients with coronary heart disease tend to have high vWF concentration, and have more ultralarge multimers than healthy people (24). VWF is exposed on the endothelium following vascular damage, which can occur acutely or chronically by several pathophysiological mechanisms, allowing it to interact with GPIb (3, 25). GPIb-IX-V binds vWF in a bidentate interaction, initiating platelet aggregation and leading to thrombus formation at high shear rates in flowing blood (3, 4). VWF binding to GPIb-V-IX involves vWF A1 domain and the 45 kDa extra-cellular N-terminal domain of GPIbα (Figure 1.1) (2, 12). The co-crystal structures of GPIb (residues 1–267) and the vWF A1 domain (residues 478–705) show involvement of N-terminal (-hairpin) and C-terminal (“-switch”) flanking sequences. GPIb residues in the N-terminal flank, the LRRs, and the C-terminal flank directly interact with vWF A1. The LRR sequence 59–128 is critical for vWF binding under static or shear conditions using human or canine chimaeras of GPIb expressed on CHO cells (2). The A1 domain of activated soluble vWF requires a conformational change to increase its affinity for GPIb-V-IX. Under physiological conditions, this change occurs when vWF is bound in the extracellular matrix, or under hydrodynamic shear stress (12).

GPIb-vWF Interaction

Platelets become activated after adhering to vWF and undergo cytoskeletal rearrangement associated with shape change, spreading and secretion of platelet agonists, for example adenosine di-phosphate (ADP), which recruits additional platelets to the developing thrombus (2, 26). VWF can simultaneously bind different GPIb-V-IX receptors on adjacent platelets, possibly contributing directly to receptor clustering and activation, and aggregate formation (11,12). GPIbα is involved in the transient binding of platelets to activated vWF at sites of vascular injury (4). GPIbα binding to vWF initiates platelet tethering to the subendothelial matrix, as well as transient platelet translocation across the matrix surface (9). This binding reduces platelet velocity in flowing blood, causing “rolling” of platelets. This allows vWF to efficiently capture platelets, initiating platelet deposition and thrombus formation (10, 12). Using a parallel plate flow chamber model, *ex vivo* platelets adhere transiently and roll on immobilized vWF under flow conditions. Rolling platelets become arrested on the vWF matrix when shear stress increased dramatically. This shear stress model is representative of arterial stenosis *in vivo* (27). Low GPIbα receptor density has been associated with increased cell velocity during this rolling phenomenon, compared to cells with high receptor density. GPV may also be necessary for optimum binding of vWF and GPIbα (9). Intracellular signal transduction leads to mobilisation of a src kinase and phospholipase Cγ2 activation. Release of internal Ca^{2+} stores follows, which causes rearrangement of the cytoskeleton, and platelet shape change with filipodia extension (28, 29). The platelet surface integrin GPIIb/IIIa (αIIbβ3) is also activated, binding fibrinogen and mediating platelet aggregation (2, 26, 29). GPIb-V-IX is also an indirect collagen receptor through the A3 domain of vWF, and possibly the A1 domain (15).

The surface expression of the GPIb-V-IX complex is downregulated following platelet activation. This could possibly be a mechanism to prevent excessive thrombus growth (15). Thrombus growth and thrombolysis are dynamic processes and may occur simultaneously. Inhibition of the GPIb-vWF

interaction may stop platelet recruitment and shift thrombus growth toward dissolution under shear flow. The GPIb-vWF interaction leads to ‘outside-in’ signaling via the GPIb-IX-V complex, stimulating GPIIb/IIIa activation in the thrombus (4, 26). This is necessary for platelet aggregation mediated by fibrinogen, and other integrin ligands (26).

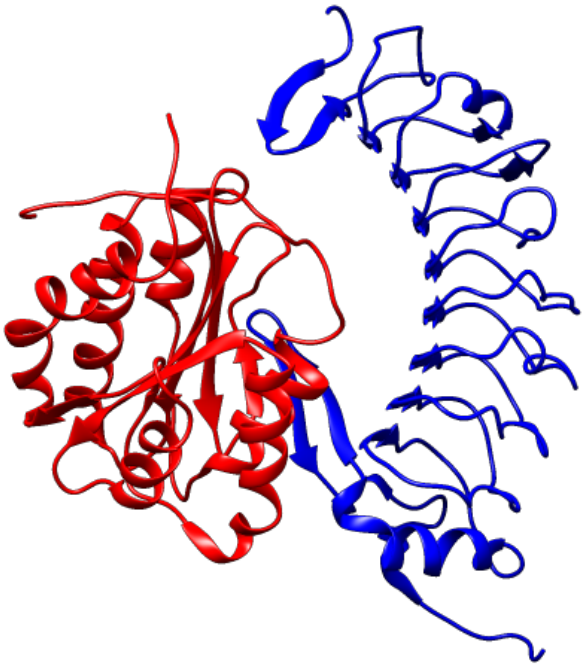

Figure 1.1. The structure of the vWF A1 domain (shown in red) and GPIbα (shown in blue), (PDB: 1M10)

GPIIb/IIIa

Fibrinogen, the main ligand for GPIIb/IIIa, is a plasma glycoprotein that plays a critical role in platelet aggregation as well as in the formation of a fibrin clot at the site of vascular injury. When the coagulation cascade is triggered, the resulting thrombin activates platelets and converts fibrinogen into fibrin monomers. GPIIb/IIIa changes conformation following platelet activation allowing binding of fibrinogen. This process is the final common pathway for platelet aggregation stimulated by ADP, thrombin, or collagen. GPIIb/IIIa contains two RGD sequences in the α-chain (residues 95-97), and a C-terminal dodecapeptide on the γ-chain (residues 400–411). RGD peptides bind to residues 109–171 of GPIIIa, and γ-chain dodecapeptides bind to residues 294–314 of GPIIb. The RGD sequences and the AGDV sequence from the dodecapeptide share the same binding site in GPIIb/IIIa, spanning both chains IIb and IIIa (31). There are between 60,000 and 80,000 GPIIb/IIIa receptors per platelet (30). GPIIb/IIIa levels are lower in patients with Glanzmann's thrombasthenia compared to healthy people, resulting in abnormal clot retraction, prolonged bleeding time, and the absence of platelet aggregation (32). These interactions are central in vascular inflammation and thrombosis.

Shear Conditions in Flowing Blood

Under high shear stress, as found in the arteries, large particles, including erythrocytes, accumulate in the centre of blood flow, forcing the smaller platelets to the periphery, allowing them to interact with the endothelium (33). The interaction of the GPIb-IX-V complex with vWF has a vital role in the initiation of platelet adhesion, particularly at high shear rates (> 1,200 s^{-1}), and is necessary for complete vessel occlusion above a limiting shear rate (approximately 10,000 s^{-1}). At sites of vascular stenosis, blood flow velocity increases due to lumen restriction, thus increasing the shear stress on platelets. Complete thrombotic occlusion of vessels initiated by the GPIb-vWF interaction, and completed by GPIIb/IIIa mediated thrombus formation and adhesion, causes blood flow obstruction and tissue damage (26). The GPIb-V-IX interaction with vWF can efficiently take place under high shear flow in arterioles and small arteries (10 - 50 Angstrom in diameter), where the shear rate varies from 500 s^{-1} to 5,000 s^{-1} (9, 12). Under this high shear stress, GPIbα has limited time to bind to vWF, and requires a strong bond to support and maintain adhesion (9). On the other hand, platelet surface integrin GPIIb/IIIa and its ligands, including fibrinogen, mediate platelet aggregation and contribute to platelet adhesion at low shear stress in larger arteries and veins (9, 12, 26).

In Vitro Activation of GPIb

Soluble vWF can be activated artificially *in vitro* to bind GPIb-V-IX in the absence of shear stress or vWF immobilisation by ristocetin and botrocetin. Ristocetin is an antibiotic glycopeptide isolated from the soil bacterium *Nocardia lurida*, and botrocetin is derived from a snake venom toxin (12, 34). Ristocetin binds to proline rich sequences contained in the anionic sequences of the A1 domain of vWF (Cys1237-Pro1251 and Glu1463-Asp1472) (12, 34). Botrocetin binds to one or more sequences within the disulphide loop (residues 539-643) of the A1 domain (12). The sulfated tyrosine residues are important for the botrocetin-induced reaction with GPIb and vWF (35). Both ristocetin and botrocetin change the conformation of the vWF A1 domain to allow binding of GPIb, in turn causing platelet aggregation *in vitro* (26). The action of ristocetin more closely mimics the GPIb-vWF interaction induced by shear stress *in vivo* (9, 34). Ristocetin- and botrocetin- induced binding of GPIb to vWF leads to signal transduction and GPIIb/IIIa activation (4).

Monoclonal Antibodies Directed Against GPIbα

Expression of GPIbα on the platelet surface can be detected by flow cytometry analysis after incubation with labelled monoclonal antibodies. Several monoclonal antibodies directed against GPIbα are commonly used.

AN51 is directed against amino acids 1-35 in the N-terminal of GPIbα. It blocks vWF binding to GPIbα (10). Binding studies using AN51 in platelet rich plasma (PRP) indicate 22,000 ± 2,700 binding sites (6), though this number tends to vary widely depending on the donor (9). AN51 is sensitive to reduction, suggesting that it is dependent on protein conformation (35). It has no effect on vWF binding induced by botrocetin or ristocetin (35). AN51 also reacts with monocytes (32).

WM23 is an anti-GPIb murine monoclonal antibody, which binds to the macroglycopeptide region of GPIbα, without affecting binding to vWF (9, 11, 36). It detects 21,000 ± 3,400 binding sites on platelets (6).

SZ 2 binds to GPIbα (residues 276-282) in the highly negatively charged domain on GPIbα, which contains three sulfated tyrosine residues (8). This region is involved in thrombin binding. SZ 2 prevents thrombin generation by the prothrombinase complex, but does not significantly affect the numbers of, or mean velocity of, rolling mammalian cells across glass slides coated with vWF (4, 9, 35). SZ 2 blocks botrocetin-induced binding of vWF to GPIb, and weakly inhibits the ristocetin-induced reaction (9, 35). It is not sensitive to protein reduction (35).

VM16d is directed towards the thrombin-binding site on GPIbα. It inhibits thrombin binding, but not vWF binding. It also blocks the procoagulant response of platelets to low doses, but not to high doses, of thrombin. VM16d does not prevent ristocetin-induced binding of vWf to GPIb when measured using flow cytometry (4).

ALMA 19 is a mouse monoclonal antibody that binds to GPIbα on human platelets and megakaryocytes. The exact binding site has not yet been established (19).

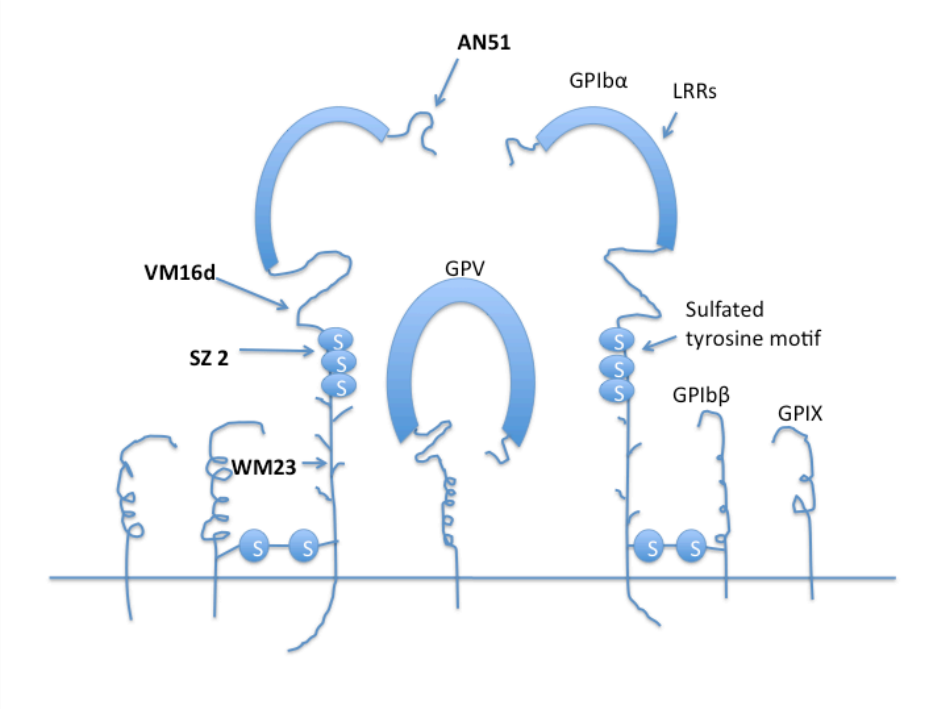

Figure 1.2. Binding of anti-GPIbα antibodies. AN51 binds to the N-terminal (residues 1-35), where vWF binds. VM16d binds to the thrombin-binding site. SZ 2 binds to residues 276-282 in the sulfated tyrosine motif. WM23 binds to the macrogylycopeptide region. ALMA 19 binds to GPIbα, though the exact binding site is unknown.

Disorders of GPIb

Bernard-Soulier syndrome (BSS) is an autosomal recessive bleeding disorder that occurs when a patient has deficient, absent, or dysfunctional GPIb-IX-V, or impaired maturation or processing of the complex (4, 8, 18, 37). Thrombocytopenia (a decrease in circulating platelet levels) and impaired platelet adhesion to vWF is observed in BSS patients, which may lead to severe and possibly fatal bleeding, and may require blood transfusion (8,18). BSS platelets are typically large, indicating a role for GPIb-IX-V in the regulation of platelet size (2). BSS platelets are round, and have abundant vacuoles (8). BSS patients show defective procoagulant activity, with high basal levels, and decreased levels when stimulated by agonists compared to healthy people. This may be due to reduced stability of the membrane (4). BSS patients have low sensitivity to thrombin, but platelets can still be activated at high doses of thrombin (4, 18, 20).

BSS can be caused by sense or missense mutations in the GPIbα, GPIbβ or GPIX genes. The majority of reported cases involve a defect in the GPIbα gene, leading to a structurally modified or truncated protein with inhibited or absent expression of GPIbα. This is followed by reduced expression of GPIX and GPV on the platelet surface. One such mutation is the Cys^{209} to Ser missense mutation (8). The Bolzano variant of BSS is hereditary, caused by a point mutation of A156 to V in the 6^{th} LRR of GPIbα. In this variant, mutated GPIbα cannot bind vWF, but thrombin binding is unaffected (13, 27). LRRs 2, 3, and 4 of GPIbα are critical for vWF binding and platelet adhesion, and mutations within the LRRs 2, 5, 6, and 7 of GPIbα have been associated with BSS. Mutation of the three sulfated tyrosine residues strongly reduces vWF and thrombin binding (12). Deletion of Leu^{179} in the seventh LRR of GPIbα causes BSS (variant Nancy I) (8). A BSS variant with one amino acid substitution in the extracellular domain of GPIbβ affects maturation of GPIbα and GPIX stability (8).

GPV does not seem to be a cause of BSS as deficiency in its expression on platelets in mice does not affect platelet structure or size, or modify surface expression of GPIb or GPIX. No symptoms of BSS are present in the case of GPV deficiency (8).

Heterozygous deficiency of GPIbβ has been linked to Di George syndrome or velo-cardio-facial syndrome, which are characterised by a micro deletion in 22q11.2, an area where the GPIbβ gene maps to (8) Patients with the deletion tend to have macrothrombocytopenia, diminished ristocetin-induced aggregation, and reduced expression of the GPIB-IX-V complex (50). Patients with autoantibodies directed against GPIb have been demonstrated to develop immune thrombocytopenic purpura (38).

Platelet Antagonists

Platelets have a central role in atherosclerosis (involving plaque formation in inflamed blood vessels), myocardial infarction (MI; the cause of tissue death following occlusion of a coronary vessel by a thrombus), and other potentially fatal thrombotic disorders. Atherosclerotic plaques can rupture following platelet interactions, forming a platelet-rich thormbus which can occlude vessels in the heart or brain, causing MI or stroke respectively (1).

