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DEVELOPMENT OF NOVEL INHIBITORS OF THROMBOSIS FOR THE PREVENTION OF CARDIOVASCULAR EVENTS

BY

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Dedicated to my mum and dad

ABSTRACT

BACKGROUND

Thrombotic events driven by platelet aggregation and activation of the coagulation system remain the leading cause of global morbidity and mortality. Antiplatelet and anticoagulant agents have proven effective in the treatment and prevention of these events in at-risk patients. However, bleeding remains a major concern and there is a large unmet clinical need for newer agents that can provide more effective antithrombotic protection. Novel candidates have been identified that may allow for a wider therapeutic index. Among these, platelet protease-activated receptor 4 (PAR4) and exosite 1 of thrombin have emerged as two of the most promising targets for antiplatelet and anticoagulant therapies, respectively.

OBJECTIVES

Given the central role of platelet aggregation to this thesis, we first sought to examine the feasibility of using a newer technique of platelet aggregometry, 96-well plate aggregometry (96-WPA), instead of the current gold standard, light transmission aggregometry (LTA). 96-WPA offers advantages over LTA but as yet there is almost no data regarding test-retest repeatability or agreement between the two techniques. Thereafter, the principal aims of this thesis were to explore the antiplatelet, anticoagulant and antithrombotic effects of PAR4 and exosite 1 thrombin inhibition in humans through a series of in vitro and ex vivo studies. Additional objectives included determination of whether platelet responses to PAR4 signaling are dependent on additional input from other agonist-receptor pathways or vice-versa and establishing the

safety and tolerability of intravenous PAR4 agonism in mice in order to facilitate future studies into establishing the wider role of PAR4 within the human vasculature.

METHODS AND RESULTS

Repeatability and agreement between two methods of platelet aggregometry

Test-retest repeatability (standard deviation, coefficient of variation (CV) and coefficient of repeatability) and agreement between 96-WPA and LTA were assessed constructing replicate agonist-induced dose-response curves from which peak aggregation and EC_{50} were determined. The pre-specified selected agonists were adenosine diphosphate (ADP), arachidonic acid (AA) and PAR4-activating peptide (PAR4-AP). Within-day CV for 96-WPA was 3.4%, 3.6% and 3.6% for peak aggregation and 9.6%, 7.1% and 6.9% for EC_{50} with ADP, AA and PAR4-AP, respectively. Corresponding values for between-day CV were 8.7%, 5.4%, and 6.6% for peak aggregation and 23.4%, 28.5%, and 23.1% for EC_{50