The most commonly used anti-thrombotic drugs inhibit GPIIb/IIIa-mediated platelet aggregation, including fibrinogen receptor antagonists, acetylsalicylic acid (aspirin), and $P2Y_{12}$ antagonists, for example clopidogrel. Recent clinical data with intravenous fibrinogen receptor antagonists and other anti-platelet drugs such as Prasugrel, demonstrated platelet inhibition and prevention of ischaemic complications, but were associated with increased bleeding complications. Inhibitors of GPIIb/IIIa are effective anti-thrombotic agents in acute situations, but can lead to thrombocytopenia and potentially life threatening bleeding complications, such as haemorrhages in the cerebral, alveolar, and gastrointestinal systems (15, 26). Furthermore, oral GPIIb/IIIa inhibitors enhanced cardiovascular events in clinical trials. Lower dosage of the oral inhibitors was used so that they could be used for chronic treatment to prevent thrombosis. However, lower dosage lead to less inhibition, along with low bioavailability of the drug. The GPIIb/IIIa antagonists induced conformational changes in the receptor, and also acted as partial agonists, causing an increase in platelet aggregation (15, 51).

Aspirin effectively prevents thrombotic disorders, such as MI and stroke, by irreversibly inhibiting cyclooxygenase 1 (COX-1), an enzyme important in generating thromboxane A_2, a potent platelet agonist (33). Thienopyridine anti-platelet drugs, including ticlopidine, prasugrel and clopidogrel, are pro-drugs that block the $P2Y_{12}$ ADP receptor, a receptor that normally augments platelet aggregation (33). Ticagrelor is an orally administered $P2Y_{12}$ blocker, but is not a prodrug, meaning it does not require hepatic activation. Goto *et al.* showed that ticlopidine also prevented stabilization of vWF bound to GPIb

under high shear stress *ex vivo,* causing dissociation of thrombi (39). For long-term anti-platelet treatment, combined aspirin and clopidogrel treatment has limited side effects, but increased reports of treatment resistance emphasise the need for new anti-thrombotic agents. Aspirin resistance may be due to alterations in enzyme expression and regulation. Clopidogrel resistance may be caused by low rates of activation of the pro-drug to the active species due to genetics, blockage of, or competition for cytochrome P450 by food components or other medicines (40). It is likely that poor response to aspirin is due to inappropriate dosage. Weight is associated with reduced response to enteric-coated aspirin (52).

Small molecule GPIIb/IIIa antagonists, including the monoclonal antibody abciximab (Reopro), the small molecule non-peptide inhibitor tirofiban, and eptifibatide (Integrillin), can reduce the incidence of acute thrombotic complications after coronary intervention by up to half. These agents block platelet aggregation and are used to prevent acute thrombotic complications after coronary intervention in patients with acute coronary syndromes and stable effort angina. However, abciximab has not been shown to have any extra benefit over dual treatment with aspirin and heparin in patients with acute coronary syndromes that did not undergo coronary intervention (33).

Bleeding complications are the main and most serious side effects associated with GPIIb/IIIa antagonists. Coronary artery reocclusion is also a serious problem with many thrombolytic treatments. Monoclonal antibody treatment is not ideal as it could lead to immunogenicity, and it may not be reversible (25). There is demand for safe anti-platelet agents that can prevent or reduce thrombosis and atherosclerosis, while minimally affecting the normal haemostatic process, therefore preventing bleeding complications.

GPIb as an Anti-Thrombotic Agent

There is great interest in targetting GPIb as an anti-thrombotic therapeutic as it is an easily accesible surface receptor restricted to platelets and has a major role in thrombotic diseases including MI and stroke (2, 9, 12). The interaction between GPIb and vWF initiates thrombus formation under high shear stress, unlike the GPIIb/IIIa interaction with fibrinogen which also takes place at lower shear rates found in the peripheral venous system. Inhibiting this interaction would be more effective in the arterial system compared to the venous system. This could possibly reduce bleeding complications compared to current anti-thrombotic agents such as aspirin (12, 41).

GPIb has not been fully investigated as an anti-thrombotic mainly due to the differences between various species in the GPIbα receptor, making it difficult to test human blocking antibodies in small animal models. This problem did not occur with GPIIb/IIIa antagonists, which made the latter antagonists easier to develop (40).

Several studies indicate that GPIb-IX-V complex inhibition would be an effective treatment for thrombosis. In patients with acute coronary syndromes, high shear stress induced platelet aggregation was increased when plasma vWF levels increased (41). Treatment of arteries with ferric chloride ($FeCl_3$) caused endothelial cells to detach from the sub-endothelium, exposing collagens, vWF and other sub-endothelial matrix proteins. In such models using non-human primates, inhibition of GPV, $\alpha_2\beta_1$ (collagen receptor) or GPIbα reduced thrombus size with little effect on bleeding time (15). GPIb-IX-V causes pathological arterial thrombosis upon binding to vWF exposed on the sub-endothelium after vascular damage. Blocking this interaction has been shown to reduce thrombus formation, and prevent long-term restenosis of narrowed vessels (9). It has been established that complete inhibition of GPIb by specific monoclonal antibodies *in vivo* in guinea pigs and baboons does not induce a haemorrhagic state (37, 38).

In vWF knockout mice, arterial thrombosis still occurs but complete vessel occlusion is either prevented or delayed. However, thrombus formation is completely inhibited in GPIb knockout mice, suggesting that GPIb receptor ligands, other than vWF, are important in platelet aggregation and thrombus formation (26). Inhibition of vWf and ADAMTS13 by monoclonal antibodies prevented thrombocytopenia and haemolytic anaemia in a baboon model of thrombotic thrombocytopenic purpura. However, partial inhibition of vWF was not effective (53). Since GPIb deficiency impairs thrombosis more effectively than vWF deficiency, GPIb is a more attractive target for antithrombotic therapy than vWF. There is a possibility that blocking vWF could affect its interaction with bound FVIII, which could affect the coagulation cascade. Targeting GPIb would not affect this interaction (26).

No GPIb antagonists have been successfully used in clinical practice, although several potential candidates have been investigated (26, 41). VCL peptide (the A1 domain of vWF) inhibited platelet adhesion to vWF under static and shear conditions, and prevented thrombus formation *in vivo* (15). Specific proteases have been used to cleave GPIbα, leaving other platelet receptors intact. The two classes of proteases used are bacterial O-sialylglycopeptide endopeptidases, which cleave GPIbα to liberate a 45 kDa fragment containing the ligand binding sites, and snake venom metalloproteases, which cleave GPIbα in the anionic peptide region to liberate a slightly smaller fragment. One such snake venom is mocarhagin. However there are some issues with using these proteases. Snake venom metalloproteases cleave GPIbα, but some can also affect other proteins such as vWF. Also, there is variability in cleavage completion depending on the donor and the platelet preparation (15).

Antibodies to GPIbα, or their Fab fragments, effectively prevented thrombosis in non-human primates without causing bleeding complications. Kleinschnitz *et al.* showed that an anti-GPIbα antibody (Fab fragments) was more effective than depleting GPVI (the main signaling receptor for collagen) or than using an anti-GPIIb/IIIa antibody (Fab fragments) in a mouse middle cerebral, artery occlusion model, without increased bleeding complications (15).

Snake Venoms

Snake venoms contain many polypeptides and proteins that can alter thrombosis and haemostasis. The use of venoms as a therapeutic for thrombotic disorders has become an interesting option, as they could possibly alter the platelet response that can lead to cardiovascular and cerebrovascular pathology (31, 42).

Snaclecs (snake venom C-type lectins) are a subset of non-enzymatic proteins isolated from snake venom, which include C-type lectins, and related proteins including C-type-like lectins and IX/X-bp like proteins. IX/X-bp like protein is a class of anticoagulant venoms, including two-chain botrocetin, convulxin, and mamushigin (42). The venoms of this class are heterodimers linked by an inter-subunit disulfide bond. The molecular weight of these proteins is approximately 30 kDa. N-terminal amino acid sequences in these proteins are highly conserved and extremely similar to C-type lectins. Despite their structural similarity, they all display different biological activities and mechanisms of action (31, 42).

Snake venom C-type lectins and C-type-like lectins are important GPIb antagonists. They are widespread and found in nearly all Viperidae and Crotalidae, and some Elipidae, venoms. Most of these are part of the simple heterodimeric class, consisting of α- and β- subunits linked by swapping loops and a disulphide bridge (43).

C-type-like lectins proteins are important haemorrhagic proteins derived from snake venom. Despite the similar structure of compounds within the class, their biological activity varies. They bind many diverse proteins including Factor IX, Factor X, GPIb, GPVI, CLEC-2 and $\alpha_2\beta_1$ (45). Agkistin and rhodocetin are two C-type-like lectins. Agkistin blocks the vWF-GPIb interaction, and inhibits angiogenesis *in vivo* (44). Rhodocetin binds the collagen receptor $\alpha_2\beta_1$, preventing collagen-induced platelet activation, similar to the effect of collagen-blocking antibodies (40).

C-type lectins form a large concave surface that is thought to bind to the outer domains of GPIbα, blocking binding to vWF, and in some cases thrombin (15). Some C-type lectins can bind to vWF, and GPVI (44). The C-type lectin jararhagin interacts with the $\alpha_2\beta_1$. Some snake C-type lectins, for example echicetin, interact with the thrombin binding site on GPIbα, but have been shown to have no effect on the platelet response to thrombin, especially at low doses (< 0.5 U/ml) (4). Some of these proteins bind to more than one ligand, for example convulxin, alboaggregin A, and alboluxin all bind GPIbα and GPVI (44). Eptifibatide is a GPIIb/IIIa inhibitor based on barbourin, a disintegrin isolated from snake venom. It is a competitive inhibitor for fibrinogen *in vivo* (30).

Snake venoms that bind GPIb can be agonists, for example botrocetin, or antagonists, such as echicetin purified from *Echis carinatus* (41). Crystallographic studies show that botrocetin, a snake venom often used to cause platelet aggregation *in vitro*, and bitiscetin, bind to both the 45 kDa outer domain of GPIbα and to the A1 domain of vWF, holding them together and enhancing their interaction. Therefore, molecules targeting GPIb may inhibit or activate platelets, depending on their mode of action (15).

Anfibatide

Anfibatide is a novel GPIb antagonist purified from the compound agkisacutacin (26). Agkisacutacin is a large fibrinogenlytic C-type-like lectin containing α and β chains with interchain disulphide bonds. It has no Ca^{2+}- or sugar- binding loops. It is derived from the tetrameric protein complex agglucetin, which is isolated from *Agkistrodon acutus* snake venom in the southern Anhui Province of China (31, 42).

Agkisacutacin has two heterologous subunits linked by an intersubunit disulfide bond (42). It has a molecular weight of 29,500, with two heterodimers of 14 kDa (β-subunit with 27 residues) and 15.3 kDa (α-subunit with 31 residues) (31, 42). Each subunit contains a compact lectin-like globular domain and long extended loop region (45). The N-terminal sequences of the two polypeptides of anfibatide hold more than 85% homology to that of the IX/X-bp family (31).

Early studies using flow cytometry and competitive enzyme-linked immunosorbent assay (ELISA) demonstrated that agkisacutacin binds to GPIb and inhibits human vWF binding, while also binding to factor IX and factor X (44). The binding of the monoclonal antibody SZ 2 to GPIbα on platelets was blocked by agkisacutacin. Agkisacutacin did not block binding of the anti-GPIIIa monoclonal antibody SZ 21 or the anti-GPIIb monoclonal antibody SZ 22 to human platelets (44). After studying the crystal structure of the compound, it was suggested that each agkisacutacin αβ-heterodimer molecule binds to one GPIb molecule and inhibits the GPIb-vWF interaction without causing GPIb clustering, unlike flavocetin-A (26, 45). Flavocetin-A and echicetin are two C-type lectins with similar structures to anfibatide. They inhibit vWF-GPIb binding, and prevent platelet aggregation, but increase platelet agglutination by forming tetramers, or by binding to immunoglobulin Mκ (IgMκ) in the blood plasma, respectively (26, 45).

Agkisacutacin inhibited platelet adhesion and aggregation *in vitro* and *in vivo,* but did not cause platelet agglutination in plasma (45). *In vivo* in canines, agkisacutacin (0.9-3.6 μg/kg) significantly inhibited platelet aggregation (44). Agkisacutacin did not inhibit ADP- or collagen- induced PRP aggregation, and had little effect on thrombin induced aggregation (44). It significantly prolonged clotting time, prothrombin time and activated partial thromboplastin time, and showed dose-dependent anticoagulation *ex vivo* in human blood (44). Agkisacutacin did not cause significant platelet activation (as measured by P-selectin expression and GPIIb/IIIa activation) *ex vivo*, prolong tail-bleeding time *in vivo*, or cause spontaneous bleeding in mice (26). Agkisacutacin was shown to be stable at -70°C for up to 46 days (41).

Agkisacutacin had a linear relationship between protein concentrations and fibrinolytic activity, significantly and directly reducing plasma levels of fibrinogen in a dose-dependent manner. It cleaved the fibrinogen α-chain and β-chain slowly, with little γ-chain cleavage, producing polypeptides of molecular weight 43 kDa, 40 kDa, 36 kDa, and 23 kDa. Lysis of the fibrinogen α-chain caused fibrinogen clotting *ex vivo* (26, 42). Agkisacutacin had a different cleavage site on fibrinogen than thrombin or plasmin. Unlike other fibrinolytic venom metalloproteases, agkisacutacin cleaves fibrinogen at a specific site, with little haemorrhagic or anti-coagulant activity (42). It is unknown why agkisacutacin could have fibrinolytic activity along with inhibitory effects on platelet aggregation *in vitro* and *in vivo*. The fibrinopeptides, containing RGD sequence, that result from fibrinogen cleavage may competitively inhibit fibrinogen binding to GPIIb/IIIa, thereby inhibiting platelet aggregation. In rats, agkisacutacin can significantly accelerate the lysis of pulmonary emboli, suggesting fibrin digestion either directly or indirectly, or both (31). When agkisacutacin was further purified by three-step chromatography to form anfibatide, all fibrinogenlytic activity was lost (44).

Agkisacutacin was used in ten healthy Chinese volunteers to assess its pharmacokinetics. Agkisacutacin protein was dissolved in sterile normal saline for intravenous bolus administration at a concentration of 5 μg / 60 kg. The peak time for agkisacutacin concentration in plasma was eight hours and the terminal half-life was 10.5 hours. No antibodies to agkisacutacin were formed in the volunteers, and there were no changes in haematological or coagulation parameters. Bleeding time was not significantly prolonged (41).

Anfibatide, the purified form of agkisacutacin, inhibited ristocetin-induced aggregation in washed murine platelets with recombinant murine vWF (26, 44). It blocked botrocetin-induced binding of vWF from murine plasma to recombinant human GPIbα as measured by ELISA. It did not inhibit botrocetin-induced aggregation in murine PRP. Anfibatide inhibited ristocetin-induced, but not botrocetin-induced, aggregation using human PRP, indicating a different binding site than other snake venom-derived GPIb antagonists. In an ongoing clinical trial, anfibatide inhibited ristocetin-induced aggregation in human PRP, confirming the difference in tertiary interactions between anfibatide, and human and mouse GPIb. In a rat arterio-venous shunt model anfibatide significantly inhibited thrombus formation in a dose-dependent manner (26).

Anfibatide strongly inhibited platelet adhesion, aggregation, and thrombus formation in perfusion chambers, at high shear rates (1,800 s^{-1}), and dissolved preformed thrombi *ex vivo*. Anfibatide inhibited thrombus formation at low shear rates (300 s^{-1}), though less than at high shear rates. As shear stress increased incrementally from 100 s^{-1} to 5,000 s^{-1} in perfusion chambers, inhibition of thrombus formation by anfibatide increased. The inhibition was approximately 100 times stronger at high shear (1,800 s^{-1}) compared to low shear (300 s^{-1}). In anfibatide-treated platelets at 5,000 s^{-1}, adhesion and aggregation on collagen was undetectable (26). Anfibatide may interact with other plasma proteins that favour thrombolysis, possibly explaining how anfibatide induces thrombus dissolution *ex vivo* (26).

Anfibatide markedly inhibited thrombosis in laser-injured cremaster vessels and prevented vessel occlusion in $FeCl_3$-injured mesenteric vessels in mice. In anfibatide-treated mice, thrombus formation was delayed, unstable, easily dissolved, and not occlusive. In vWF-deficient mice that had impaired laser-induced thrombosis compared to wild-type (WT) mice, anfibatide inhibited thrombus formation further than knockout alone. This suggests that anfibatide inhibits thrombotic pathways mediated by GPIbα, aside from the GPIb-vWF interaction (26).

Lee's Pharmaceutical Limited is currently recruiting participants in China for a phase Ib-IIa clinical trial. The trial will investigate the safety and efficacy of anfibatide in non-ST segment myocardial infarction patients. It is a multi-center, randomised, double-blind, placebo-controlled trial. The primary outcome measure is thrombosis formation 48 hours after infusion during stent implantation. Secondary outcome measures are mortality (30 days after treatment), bleeding events and thrompbocytopenia. The study is due to finish in February 2015 and it is hoped that 90 patients will participate. A bolus injection with different doses of anfibatide will be administered immediately when the guidewire passes through the first stenosed vessel, along with an intravenous infusion at 0.002 IU/kg/h for 48 hours afterwards. Saline will be used as a placebo. The study aims to establish the efficacy of different doses of anfibatide so that Phase III trials can progress. The study will accept males or females aged between 18 and 70 years. Patients must show an increase in markers of myocardial damage, ischaemic symptoms, and must give informed consent (46). Phase I Clinical trials were completed in March 2011. Anfibatide was administered to 94 healthy volunteers. The primary outcome measure was the number of adverse events in patients with single or multiple intravenous injections or infusions. The secondary outcome measure was the observation of area under curve characteristics of anfibatide in single or multiple dose groups. A dose of 0.33 – 5 μg/60 kg was administered in a single dose (47). The Phase I trial demonstrated that anfibatide did not significantly decrease platelet count or prolong bleeding time. There were no serious adverse events or deaths during the study, and all results indicated

that anfibatide is a safe and potent anti-platelet reagent, and a potential anti-thrombotic therapy (48).

Bacterial-Platelet Interactions

A major cause of MI, stroke, and other conditions involving dysregulated platelet function, is pathogenic bacteria circulating in the blood. Many strains of bacteria can cause platelet activation, aggregation, and thrombocytopenia. *Staphylococcus aureus* is the most common cause of infective endocarditis, and acts through GPIIb/IIIa. *Streptococcus sanguinis* and *streptococcus gordonii* act through the GPIb receptor, at the N-terminal (1-225 residues) of GPIbα, to cause direct platelet aggregation. The ability of *S. sanguinis* to adhere to and to activate platelets corresponds to disease severity. GPIb interacts with a glycoprotein, serine-rich protein A (SrpA) on *S. sanguinis* to cause strong adhesion and rapid platelet aggregation. Anti-GPIb antibodies can inhibit the aggregation response to *S. sanguinis*. *S. gordonii* binds GPIb through the surface-anchored GspB to stimulate platelet activation. *Heliobacter pylori* strains also interact with GPIb, as indicated when anti-GPIb antibodies prevented platelet aggregation in its presence (1). In this study we investigate the effect of anfibatide on *S. sanguinis*-induced platelet aggregation, in order to determine if it would be useful as a therapeutic agent in treating or preventing bacterial-induced cardiovascular events.

The Assay

As anfibatide progresses through clinical trials, it is necessary to develop a tool to accurately measure the exact occupancy of GPIb receptors by anfibatide. An assay that measures receptor occupancy needs to be simple to learn, and accessible in hospital laboratories so that it can be widely used wherever patients are being treated. By measuring receptor occupancy of anfibatide, clinicians can precisely control dosage of the drug, thereby providing optimal therapeutic benefit, while limiting side effects.

There are three main approaches to measuring drug levels in patients: measuring plasma levels of the drug, measuring receptor binding, or functional analysis. Measuring plasma levels of drugs is not always accurate, particularly in the case of drugs that have high affinity binding to their receptor. For example, even when aspirin levels are low, antiplatelet effects are still seen as the drug is bound to platelets rather than circulating in the plasma (54). Functional analysis for anti-platelet agents generally involves measuring platelet aggregation. This involves isolating PRP from patient blood samples, and the assay may be long and difficult. Furthermore, most hospitals do not have access to platelet aggregometers, and the majority of health professionals are not trained in the technique. There is also wide variation in the results obtained from different platelet functional tests (24). Receptor binding is usually difficult to measure due to the tissue where receptors are present being inaccessible. This is not the case with anti-platelet agents, as platelets are easily accessible in the blood. The receptor occupancy method has previously been used to evaluate binding of abciximab to GPIIb/IIIa (55).

We have developed a novel GPIb receptor occupancy assay based on a GPIIb/IIIa receptor occupancy assay previously developed in this lab (55). A platelet calibrator kit, marketed by Biocytex, was used. The kit is normally used to measure platelet glycoprotein expression levels. We have optimised the assay using GPIb monoclonal antibodies. This assay is accessible to any clinician who has access to a flow cytometer. The development of this assay

is particularly important when studying the pharmacokinetics of anfibatide in Phase Ib-IIa trials. It will help clinicians assess the receptor occupancy of anfibatide, and therefore it could be used to help determine the appropriate dose in patients based on these trials.

Aims

1. To develop a quantitaive flow cytometry based receptor occupancy assay for the novel GPIb receptor antagonist anfibatide.
2. To validate this assay in healthy donor platelets *ex vivo.*
3. To use this assay to investigate the efficacy of Anfibatide in healthy donor blood *ex vivo.*
4. To investigate the effect of Anfibatide on *S. sanguinis* induced platelet aggregation *ex vivo***.**

Chapter 2: Materials and Methods

Materials

Materials used are summarised in table 2.1 below.

Table 2.1: Summary table of materials and suppliers

Anti-anfibatide antibody 1B9 Anfibatide	Lee's Pharmaceutical Limited, Hong Kong
PLATELET calibrator kit PLATELET GPIIb/IIIa Occupancy kit CD42b clone ALMA 19 CD42b clone SZ 2	Biocytex, Marseilles, France
CD62P (P-selectin), PE format	BD Biosciences, Oxford, UK
Round bottom FACS test tubes	VWR International Ltd, Dublin, Ireland
Sodium Citrate Brain Heart Infusion (BHI) Broth BHI Agar Phosphate buffered saline (PBS) All other reagents	Sigma Aldrich Ireland Limited, Co. Wicklow, Ireland
Adenosine di-phosphate (ADP) Arachidonic acid AggRecetin (ristocetin) Aggregation test tubes Micro-magnetic stir bars	Bio/data Corp, PA, USA
Multiplate test cells	Roche Diagnostics, West Sussex, UK
VM16d anti-CD42b antibody	Abcam, Cambridge, UK
Mouse anti-human monoclonal antibody, IgG2a isotype, AN51	Fischer Scientific Ireland, Dublin, Ireland
S. aureus Newman	Gift from Prof. Timothy J Foster, TCD
S. sanguinis NCTC 7863	National Collection of Type Cultures, UK
S. sanguinis 133-79	Gift from Dr. Mark Herzburg

Methods

1. *Preparation of Blood*

Whole blood was collected in 3.8% sterile sodium citrate, in the ratio 9:1, from healthy volunteers who had abstained from using anti-platelet agents in the previous 10 days. This study was approved by the RCSI Research Ethics Committee, approval number REC676. To prepare platelet rich plasma (PRP), whole blood was aliquoted and centrifuged at 150 rpm for 10 minutes. PRP was collected. To prepare platelet poor plasma (PPP), 1 ml of PRP was centrifged for a further 5 minutes. PPP was collected and the platelet pellet was discarded.

2. Quantitative Flow Cytometry

Quantitative flow cytometry was performed using a BD FACS Canto II flow cytometer (Becton Dickinson, UK). A platelet calibrator kit (Biocytex, France) was used, and the accompanying instructions were followed. Briefly, platelets were stained with specific monoclonal antibodies by no wash indirect immunofluorescence. Whole blood (10 μl) was diluted 1:10 with diluent from the kit. Diluted samples were incubated with either a negative control from the kit, or a specific chosen antibody at a final concentration of 10 μg/ml for 20 minutes at room temperature. Anti-mouse IgG-FITC (10 μl) was added for 10 minutes. This antibody was previously optimised and provided in the kit. Beads coated with an increasing and specifically known amount of mouse IgG were labelled with IgG-FITC for 10 minutes, and used to create a calibration curve. The samples were then diluted with 1 ml of diluent from the kit. The samples were analysed using quantitative flow cytometry. Platelets were gated, 10,000 events were recorded, and the FITC-A geomean was used for analysis.

3. 1B9 Concentration Optimisation

A mouse monoclonal anti-anfibatide antibody, 1B9, was developed in China (41). The optimal concentration of anti-anfibatide antibody 1B9 to be used in

the development of this assay was determined using quantitative flow cytometry. Serial dilutions of 1B9 were made using tris buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5). Whole blood was incubated with anfibatide (final concentration of 6.24 μg/ml) for 30 minutes at room temperature. Of each dilution of 1B9, 10 μl was incubated at room temperature for 20 minutes with 10 μl of anfibatide-treated whole blood. Mouse anti-IgG FITC (10 μl) was added for 10 minutes at room temperature. The sample was diluted using 1 ml diluent from the platelet calibrator kit (Biocytex, France). The sample was analysed using quantitative flow cytometry on a FACS machine as described above.

4. Flow Cytometry based Dose Response of Anfibatide in Whole Blood

A platelet calibrator kit (Biocytex, France) was used to determine the optimal concentration of anfibatide that causes maximum binding to platelets. Platelets in whole blood were stained by no wash indirect immunofluorescence with the optimal dose of 1B9, as determined previously in section 3. Whole blood was incubated with varying doses of anfibatide at room temperature for 30 minutes. Each sample was diluted 1:10 with diluent from the kit. Diluted whole blood (10 μl) was labelled with 10 μl of 1B9 (final concentration: 3.68 μg/ml) for 20 minutes at room temperature. Mouse anti-IgG FITC was added (10 μl) for 10 minutes at room temperature. Sample was then diluted using 1 ml diluent from the kit, and analysed as described in section 2.

5. Effect of Anfibatide on Various Anti-GPIb Monoclonal Antibodies

Anfibatide treated whole blood was incubated with various anti-GPIb monoclonal antibodies (AN51, SZ 2, VM16d, ALMA 19, and WM23) at a final concentration of 10 μg/ml, and protocol was carried out as described in section 2.

6. Effect of Anfibatide on Anti-GPIIb/IIIa Monoclonal Antibodies

A platelet GPIIb/IIIa occupancy kit (Biocytex, France) was used to determine the effect of anfibatide on GPIIb/IIIa. Whole blood was incubated with anfibatide (final concentration: 10 μg/ml) for 30 minutes at room temperature. Anfibatide-treated, and control whole blood was incubated with MAb1 and MAb2, two anti-GPIIb/IIIa antibodies provided in the kit. Protocol as described in section 2.

7. Platelet Activation by Anfibatide

Whole blood was incubated varying concentrations of anfibatide at room temperature for 30 minutes. This whole blood (10 μl) was added to 10 μl CD62-PE for 10 minutes. Whole blood incubated with 20 μM thrombin receptor activating peptide (TRAP) acted as a positive control. Each sample was diluted with 1 ml PBS, and analysed using a FACS machine. PE geomean was recorded and used for analysis.

8. Loss of Single Platelets in PRP

PRP was prepared as described in section 1. PRP was incubated with various concentrations of anfibatide for 30 minutes at room temperature. Platelet count was measured using a Sysmex haematology analyser (Kobe, Japan).

9. Effect of Anfibatide on Whole Blood Aggregation and PRP Aggregation Induced by S. aureus *Newman and* S. sanguinis *133-79*

S. aureus Newman, and *S. sanguinis* 133-79 bacterial strains were grown overnight in brain heart infusion (BHI) broth at 37°C . Innoculated broth was centrifuged at 5,000 rpm for 10 minutes. Broth was removed and the bacterial pellet was resuspended in 50 ml sterile PBS. The bacterial solution was centrifuged at the same settings again. PBS was removed and the final pellet was resuspended in 1 ml PBS. Bacteria was adjust to an optical density (OD_{600}) of 1.6 using an Ultraspec III spectrophotometer (Pharmacia Biotech, UK).

For whole blood aggregometry, a Multiplate analyser (Roche Diagnostics, UK) was used. The Multiplate test cell consists of two sensors onto which platelets can adhere and aggregate when activated by an appropriate agonist. This adhesion and aggregation causes an increase in impedance, which is then recorded in arbitrary aggregation units and plotted against time on the analyser. The area under the aggregation curve (AUC) is the commonly used parameter. This takes the total height of the aggregation curve, and the slope of the curve, into account. Whole blood was incubated with 32 μg/ml Anfibatide at room temperature for 30 minutes. Anfibatide-treated whole blood (300 μl) was diluted with 300 μl PBS and allowed to rest at 37°C for 5 minutes in a Multiplate test cell. Bacteria (1:10) was added to the test cell and aggregation was recorded for 20 minutes. All aggregations took place in the Multiplate analyser (Roche Diagnostics, UK), at 37°C, and samples were mixed using disposable magnetic stir bars. Anti-GPIb antibody AN51, at a final concentration of 12.5 μg/ml, was incubated with whole blood for 10 minutes, prior to aggregation.

For PRP aggregation, PRP and PPP were both isolated as outlined in section 1. A PAP-8 platelet aggregometer (Biodata, USA) was used to carry out light transmission aggregometry. PRP was incubated with anfibatide for 30 minutes at room temperature. PPP (250 μl) was used to blank the machine. Bacteria was then added to PRP (1:10 dilution) and aggregation was measured for 30 minutes. Magnetic stir bars were used to mix the samples druing aggregation, and all aggregations took place at 37°C.

10. The Effect of Anfibatide on Ristocetin Induced Whole Blood Aggregation

A dose response to ristocetin in a healthy volunteer using a Multiplate analyser found that 0.5 mg/ml ristocetin was the minimum dose of ristocetin to induce aggregation in whole blood. Following this, whole blood was incubated with various doses of anfibatide for 30 minutes at room temperature. Aggregations were carried out as described in section 8, inducing aggregation by adding ristocetin at a final concentration of 0.5 mg/ml.

11. Effect of Anfibatide on Aggregation in PRP Induced by Various Agonists

PRP was incubated with anfibatide at a final concentration of 10 μg/ml for 30 minutes at room temperature. Various agonists were added to PRP and aggregation was recorded using light transmission aggregometry (LTA) with a PAP-8 platelet aggregometer for 10 minutes. Agonists included low dose thrombin (0.05 U/ml), high dose thrombin (0.5 U/ml), ADP, arachidonic acid and ristocetin (1 mg/ml).

12. Statistical Analysis

Results were analysed using ANOVA and Student's T-test, paired and unpaired. Data was considered statistically significant when $p<0.05$.

Chapter 3: Results

3.1. 1B9 optimisation

In order to develop an assay to measure the occupancy of the GPIb receptor on platelets by the novel drug anfibatide, an antibody directed against anfibatide was developed in China and named 1B9. The optimal concentration of 1B9 to be used, that is the concentration of 1B9 that maximally detects anfibatide binding, was determined using quantitative flow cytometry. A high dose of 10 μg/ml anfibatide was chosen from previous literature (26). Whole blood was labelled with various concentrations of 1B9, and the optimal dose was determined to be 3.68 μg/ml. At this concentration, 54,784 ± 7,592 molecules of anfibatide were detected. As 1B9 concentration increased past this point, binding to anfibatide decreased (Figure 3.1.1).

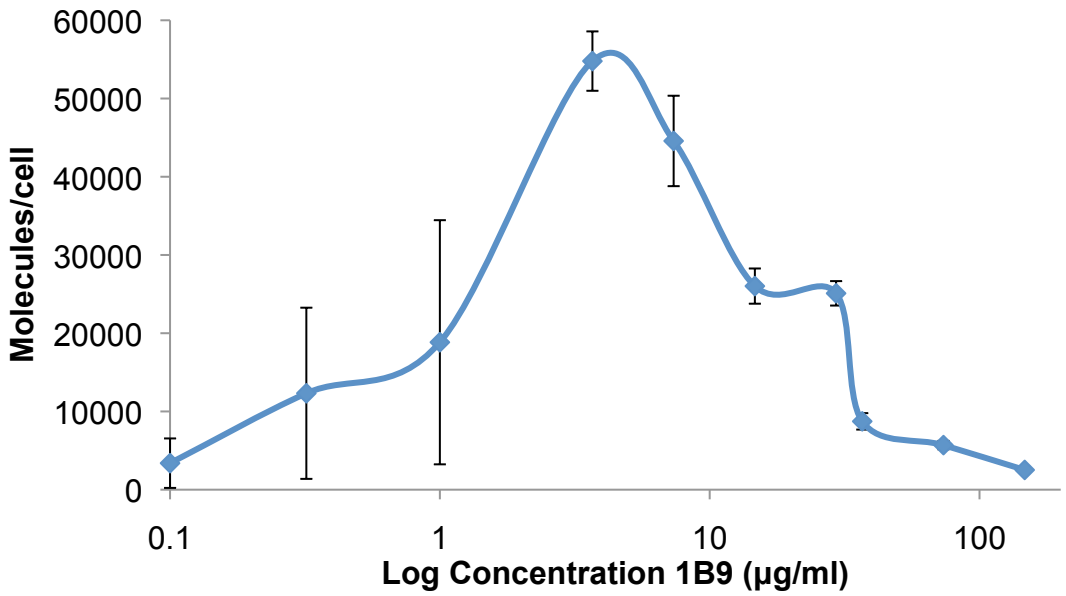

Figure 3.1.1. The optimum concentration of anti-anfibatide antibody 1B9 that detects 10 μg/ml anfibatide, in whole blood, using quantitative flow cytometry, is 3.68 μg/ml. Number of anfibatide molecules bound to platelets as measured by various doses of anti-anfibatide antibody 1B9 in whole blood samples from healthy donors using quantitative flow cytometry, n=3-4, error bars represent SEM.

3.2. Anfibatide dose response

Following the determination of the optimal dose of 1B9 (3.68 µg/ml) to be used in this assay, binding of anfibatide to healthy donor platelets was measured. Various doses of anfibatide were incubated with whole blood for 30 minutes at room temperature. In this dose response, maximal binding was detected at 16 µg/ml, showing 69,181 ± 6,242 binding sites per platelet (Figure 3.2.1).

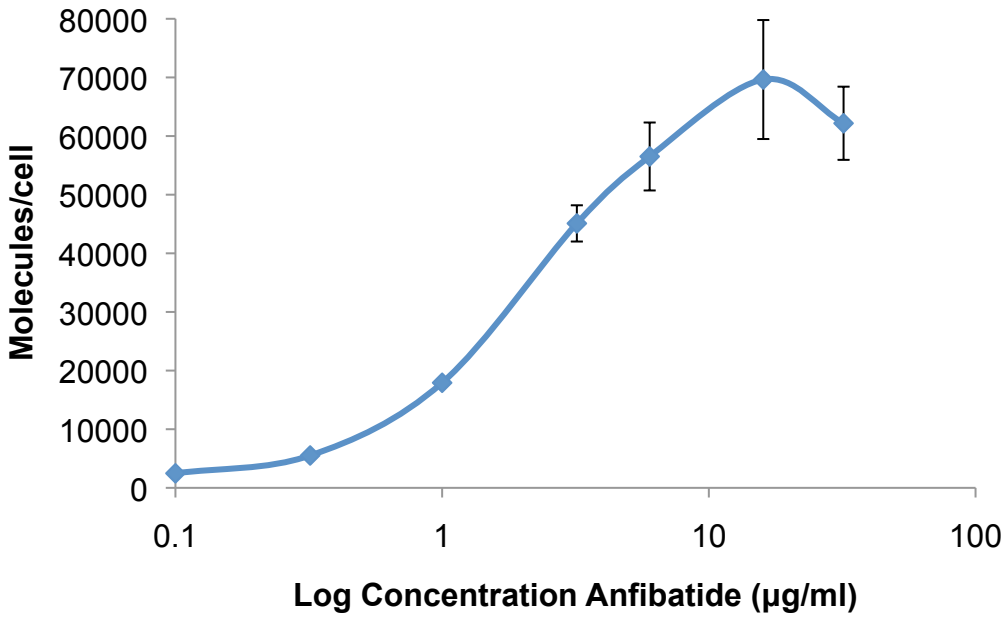

Figure 3.2.1. Anfibatide dose response, using quantitative flow cytometry, with whole blood and 3.68 µg/ml anti-anfibatide antibody 1B9. Number of anfibatide molecules bound to platelets varying with dose, as measured using quantitative flow cytometry, with anti-anfibatide antibody 1B9 (3.68 µg/ml) in whole blood samples from healthy donors, n=3, error bars represent SEM.

The results from figure 3.1.1 suggested that the optimal concentration for 1B9 use was 3.68 µg/ml, after which detection decreased. In order to ensure that this was the optimum dose of 1B9, 3.68 µg/ml and a higher dose of 7.35 µg/ml were tested against a range of Anfibatide concentrations. At 3.68 µg/ml, 1B9 detects significantly more binding sites at high doses of anfibatide compared to 7.35 µg/ml 1B9, p=0.016 (Figure 3.2.2).

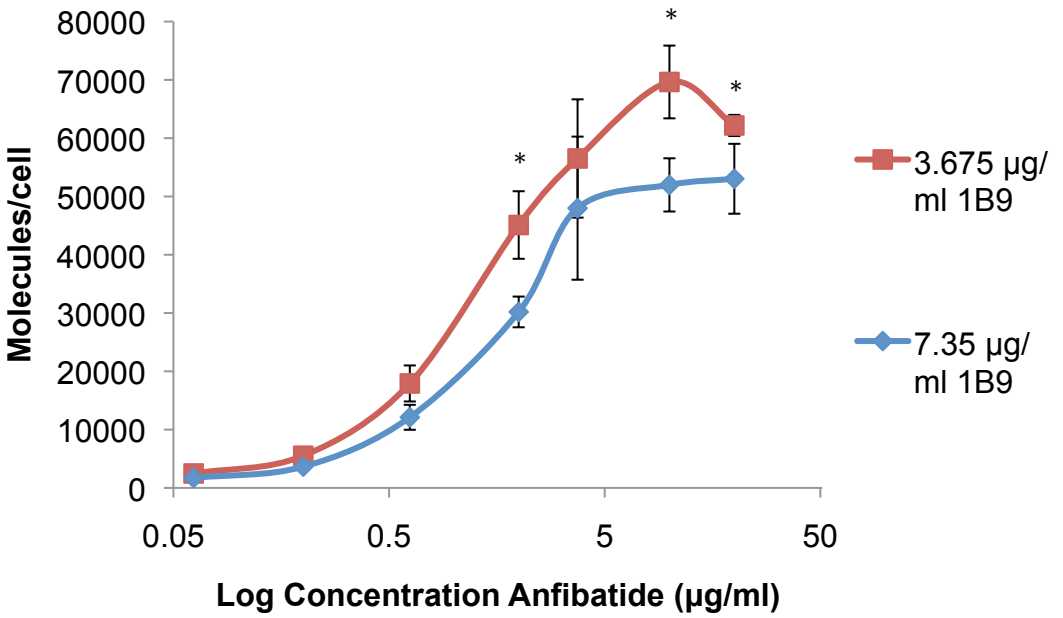

Figure 3.2.2. 3.68 µg/ml 1B9 detects more binding sites to anfibatide than 7.35 µg/ml 1B9, as measured using flow cytometry. Number of anfibatide molecules bound to platelets varying with dose, using quantitative flow cytometry, comparing 3.68 µg/ml 1B9 and 7.35 µg/ml 1B9 in whole blood samples form healthy donors, n=3. There is a significant decrease in receptor numbers detected by 1B9 at 7.35 µg/ml compared to 3.68 µg/ml, p=0.016, paired t-test, error bars represent SEM.

3.3. Effect of anfibatide on anti-GPIb monoclonal antibodies

An anti-GPIb antibody that is not inhibited by anfibatide is required to measure total GPIb receptor numbers per platelet. The effect of anfibatide on binding of five anti-GPIb antibodies to platelets was measured using quantitative flow cytometry. These monoclonal antibodies were also used to determine the binding site of the drug. All antibodies were used at a final concentration of 10 µg/ml. Each anti-GPIb antibody showed a different number of binding sites per platelet (Figure 3.3.1). AN51 had the lowest number of binding sites (14,325 ± 1,949 binding sites), ALMA 19, SZ 2, and WM23 detected between 27,000 and 33,000 receptors, and VM16d had 40,880 ± 8,931 binding sites. There was a significant difference between GPIb receptor numbers as measured by AN51 compared to WM23, SZ 2, and ALMA 19 ($p<0.05$).

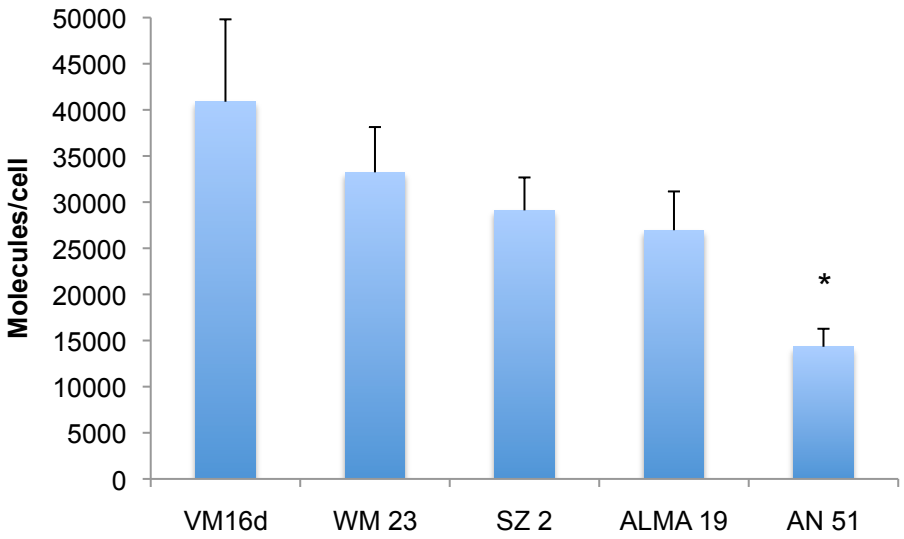

Figure 3.3.1. GPIb receptor numbers vary depending on the anti-GPIb monoclonal antibodies used in quantitative flow cytometry. Whole blood was labelled with various anti-GPIb monoclonal antibodies and receptor numbers were measured using quantitative flow cytometry. All antibodies were used at a final concentration of 10 µg/ml. There was a significant difference in receptor numbers as measured by AN51 compared to WM23, SZ 2, and ALMA 19, $p<0.05$, multiple paired t-tests, error bars represent SEM.

Binding of WM23, AN51, and ALMA 19 antibodies was not significantly affected by anfibatide (p>0.7). Binding of VM16d was inhibited by 52 ± 2% by 10 µg/ml anfibatide pre-incubated with whole blood for 30 minutes at room temperature, p=0.036. SZ 2 was inhibited 23 ± 1% by 10 µg/ml Anfibatide, p=0.012 (Figure 3.3.2).

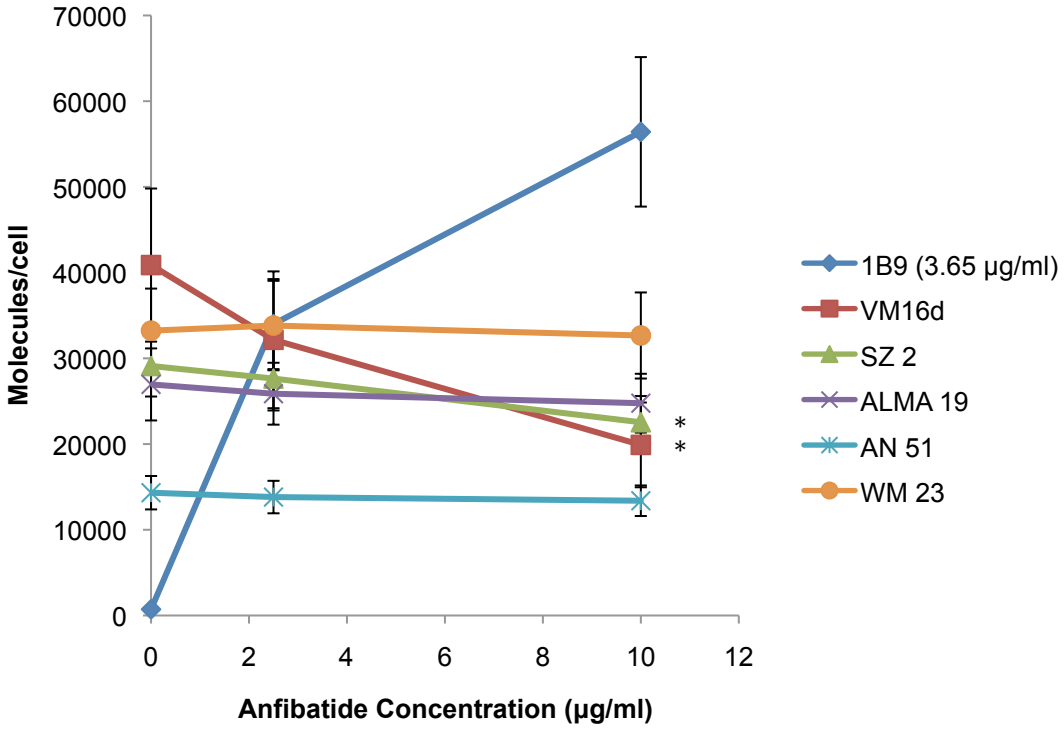

Figure 3.3.2. Anfibatide partially inhibits VM16d and SZ 2 at 10 µg/ml. Whole blood was incubated with various doses of anfibatide for 30 minutes. Binding of five anti-GPIb antibodies (VM16d, SZ 2, ALMA 19, AN51, and WM23) to healthy donor platelets was measured using quantitative flow cytometry. Binding of two antibodies was significantly inhibited by anfibatide compared to the control group which was not incubated with anfibatide: VM16d, p=0.036, and SZ 2, p=0.012, one-way ANOVA, error bars represent SEM, n=3.

The effect of anfibatide on high dose VM16d (20 µg/ml) was also tested (Figure 3.3.3). Anfibatide did not significantly inhibit VM16d at 20 µg/ml binding in the presence of 10 µg/ml anfibatide (p>0.05). VM16d at 10 ug/ml was partially inhibited by 10 µg/ml anfibatide (p=0.0123).

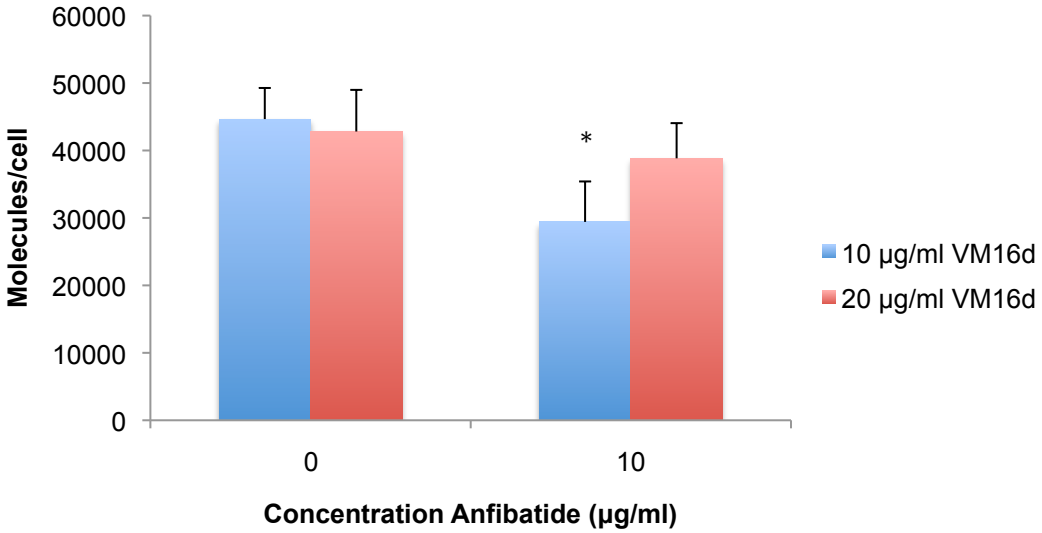

Figure 3.3.3. High dose VM16d (20 µg/ml) is not inhibited by anfibatide. Anfibatide was incubated with whole blood for 30 minutes at room temperature. Binding to 20 µg/ml and 10 µg/ml VM16d was measured using quantitative flow cytometry and compared to the control blood samples, which had not been incubated with anfibatide. No significant inhibition of binding was detected with 20 µg/ml VM16d, p>0.05. VM16d at 10 µg/ml was significantly inhibited by 10 µg/ml anfibatide, p=0.0123, paired t-test, n=3-6, error bars represent SEM.

GPIb receptor numbers were measured using VM16d at 20 µg/ml, and 1B9 at 7.35 µg/ml directed against two doses of anfibatide. Both antibodies gave similar receptor numbers (40,000 – 50,000 binding sites) and were not inhibited by increasing anfibatide dosage (p>0.3).

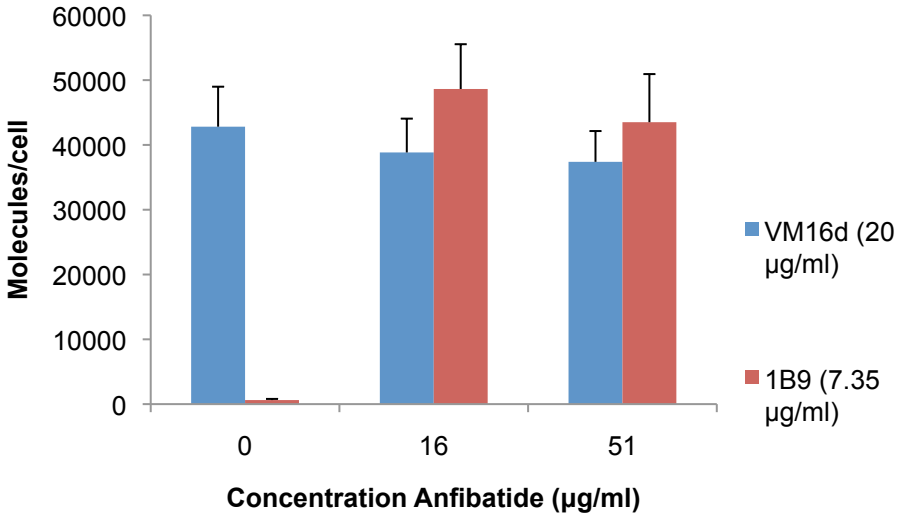

Figure 3.3.4. Number of GPIb receptors detected, by quantitative flow cytometry, on platelets by VM16d and 1B9 are not statistically different following incubation of whole blood with anfibatide. Whole blood was incubated with two doses of anfibatide for 30 minutes at room temperature. GPIb receptor binding was detected using quantitative flow cytometry. VM16d (20 µg/ml) and 1B9 (7.35 µg/ml) were used. Control samples were not incubated with anfibatide. There was no significant difference between receptor numbers when incubated with 16 µg/ml, or 51 µg/ml anfibatide, as detected by VM16d, and 1B9, p>0.3, two-way ANOVA and multiple t-tests, n=3, error bars represent SEM.

3.4. Effect of anfibatide on anti-GPIIb/IIIa monoclonal antibodies

The effect of anfibatide on binding of monoclonal antibodies directed against the GPIIb/IIIa receptor was examined to confirm that GPIIb/IIIa was not the binding site of anfibatide. Anfibatide at a final concentration of 10 µg/ml did not inhibit binding of two monoclonal antibodies, MAb1 and MAb2, as measured by quantitative flow cytometry ($p>0.999$). Both antibodies were from the GPIIb/IIIa receptor occupancy kit (Biocytex, France).

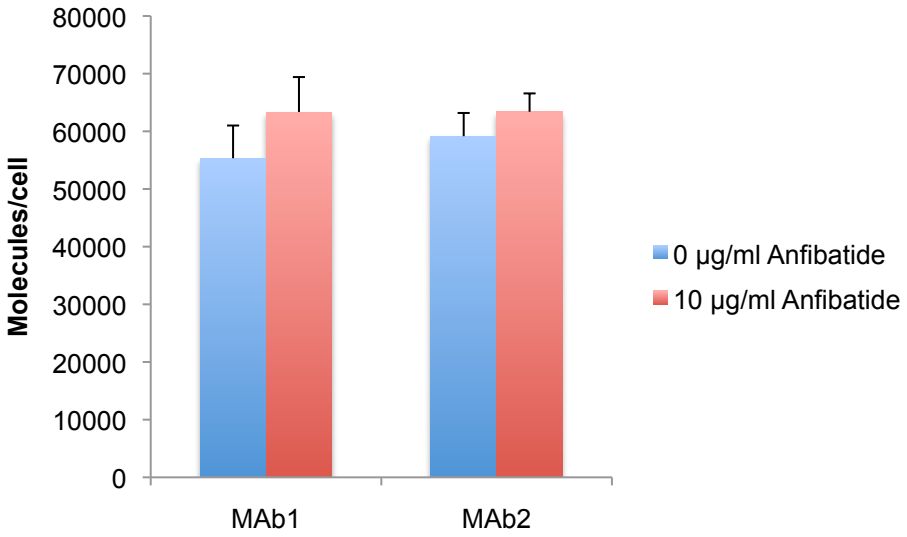

Figure 3.4.1. Anti-GPIIb/IIIa monoclonal antibodies, MAb1 and MAb2 (Biocytex, France), are not affected by anfibatide. Anfibatide does not significantly affect binding of the anti-GPIIb/IIIa monoclonal antibodies, MAb1 and MAb2 in whole blood samples using flow cytometry, compared to control samples which were not incubated with anfibatide, n=3, p=>0.999, paired t-test, error bars represent SEM.

3.5. Effect of anfibatide on platelet activation

The effect of anfibatide on platelet activation was measured using quantitative flow cytometry. Platelet activation was quantified by measuring CD62 (P-selectin) levels on the platelet surface. CD62 is released from α-granules in platelets and displayed on the cell surface following activation, for example by TRAP. TRAP activated platelets were used as a positive control to measure CD62 expression. Anfibatide at 6 µg/ml, and 25.5 µg/ml, did not cause platelet activation after incubation for 30 minutes at room temperature.

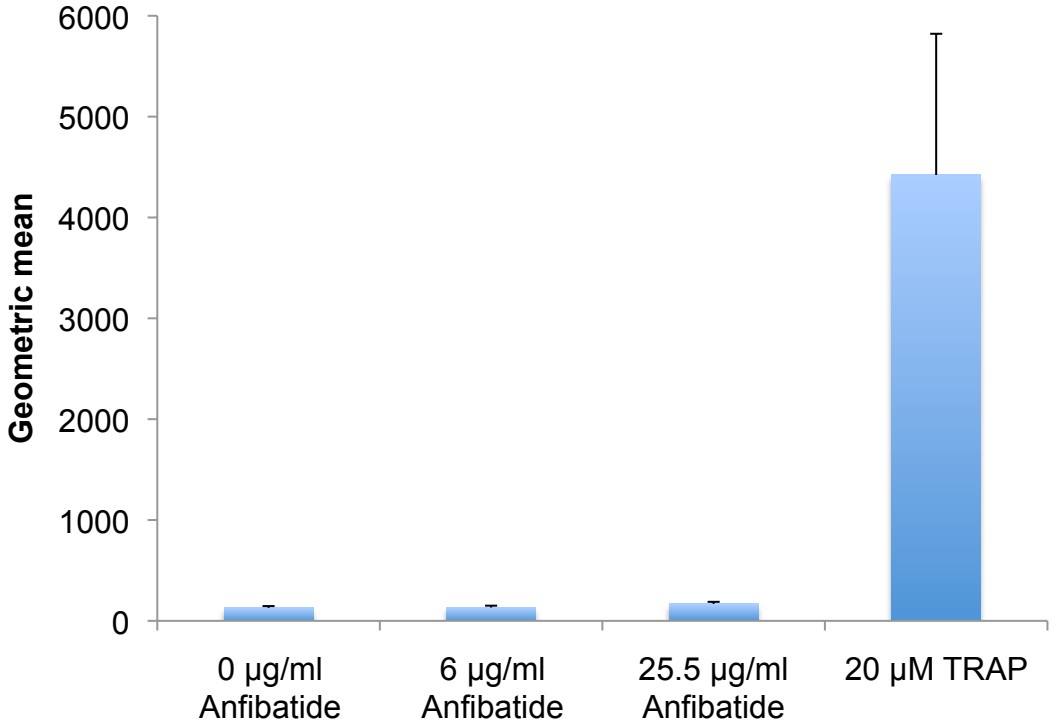

Figure 3.5.1. Anfibatide does not activate platelets (CD62P) as measured by flow cytometry in whole blood. Measurement of platelet activation by anfibatide as measured by CD62 levels using flow cytometry. Anfibatide did not cause platelet activation compared to control sample incubated with 20 µM TRAP in whole blood samples from healthy donors, n=3, error bars represent SEM.

3.6. Loss of single platelets following anfibatide treatment

The effect of Anfibatide on platelets was examined using a loss of single platelets study. Following incubation with anfibatide for 30 minutes at room temperature, platelet count was measured using a Sysmex haematology analyser. Recorded platelet numbers did not significantly differ following anfibatide treatment (p=0.27) indicating that anfibatide does not cause a loss of single platelets *in vitro* (figure 3.6.1).

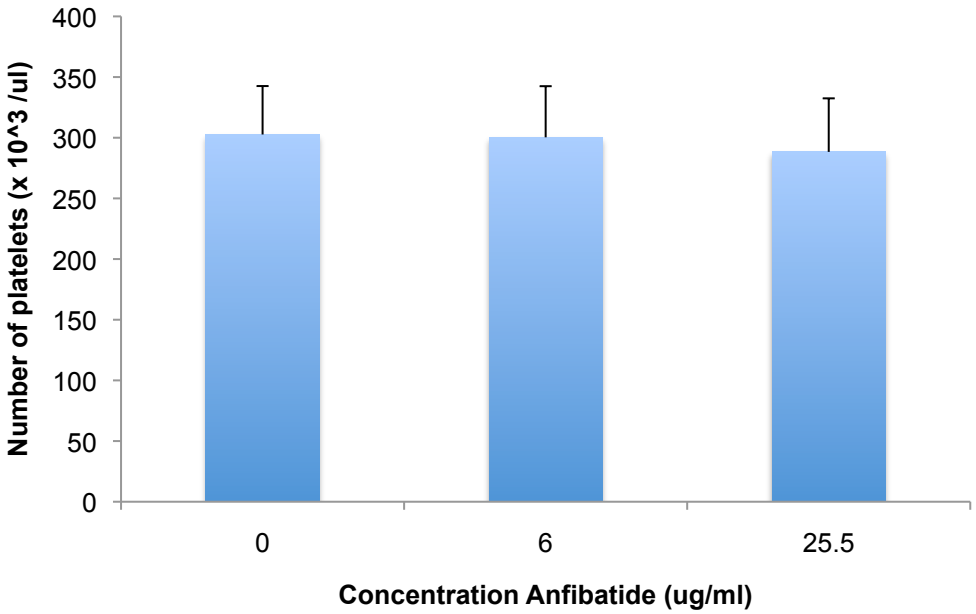

Figure 3.6.1. Anfibatide does not cause a loss of single platelets. Various concentrations of anfibatide do not cause a decrease in the average number of platelets in PRP samples incubated with anfibatide at room temperature for 30 minutes, compared to a sample that was not incubated with anfibatide, p=0.27, ANOVA, n=3, error bars represent SEM.

3.7. The effect of anfibatide on ristocetin-induced platelet aggregation in whole blood

Ristocetin was used to induce platelet aggregation in whole blood, and the effect of anfibatide on this aggregation was examined. Anfibatide inhibited ristocetin-induced aggregation in whole blood in a dose dependent manner (p=0.043). On average, a dose of 10 µg/ml anfibatide inhibited aggregation by 77.5% (figure 3.7.1). IC_{50} is 2.39, 95% CI 1.08 – 5.31, R^2=0.84.

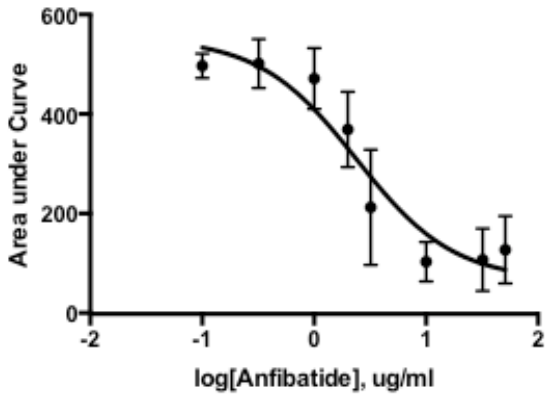

Figure 3.7.1. Anfibatide inhibits whole blood aggregation induced by 0.5 mg/ml ristocetin in a dose dependent manner. Anfibatide inhibited whole blood aggregation induced by 0.5 mg/ml ristocetin in a dose dependent manner, compared to aggregation occuring in the absence of anfibtide, as measured using a Multiplate analyser, n=3, p=0.043, ANOVA, error bars represent SEM.

3.8. The effect of anfibatide on ristocetin-induced platelet aggregation in PRP

The effect of anfibatide on platelet aggregation induced by 1 mg/ml ristocetin in PRP was measured using a PAP-8 aggregometer. Anfibatide significantly inhibited platelet aggregation in a dose dependent manner (p=0.019). Anfibatide at a final concentration of 5 µg/ml inhibited aggregation by 94 ± 4% (figure 3.8.1).

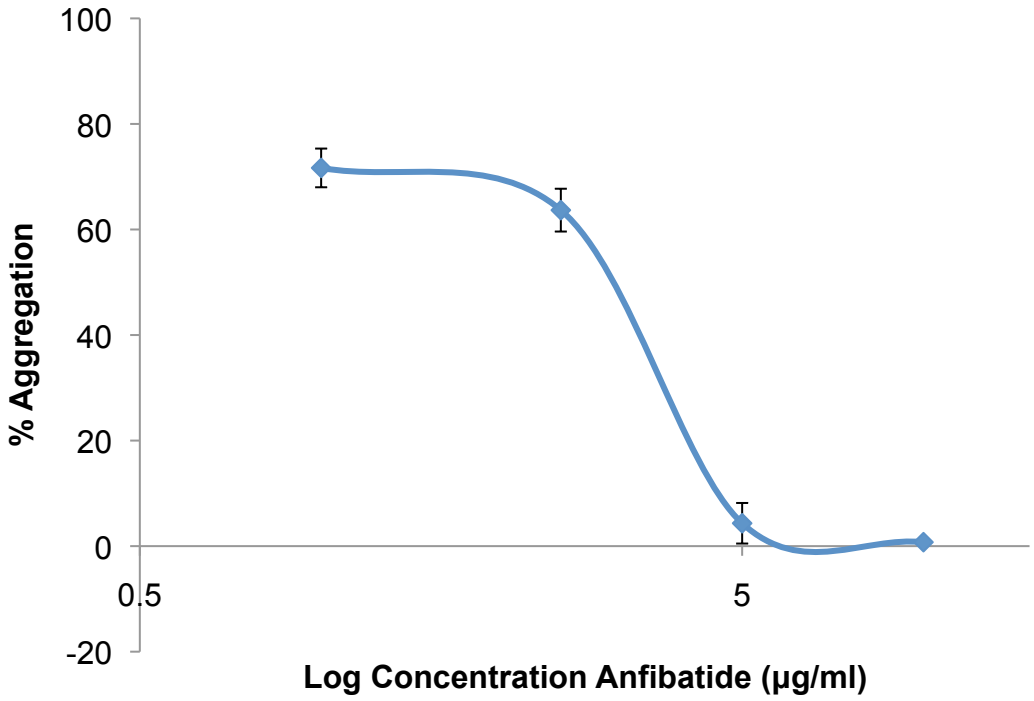

Figure 3.8.1. Anfibatide inhibits ristocetin induced aggregation in PRP in a dose dependent manner. Anfibatide inhibited aggregation induced by 1 mg/ml ristocetin in PRP as measured by light transmission aggregometry. The inhibition was dose dependent, p=0.092, ANOVA, n=3-4, error bars represent SEM.

3.9. GPIb receptor occupancy compared to inhibition of ristocetin-induced aggregation in whole blood by anfibatide

As shown in figure 3.3.4, 20 µg/ml VM16d detects a similar number of binding sites to high dose anfibatide. VM16d was used to measure total GPIb receptor numbers. A flow cytometry based dose response to anfibatide was carried out using 7.35 µg/ml 1B9. Each dose was compared to the total receptor number as measured by VM16d and receptor occupancy was measured as a percentage. On the same day, using the same healthy donor whole blood samples, a dose response of ristocetin-induced platelet agregation to anfibatide was carried out. The inhibition of aggregation was expressed as a percentage and plotted with receptor occupancy as measured by VM16d (figure 3.9.1). Inhibition matched of aggregation closely matched GPIb receptor occupancy by anfibatide in each donor. IC_{50} for receptor occupancy is 5.295, and 95% CI is 1.96 – 14.29. IC_{50} for inhibition of aggregation is 4.33. 95% CI is 1.43 – 13.14. R^2 = 0.97, 0.95 respectively.

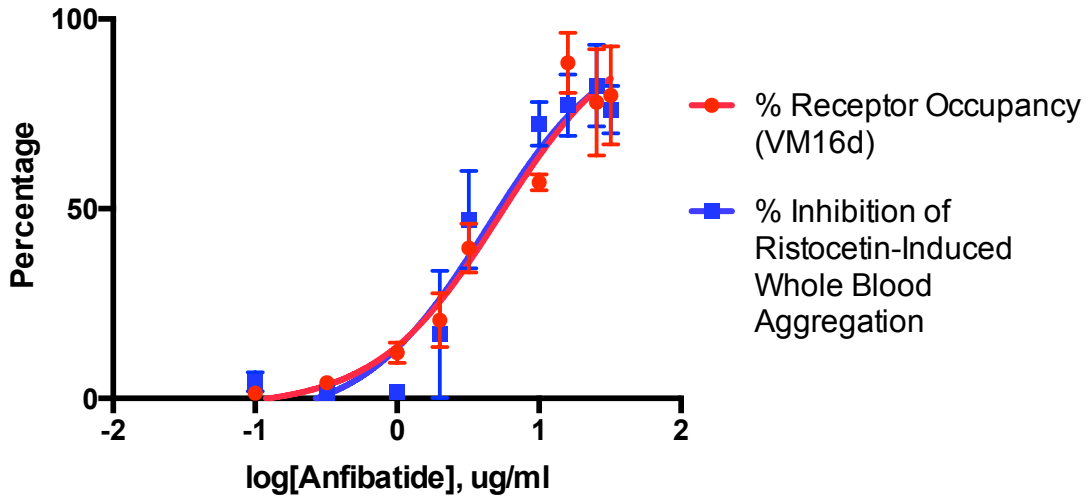

Figure 3.9.1. Receptor occupancy by anfibatide correlates with inhibition of whole blood aggregation by anfibatide. Total GPIb receptor number is measured using 20 µg/ml VM16d, and GPIb receptor occupancy by anfibatide is measured with 7.35 µg/ml anti-anfibatide antibody 1B9, using quantitative flow cytometry. This is compared to the inhibition of aggregation induced by 0.5 mg/ml ristocetin in whole blood samples incubated with varying doses of anfibatide for 30 minutes at room temperature, n=3, error bars represent SEM.

Receptor occupancy was also measured using a lower doses of VM16d (10 µg/ml), and 1B9 (3.675 µg/ml), and compared to inhibition of aggregation by anfibatide at 0.02 µg/ml and 10 µg/ml (figure 3.9.2). Receptor occupancy and inhibition of aggregation also matched at these concentrations.

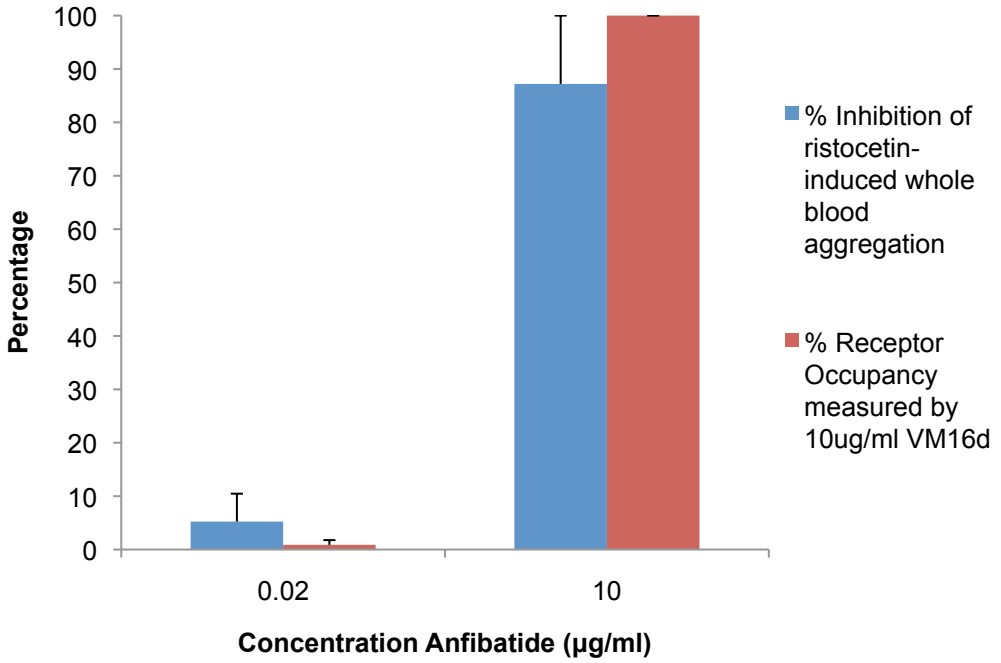

Figure 3.9.2. GPIb receptor occupancy by anfibatide correlated with inhibition of ristocetin-induced whole blood aggregation by anfibatide. Total GPIb receptor number is measured using 10 µg/ml VM16d, and GPIb receptor occupancy by anfibatide is measured using 3.675 µg/ml anti-anfibatide antibody 1B9. This is compared to the inhibition of aggregation induced by 0.5 mg/ml ristocetin in whole blood samples incubated with varying doses of anfibatide for 30 minutes at room temperature, n=3, error bars represent SEM.

3.10. The effect of Anfibatide on aggregation induced by various agonists

The effect of high dose anfibatide (10 µg/ml) on platelet aggregation induced by various agonists in PRP was tested using a PAP-8 platelet aggregometer. Anfibatide did not significantly affect aggregation induced by high dose thrombin, low dose thrombin, ADP or arachidonic acd. Anfibatide fully inhibited aggregation induced by 1 mg/ml ristocetin (p=0.0002).

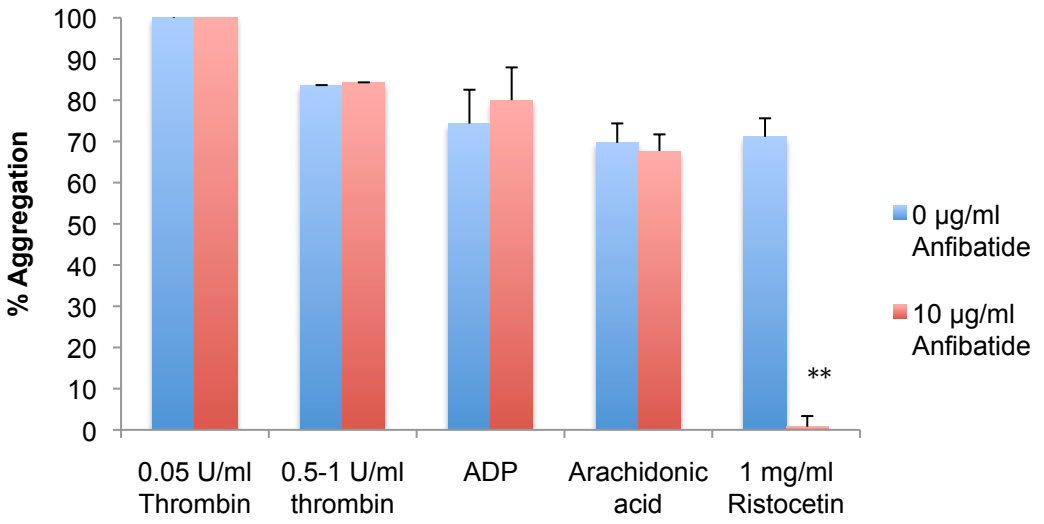

Figure 3.10.1. 10 µg/ml anfibatide does not effect aggregation induced by high dose or low dose thrombin, ADP or arachidonic acid in PRP, but does inhibit ristocetin induced aggregation, as measured by light transmission aggregometry. Anfibatide did not significantly affect aggregation induced in PRP by low dose thrombin (0.05 U/ml), high dose thrombin (0.5 U/ml), ADP, or arachidonic acid, compared to control samples that were not incubated with anfibatide. Anfibatide completely inhibits ristocetin-induced aggregation in PRP, p=0.0002, multiple paired t-tests, n=3-5, error bars represent SEM.

3.11. The effect of anfibatide on bacteria-induced platelet aggregation in whole blood

Two bacteria strains were used to induce platelet aggregation in whole blood samples from healthy donors: *Staphylococcus aureus* Newman, and *Streptococcus sanguinis* 133-79. *S. aureus* Newman causes platelet aggregation by interacting with the FcγRIIa receptor and GPIIb/IIIa. Since anfibatide does not interact with GPIIb-IIIa as measured by a monoclonal antibody study (figure 3.4.1), it is expected to have no effect on *S. aureus* Newman induced platelet aggregation. As hypothesised, high dose anfibatide (32 µg/ml) had no significant effect on *S. aureus* induced aggregation in whole blood, n=3, analysed using a Multiplate analyser (p=0.104).

S. sanguinis 133-79 induces platelet aggregation through the GPIb receptor. It was expected that the GPIb antagonist anfibatide would inhibit aggregation induced by this bacterial strain. Whole blood from six healthy donors was tested. In three of these donors, anfibatide at 3.2 µg/ml, 10 µg/ml, and 32 µg/ml had no significant effect on platelet aggregation (p=0.156). In the remaining three donors, anfibatide partially inhibited whole blood aggregation in a dose dependent manner (figure 3.11.1). Anfibatide at a final concentration of 32 µg/ml significantly inhibited aggregation by 49 ± 17% ($p<0.05$). AN51, an anti-GPIb antibody, partially and significantly inhibited aggregation by 53 ± 17% (p=0.04), indicating that *S. sanguinis* 133-79 does cause aggregation through the GPIb receptor, at least in part (figure 3.11.2).

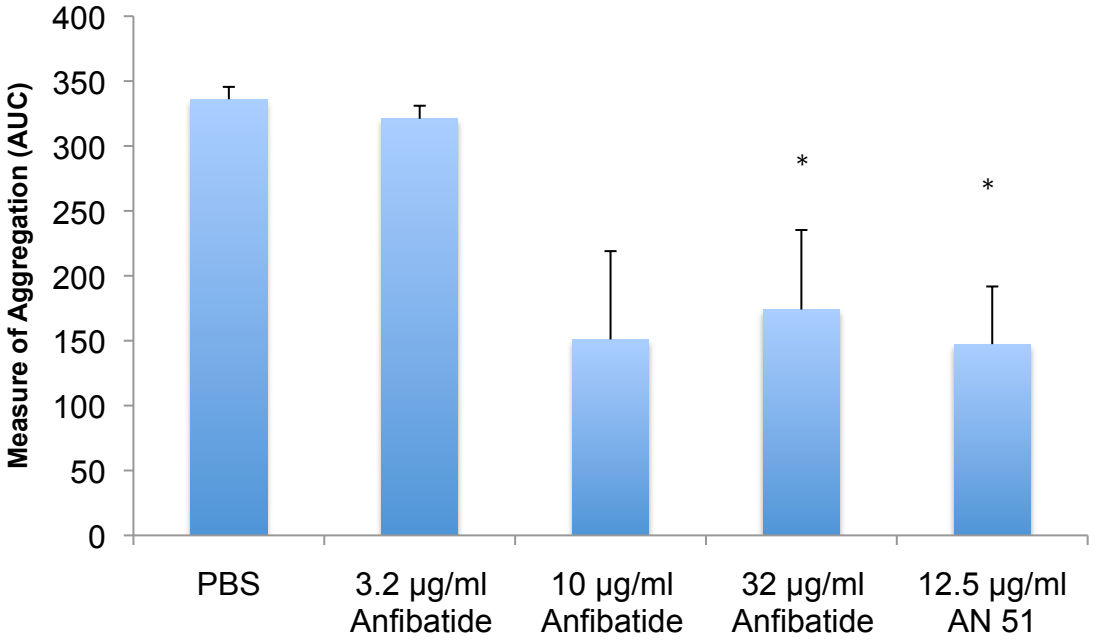

Figure 3.11.1. Anfibatide inhibits *S. sanguinis* 133-79 induced aggregation in whole blood samples from half of healthy volunteers by 50%. In 3 of 6 healthy volunteers, 32 µg/ml anfibatide inhibited whole blood aggregation by 49 ± 17%, $p<0.05$, compared to control samples which were incubated with PBS. In these three donors, the anti-GPIb antibody AN51 significantly inhibited platelet aggregation by 53 ± 17%, $p<0.05$, ANOVA, n=3, error bars represent SEM.

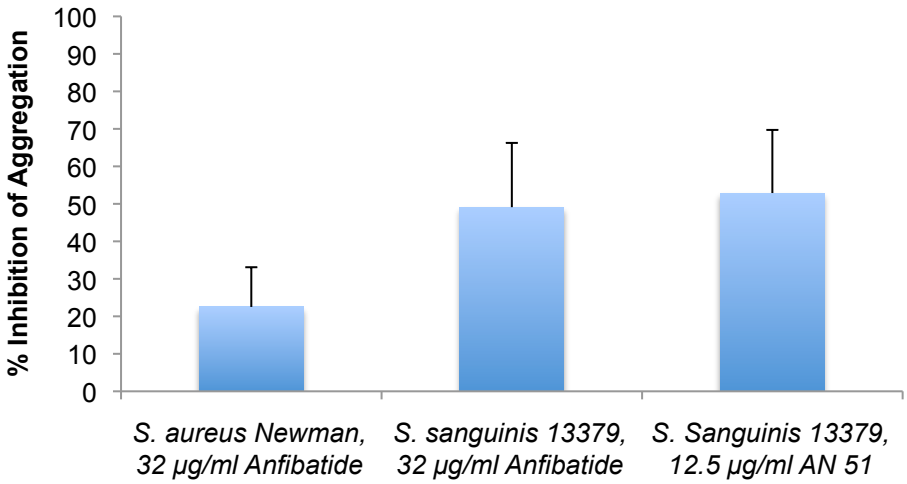

Figure 3.11.2. *S. sanguinis* 133-79 is inhibited by AN51 and high dose anfibatide, *S. aureus* Newman induced platelet aggregation is not inhibited by anfibatide in whole blood. High dose anfibatide (32 µg/ml) and the anti-GPIb antibody AN51 (12.5 µg/ml) both inhibit *S. sanguinis* 133-79 induced platelet aggregation in whole blood to the same extent. *S. aureus* Newman induced platelet aggregation is not significantly inhibited, compared to control samples incubated with PBS instead of anfibatide, $p=0.104$, ANOVA, n=3, error bars represent SEM.

3.12. The effect of anfibatide on bacterial induced aggregations in PRP

The effect of anfibatide on bacterial induced platelet aggregation in PRP was measured using two strains of *S. sanguinis*: *S. sanguinis* NCTC 7863, and *S. sanguinis* 133-79, both of which act through the GPIb receptor to cause platelet aggregation. Anfibatide at a final concentration of 10 µg/ml had no significant effect on platelet aggregation in PRP induced by either strain (figure 3.12.1).

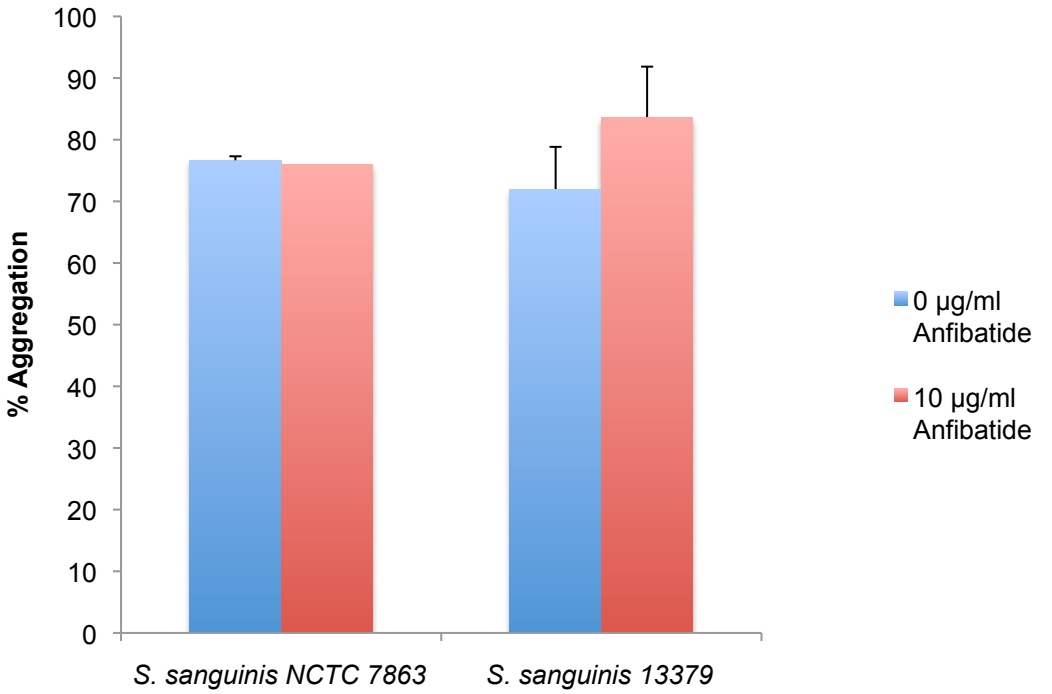

Figure 3.12.1. High dose anfibatide did not effect *S. sanguinis* induced aggregations in PRP, as measure by LTA. High dose anfibatide (10 µg/ml) did not significantly effect aggregation induced by *S. sanguinis* 133-79 (p=0.144) or *S. sanguinis* NCTC 7863 (p=0.802), compared to control samples which were not incubated with anfibatide, as measured by light transmission aggregometry, paired t-test, n=3-4, error bars represent SEM.

The lag time for *S. sanguinis* NCTC 7863 was 8.4 ± 0.8 minutes, and was not significantly affected by anfibatide treatment (p=0.356). The lag time for *S. sanguinis* 133-79 was 3.2 ± 0.6 minutes, and was not significantly altered by anfibatide (p=0.547) (figure 3.12.2).

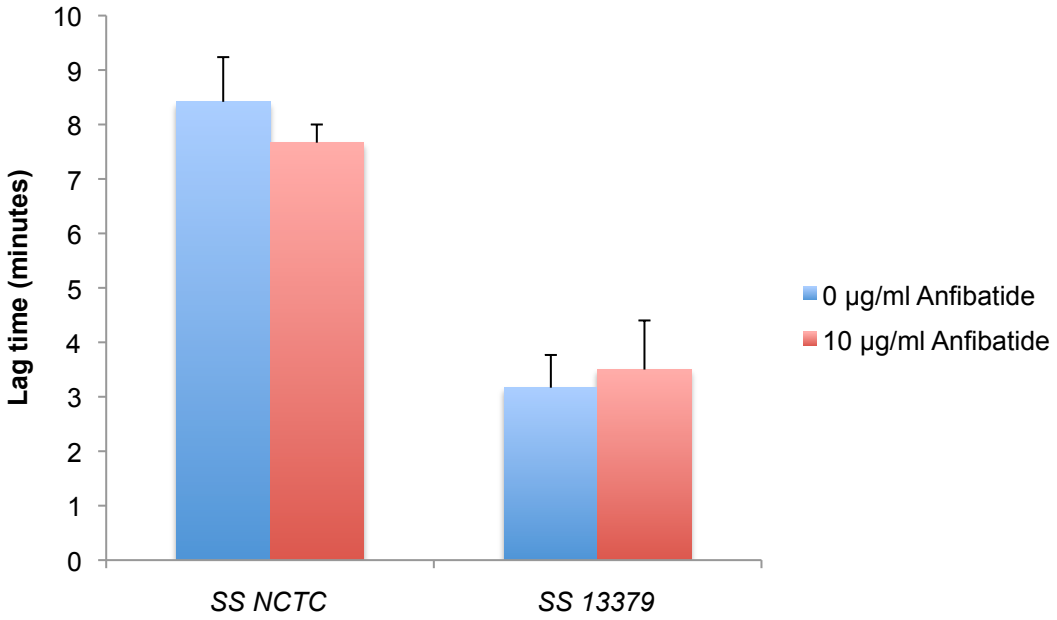

Figure 3.12.2. Anfibatide did not significantly affect lag time in bacterial induced aggregation in PRP. High dose anfibatide (10 µg/ml) did not significantly affect the lag time of *S. sanguinis* NCTC 7863 (p=0.356), or *S. sanguinis* 133-79 (p=0.547), compared to control samples which were not incubated with anfibatide, as measured by light transmission aggregometry, paired t-tests, n=3-4, error bars represent SEM.

Chapter 4: Discussion

Anfibatide is a novel anti-GPIb antagonist that is entering Phase Ib-IIa trials in early 2015. This trial will investigate the pharmacokinetics of the compound so that an effective dose can be chosen with which to treat patients. For the compound to be used effectively in patients with non-ST segment myocardial infarction, or possibly in the future in patients suffering from other thrombotic disorders, the extent of drug binding needs to be established. This is particularly important when targeting the GPIb receptor as not only do platelet numbers vary between patients, but receptor numbers per platelet also vary.

In a clinical setting, it is necessary to monitor the levels of many pharmaceutical agents in patients. For example, in warfarin treatment, drug levels and activity in patients must be constantly monitored in order to control bleeding risk and optimise drug efficacy. Warfarin is a commonly used anticoagulant that can prevent thrombosis and thromboembolism (49). There are three main approaches to measuring drug levels in patients: measuring plasma levels of the drug, measuring functional analysis, or measuring receptor binding. Measuring the plasma levels of a drug is not always accurate, particularly in the case of drugs that have high affinity binding to their target receptor. For example, even when aspirin levels are low, antiplatelet effects are still seen as the drug is bound to COX-1 in platelets rather than freely circulating in the plasma (54). Anfibatide is expected to have high affinity binding to the GPIb receptor, making this an inappropriate tool. Functional analysis for anti-platelet agents generally involves measuring platelet aggregation. Most aggregometers require manipulation of blood, which is time consuming and can lead to platelet activation. Furthermore, most hospitals do not have access to platelet aggregometers, and the majority of health professionals are not trained in the technique. There is also wide variation in the results obtained from different platelet functional tests, making them unreliable and inconsistent (24). Receptor binding is the most appropriate way to measure anfibatide levels in patients in clinical trials.

A receptor binding assay was previously developed in this lab for GPIIb/IIIa (55), and it is on this principle that the current GPIb receptor occupacy assay is based. The assay that we have developed measures total GPIb receptor numbers per platelet for each patient, and measures binding of anfibatide to the platelet with the anti-anfibatide antibody 1B9. Therefore, the percentage of GPIb receptors occupied by anfibatide can be calculated using our assay. This is a no-wash assay, meaning that both the primary and secondary antibodies must be used at saturating concentrations. The secondary antibody, IgG-FITC, was part of the platelet calibrator kit (Biocytex, France) and was pre-optimised, meaning that only the primary antibody had to be optimised. The primary antibody, 1B9, was found to detect optimal binding of anfibatide at 3.8 µg/ml. It is recommended that the primary antibody is not used in excess of 10 µg/ml as part of the platelet calibrator kit, which means that this dose of 1B9 is appropriate. As 1B9 concentration increased beyond 3.8 µg/ml, 1B9 detection of anfibatide decreased. This could be due to excess 1B9 binding the FITC-labelled antibody in the plasma, preventing binding to anfibatide.

In order to detect total GPIb receptor numbers, an anti-GPIb antibody was required for the assay. Five anti-GPIb monoclonal antibodies were tested. It was found that high dose VM16d (20 µg/ml) was not affected by anfibatide. Since the receptor numbers indicated in flow cytometry studies using VM16d are similar to those reported when measuring anfibatide binding using 7.35 µg/ml 1B9, this antibody was chosen to measure total GPIb receptor number. This study showed that GPIb receptor occupancy by anfibatide, calculated using the anti-GPIb antibody VM16d, was directly related to functional activity as measured by inhibition of ristocetin-induced platelet aggregation in whole blood. In a clinical setting, blood samples could be tested using this assay to determine the occupancy of GPIb receptors by anfibatide. Since the percentage correlates with functional studies, dosage of anfibatide can be adjusted in order maximise receptor occupancy, while minimising the dose so as to prevent possible side effects. In order for this assay to be used in this way, this would need to be tested in a clinical trial.

VM16d was chosen as the receptor numbers detected by it most closely matched binding sites detected by 1B9. However, since low dose VM16d binding to GPIb is partially inhibited by anfibatide, it may not be the ideal candidate to measure receptor numbers. 1B9 at 3.8 µg/ml detected approximately 60,000 binding sites, compared to only approximately 30,000 receptors detected by the anti-GPIb antibodies WM23, ALMA 19, and SZ2. Assuming that the evidence pointing to anfibatide being a GPIb antagonist is correct, there are some explanations for double the receptor numbers detected by 1B9 binding compared to normal GPIb receptor numbers. Anfibatide may be forming dimers, onto which two 1B9 molecules bind. It is unknown where 1B9 binds to anfibatide, and there may be two binding sites on 1B9 for the compound. There could potentially be an additional pool of GPIb receptors that is undetectable by the monoclonal antibodies. Since neither WM23 or ALMA 19 are affected by anfibatide, and they detect approximately half the number of binding sites that 1B9 detects with anfibatide, these could possibly be used to measure total receptor numbers, and the measured total could be doubled before calculating receptor occupancy by anfibatide.

The assay is simple to use, and is very quick. From the initial blood draw, the entire assay could be completed and analysed in two hours by someone trained in the technique. Whole blood can be used, meaning that preparation time is short. The fact that the blood does not need to be manipulated limits the possiblity of platelet activation which could affect the results. The assay is easily taught, and easily learned, and completely accessible by any laboratory or hospital facility that has access to a flow cytometer. The optimum doses of each antibody has been determined in this study, so no calculations or optimisation has to be carried out in individual facilities. These factors make it feasible for the assay to be widely used in hospitals where patients would be undergoing anfibatide treatment. The assay would have to be validated in a cinical study prior to use in the clinic.

In this study, 10 µg/ml of anfibatide resulted in maximal binding, which was matched with close to complete inhibition of ristocetin-induced whole blood

aggregation. The dose of anfibatide being used in the clinical studies in China would produce an estimated final concentration of only 2 ng/ml. In this study, the lowest concentration of anfibatide tested was 0.1 µg/ml, which had no recorded occupancy of GPIb receptors, and had no functional effect on aggregation. The concentration used in China is approximately 5,000 times more dilute than the concentration we found to have maximal effect in healthy donor blood, yet they are reporting an anti-thrombotic effect. This substantial difference in reported effective dosage raises a number of questions. The journey of anfibatide from China to Ireland may effect its potency. The protein is being transported in a lyophilised state, and is stored and prepared in the same manner as it is in China, making this an unlikely cause of the discrepancy. A second possible explanation for the discrepancy could be population differences. Chinese donors may have a higher affinity for anfibatide than Irish donors. A study comparing European and Chinese donors response to anfibatide should be carried out in one laboratory under the same conditions in an attempt to explain this difference.

Identification of the Anfibatide Binding Site

The effect of anfibatide on anti-GPIb antibodies gives an insight into the possible binding site of the drug. Anfibatide had no effect on WM 23, AN 51, and ALMA 19 binding. This indicates that anfibatide does not bind to the macroglycopeptide region, or the vWF binding site on GPIbα. Anfibatide partially inhibited both VM16d and SZ 2, which bind to the thrombin binding site and the sulfated tyrosine region respectively. Anfibatide did not inhibit aggregation induced in PRP by thrombin at low dose or high doses. This indicates that the drug does not bind directly to the thrombin binding site where VM16d does. Thus we hypothesise that anfibatide binds to GPIbα in the region between the thrombin binding site, and the sulfated tyrosine region contained in residues 276 - 282.

Estimation of GPIb Receptor Numbers

Five different anti-GPIbα antibodies were used in this study. Surprisingly, the antibodies had varying numbers of binding sites. It was anticipated that all antibodies would give similar receptor numbers, as they all bind to the same receptor, and all antibodies were tested in the same donor on the same day (n=3). WM23, SZ 2, and ALMA 19 all had a similar number of binding sites (27,000 – 30,000 receptors). Previous studies with WM23 demonstrated that there were approximately 21,000 binding sites (6). However their study indicated that there was wide variability in recepetor numbers depending on the donor, indicating that the results of our study are reasonable and in line with previous data. In our study, AN51, which binds to the vWF binding site on GPIbα, has had only 15,000 binding sites, significantly less than the other anti-GPIb antibodies. Previous studies with AN 51 indicated 22,000 binding sites, though once again there was high inter-donor variability (6, 9). VM16d had the highest receptor numbers, with an average of 40,880 binding sites. However, there was no statistical significance when comparing WM23, SZ 2, ALMA 19, and VM16d receptor numbers. Conventionaly, it is believed that there are 25,000 – 30,000 GPIb receptors per platelet, half the number of GPIIb-IIIa receptors present. This is in agreement with data from WM23, SZ 2, and ALMA 19. The higher numbers indicated by VM16d may indicate that more than one antibody binds to each GPIb receptor, or there may be clustering of the antibody on GPIb. The fact that AN51 binds half the number of receptors compared to the other antibodies suggests that perhaps AN51 can only bind one GPIbα molecule per GPIb-IX-V complex, or cleavage of glycocalicin may be occurring.

Anfibatide and Bacteria-Platelet Interactions

If anfibatide is demonstrated to be a clinically effective and safe anti-thrombotic, it could theoretically be used to inhibit bacteria that activate platelets through the GPIb receptor. *S. sanguinis* 133-79 has been shown to activate platelets by directly interacting with GPIb (57). Anfibatide inhibited *S. sanguinis* 133-79 induced whole blood aggregation in a dose dependent manner, to the same extent as the anti-GPIb antibody AN51. It did not inhibit *S. aureus* Newman induced aggregation, which is stimulated directly through GPIIb/IIIa receptor activation (56). These results indicate that anfibatide may be useful and effective in preventing or treating bacterial-induced sepsis, initiated by the interaction of certain bacterial strains with platelets. In our study however, aggregation was only inhibited in three of six healthy donors. This suggests that the treatment would not be effective in all patients, possibly due variations in GPIb structure between donors. Anfibatide also had no effect on aggregation induced in PRP by *S. sanguinis* 133-79. There is a possibility that there is some component of the whole blood that is not present in PRP that affects the anfibatide - platelet interaction. Whole blood aggregations are mosre physiologically relevant than aggregations in PRP. The PRP aggregations were only carried out in four healthy donors, so it is possible, based on the donor variation seen in the whole blood study, that the sample size was too small to analyse the true effect of anfibatide.

Whole Blood Aggregation

Ristocetin is a commonly used platelet agonist that induces platelet aggregation through the GPIb receptor, closely mimicking the *in vivo* interaction between vWF and the GPIb-IX-V complex. Anfibatide inhibited ristocetin - induced platelet aggregation in whole blood and PRP. This is further confirmation that anfibatide is a GPIb antagonist.

Whole blood aggregation is a straightforward and easily learned technique. Use of Multiplate software could allow professionals in a clinical setting to

complete functional testing, along with the flow cytometry based receptor occupancy assay, to confirm that patients receiving anfibatide treatment are being effectively treated. Whole blood aggregations require no manipulation of blood, meaning that preparation time for the assay is short, and minimising platelet activation due to inappropriate handling. Furthermore, the assay only takes a few minutes to complete, and analysis is simple and fast. Inhibition of ristocetin-induced aggregation correlates with receptor occupancy by anfibatide, meaning that it is an excellent indicator of anfibtide efficacy, and suggesting that there are no free GPIb receptors. This indicated that whole blood aggregation could be used as a functional assay alongside the flow cytometry assay.

Anfibatide Specificity

The results of this *in vitro* study indicate that anfibatide is a potentially safe anti-thrombotic therapeutic. Anfibatide did not cause platelet activation, or loss of single platelets. It had no effect on GPIIb-IIIa in a monoclonal antibody binding study, which indicates that it would not cause bleeding at low shear stress, where GPIIb-IIIa is most active. Anfibatide studies in *S. aureus* Newman aggregation, as well as the GPIIb-IIIa monoclonal antibody studies, indicate that the inhibitory activity is specific to GPIb. It also had no effect on a range of agonists, including ADP, thrombin, and arachidonic acid, suggesting no off target effects. This implies that *in vivo*, anfibatide could effectively inhibit thrombus formation at high shear stress, for example in atherosclerotic arteries, while leaving the GPIIb-IIIa interaction unffected. Since the GPIIb-IIIa interaction is predominant at low shear stress in the periphery, excessive and unwanted bleeding would be prevented, making anfibatide an enticing alternative to GPIIb-IIIa antagonists.

Future Work

Anfibatide needs to be validated in upcoming clinical trials in China. This assay will be validated in approximately 100 non-ST segment myocardial

infarction patients on site in a military hospital in Beijing, beginning in March 2015. Further investigation is also required to elucidate the exact binding site of anfibatide in order to fully understand its mechanism of action.

Conclusion

A flow cytometry based receptor occupancy assay has been developed. The technique involves measuring total GPIb receptor numbers with the monoclonal antibody VM16d, measuring anfibatide binding, and calculating total receptor occupancy, which is an indicator of functional activity. The assay is simple and quick, and can be used alongside the Multiplate analyser to measure functional activity through whole blood platelet aggregation. The assay is appropriate to use in forthcoming clinical trials on anfibatide in China, and in clinical practice in the future. The binding site of anfibatide is hypothesised to lie between the thrombin binding site and the sulfated tyrosine residues on GPIbα. Anfibatide inhibits ristocetin-induced aggregation in a dose-dependent manner, and partially inhibited *S. sanguinis* 133-79 induced aggregation in 50% of healthy donors, indicating its potential use for bacterial-induced sepsis.

References

1. Fitzgerald JR, Foster TJ, and Cox D. The interaction of bacterial pathogens with platelets. *Nature Reviews Microbiology* 2006; 4:445-457.
2. Andrews RK, Gardiner EE, Shen Y, Whisstock JC, and Berndt MC. Glycoprotein Ib-IX-V. *The International Journal of Biochemistry and Cell Biology* 2003; 35:1170-1174.
3. Cranmer SL, and Jackson SP. GPIb, filamin, and platelet activation: a view from within. *Blood* 2003; 102(6):1937.
4. Dormann D, Clemetson KJ, and Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; 96:2469-2478.
5. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, and Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcγRIIA on the platelet plasma membrane. *Journal of Biological Chemistry* 1998; 273:5331-5336.
6. Berndt MC, Gregory C, Kabral A, Zola H, Fournier D, and Castaldi PA. Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *European Journal of Biochemistry* 1985; 151:637-649.
7. Lu Q, Clemetson JM, and Clemetson KJ. Snake venoms and hemostasis. *Journal of Thrombosis and Haemostasis* 2005; 3:1791-1799.
8. Strassel C, Pasquet J-M, Alessi M-C, Juhan-Vague I, Chambost H, Combrie R, Nurden P, Bas M-J, De La Salle C, Cazenave J-P, Lanza F, and Nurden AT. A novel missense mutation shows that GPIbβ has a dual role in controlling the processing and stability of the platelet GPIb-IX adhesion receptor. *Biochemistry* 2003; 42:4452-4462.
9. Fredrickson BJ, Dong J-F, McIntire LV, and Lopez JA. Shear-dependent rolling on von Willebrand factor of mammalian cells

expressing the platelet glycoprotein Ib-IX-V complex. *Blood* 1998; 92:3684-3693.

10. Badlou BA, Spierenburg G, Ulrichts H, Deckmyn H, Martin Smid W, and Akkerman JN. Role of glycoprotein Ibα in phagocytosis of platelets by macrophages. *Transfusion* 2006; 46:2090-2099.
11. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong J-F, and Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *Journal of Experimental Medicine* 2002; 196(8):1057-1066.
12. Canobbio I, Balduini C, and Torti M. Signalling through the platelet glycoprotein Ib-V-IX complex. *Cellular Signalling* 2004; 16:1329-1344.
13. De Marco L, Mazzucato M, Masotti A, Fenton JW, and Ruggeri ZM. Function of glycoprotein Ibα in platelet activation induced by α-thrombin. *The Journal of Biological Chemistry* 1991; 266(35):23776-23783.
14. Jandot-Perrus M, Celmetson KJ, Huisse M-G, and Guillin MC. Thrombin interaction with platelet glycoprotein Ib: effect of glycocalicin on thrombin specificity. *Blood* 1992; 80(11):2781-2786.
15. Clemetson KJ, & Clemetson JM. Platelet GPIb complex as a target for anti-thrombotic drug development. *Journal of Thrombosis and Haemostasis* 2008; 99:473-479.
16. De Marco L, Mazzucato M, Masotte A, and Ruggeri ZM. Localization and characterization of an α-thrombin-binding site on platelet glycoprotein Ibα. *The Journal of Biological Chemistry* 1993; 269(9):6478-6484.
17. Afshar-Kharghan V, Gineys G, Schade AJ, Sun L, Li CQ, McIntire LV, Dong J, and Lopez JA. Necessity of conserved asparagine residues in the leucine-rich repeats of platelet glycoprotein Ibα for the proper conformation and function of the ligand-binding region. *Biochemistry* 2000; 39:3384-3391.
18. Lopez JA, Chung DW, Fujikawa K, Hagen FS, Papayannopoulou T, and Roth GJ. Cloning of the α chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich α_2-glycoprotein.

Proceedings of the National Acadamy of Sciences 1987; 84:5615-5619.

19. Perrault C, Mangin P, Santer M, Baas M-J, Moog S, Cranmer SL, Pikovski I, Williamson D, Jackson SP, Cazenave J-P, and Lanza F. Role of the intracellular domains of GPIb in controlling the adhesive properties of the platelet GPIb/V/IX complex. *Hemostasis, Thrombosis, and Vascular Biology* 2003; 101(9):3477-3484.
20. Yamamoto N, Greco NJ, Barnard MR, Tanoue K, Yamazaki H, Jamieson GA, and Michelson AD. Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* 1991; 77(8):1740-1748.
21. Cauwenberghs N, Meiring M, Vauterin S, van Wyk V, Lamprecht S, Roodt JP, Novak L, Harsfalvi J, Deckmyn H, and Kotze HF. Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2000; 20:1347-1353.
22. Nowak AA, Canis K, Riddell A, Laffan MA, and McKinnon TAJ. *O*-linked glycosylation of von Willebrand factor modulates the interaction with platelet receptor glycoprotein Ib under static and shear stress conditions. *Blood* 2012; 120:214-222.
23. Wang Q, Shorten D, Xu X, Shaw GD, Schaub RG, Shea C, Brooks J, Sako D, Wiswall E, Xu J, Szklut P, and Patel VS. Effect of von Willebrand factor on the pharmacokinetics of recombinant human platelet glycoprotein Ibα-immunoglobulin G1 chimeric proteins. *Pharmaceutical research* 2006; 23(8):1743-1749.
24. Hezard N, Metz D, Nazeyrollas P, Droulle C, Potron G, and Nguyen P. PFA-100 and flow cytometry: can they challenge aggregometry to assess antiplatelet agents, other than GPIIb/IIIa blockers, in coronary angioplasty? *Thrombosis Research* 2003; 108:43-47.
25. Mousa SA, Bozarth JM, Forsythe MS, Jackson SM, Leamy A, Diemer MM, Kapil RP, Knabb RM, Mayo MC, and Pierce SK. Antiplatelet and antithrombotic efficacy of DMP 728, a novel platelet GPIIb/IIIa receptor antagonist. *Circulation* 1994; 89:3-12.

26. Lei X, Reheman A, Hou Y, Zhou H, Wang Y, Marshall AH, Liang C, Dai X, Li BX, Vanhoorelbeke K, and Ni H. Anfibatide, a novel GPIb complex antagonist, inhibits platelet adhesion and thrombs formation *in vitro* and *in vivo* in murine models of thrombosis. *Journal of Thrombosis and Haemostasis* 2014; 111.
27. Dong J, Li C, Schade AJ, Fredrickson BJ, Sun L, McIntire LV, and Lopez JA. Mutation in the leucine-rich repeat of platelet glycoprotein Ib α results in defects in its interaction with immobilized von Willebrand factor under flow. *Chinese Medical Journal (English)* 2000; 113(8):693-8.
28. Benard SA, Smith TM, Cunningham K, Jacob J, DeSilva T, Lin L, Shaw GD, Kriz R, and Kelleher KS. Identification of peptide antagonists to glycoprotein Ibα that selectively inhibit von Willebrand factor dependent platelet aggregation. *Biochemistry* 2008; 47:4674-4682.
29. David T, Ohlmann P, Eckly A, Moog S, Cazenave J-P, Gachet C, and Lanza F. Inhibition of adhesive and signaling functions of the platelet GPIb-V-IX complex by a cell penetrating GPIbα peptide. *Journal of Thrombosis and Haemostasis* 2006; 4:2645-2655.
30. Armstrong PC, and Peter K. GPIIb/IIIa inhibitors: from bench to bedside and back to bench again. *Thrombosis and Haemostasis* 2012; 107:808-814.
31. Li S, Ji H, Cheng X, Li BXY, and Ng TB. Antithrombotic and thrombolytic activities of Agkisacutacin, a snake venom proteinase, in experimental models. *General Pharmacology* 2002; 35:179-187.
32. Bai Y, Durbin H, and Hogg N. Monoclonal antibodies specific for platelet glycoproteins react with human monocytes. *Blood* 1984; 64:139-146.
33. Goto S. Understanding the mechanism of platelet thrombus formation under blood flow conditions and the effect of new antiplatelet agents. *Current Vascular Pharmacology* 2004; 2:23-32.
34. Chen J, Ling M, Fu X, Lopez JA, and Chung DW. Simultaneous exposure of sites in von Willebrand factor for glycoprotein Ib binding

and ADAMTS13 cleavage studies with ristocetin. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2012; 32:2625-2630.

35. Shimizu A, Matsushita T, Kondo T, Inden Y, Kojima T, Saito H, and Hirai M. Identification of the amino acid residues of the platelet glycoprotein Ib (GPIb) essential for the von Willebrand factor binding by clustered charged-to-alanine scanning mutagenesis. *Journal of Biological Chemistry* 2004; 279:16285-16294.
36. Michelson AD, and Barnard MR. Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb/IIIa complex. *Blood* 1987; 70:1673-1678.
37. Becker BH, and Miller JL. Effects of an antiplatelet glycoprotein Ib antibody on hemostatic function in the guinea pig. *Blood* 1989; 74:690-694.
38. Cadroy Y, Hanson SR, Kelly AB, Marzec UM, Evatt BL, Kunicki TJ, Montgomery RR, and Harker LA. Relative antithrombotic effects of monoclonal antibodies targetting different platelet glycoprotein-adhesive molecule interactions in nonhuman primates. *Blood* 1994; 83(11):3218-3224.
39. Goto S, Tamura N, Sakakibara M, Ikeda Y, and Handa S. Effects of ticlopidine on von Willebrand factor-mediated shear-induced platelet activation and aggregation. *Platelets* 2001; 12:406-414.
40. Clemetson KJ, and Clemetson JM. Collagen receptors as potential targets for novel anti-platelet agents. *Current Pharmaceutical Design* 2007; 13:1-11.
41. Zhao Y-N, Dai X-R, Liu J-J, Li X-H, Yang J-J, Sun H, Wu P, Shen J, Lu J-P, Xie H-T, and Liu X-Q. An indirect sandwich ELISA for the determination of agkisacutacin in human serum: application to pharmacokinetic study in Chinese healthy volunteers. *Journal of Pharmaceutical and Biomedical Analysis* 2012; 70:396-400.
42. Cheng X, Qian Y, Liu Q, Li BXY, Zhang M, and Liu J. Purification, characterization, and cDNA cloning of a new fibrinogenlytic venom protein, Agkisacutacin, from *Agkistrodon acutus* venom. *Biochemical and Biophysical Research Communications* 1999; 265:530-535.

43. Andrews RK, and Berndt MC. Snake venom modulators of platelet adhesion receptors and their ligands. *Toxicon* 2000; 38:775-791.
44. Li W-F, Chen L, Li X-M, and Liu J. A C-type lectin-like protein from *Agkistrodon acutus* venom binds to both platelet glycoprotein Ib and coagulation factor IX/factor X. *Biochemical and Biophysical Research Communications* 2005; 332:904-912.
45. Gao Y, Ge H, Chen H, Li H, Liu Y, Chen L, Li X, Liu J, Niu L, and Teng M. Crystal structure of agkisacucetin, a GPIb-binding snake C-type lectin that inhibits platelet adhesion and aggregation. *Proteins* 2012; 80:1707-1711.
46. Clinical Trials Registry, a service of the U.S. National Institute of Health. 2014 Phase Ib-IIa clinical trial of anfibatide. http://clinicaltrials.gov/ct2/show/record/NCT01585259 Last updated 14/10/2014. Last accessed: 25/10/2014.
47. Clinical Trials Registry, a service of the U.S. National Institute of Health. 2012. Anfibatide Phase 1 Clinical Trial in Healthy Volunteers. http://clinicaltrials.gov/ct2/show/study/NCT01588132 Last updated: 26/04/2012. Last accessed: 25/10/2013.
48. Hou Y, Lei X, Li BX, Dai X, Yang Z, Qian F, Zhang G, Xu Z, Liu J, Liang C, Zhao Y, Shen J, Zhou H, Wang Y, Marshall AH, and Ni H. The first in vitro and in vivo assessment of anfibatide, a novel glycoprotein Ib antagonist, in mice and in a phase I human clinical trial. *Presented orally at session 332, Antithrombotic Therapy: Basic Research and Caner Trials at the 55th American Society of Hematology Annual Meeting and Exposition.* 09/12/2013.
49. Olsson SB. Executive Steering Committee of the SPORTIF III Investigatiors. Stroke prevention with the oral direct thrombin inhibitor ximelagatran compared with warfarin in patients with non-valvular atrial fibrillation (SPORTIF III): randomised controlled trial. *Lancet* 2003; 362(9397):1691-1698.
50. Liang HP, Morel-Kopp MC, Curtin J, Wilson M, Hewson J, Chen W, and Ward CM. Heterozygous loss of platelet glycoprotein (GP) Ib-V-IX variably affects platelet function on velocardiofacial syndrome (VCFS)

patients. *Journal of Thrombosis and Haemostasis* 2007; 98(6):1298-308.

51. Cox D. Oral GPIIb/IIIa antagonists: what went wrong? *Current Pharmaceutical Design* 2004; 10(14):1587-1596.
52. Peace A, McCall M, Tedesco T, Kenny D, Conroy RM, Foley D, and Cox D. The role of weight and enteric coating on aspirin response in cardiovascular patients. *Journal of Thrombosis and Haemostasis* 2010; 8(10):2323-2325.
53. Feys HB, Roodt J, Vandeputte N, Pareyn I, Mottl H, Hou S, Lamprecht S, Van Rensburg WJ, Deckmyn H, and Vanhoorelbeke K. Inhibition of von Willebrand factor-platelet glycoprotein Ib interaction prevents and reverses symptoms of acute acquired thrombotic thrombocytopenic purpura in baboons. *Blood* 2012; 120(17):3611-4.
54. Cox D. Aspirin resistance: a nebulous concept. *Journal of Clinical Pharmacology and Pharmacoepidemiology* 2008; 1(2):39-47.
55. Quinn M, Deering A, Stewart M, Cox D, Foley B, and Fitzgerald D. Quantifying GPIIb/IIIa receptor binding using 2 monoclonal antibodies: discriminating abciximab and small molecular weight antagonists. *Circulation* 1999; 99(17):2231-8.
56. Fitzgerald JR, Loughman A, Keane F, Brennan M, Knobel M, Higgins J, Visai L, Speziale P, Cox D, and Foster TJ. Fibronectin-binding proteins of *Staphylococcus aureus* mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcγRIIa receptor. *Molecular Microbiology* 2006; 59(1):212-230.
57. Plummer C, Wu H, Kerrigan SW, Meade G, Cox D, and Ian Douglas CW. A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GPIb. *British Journal of Haematology* 2005; 129(1):101-9.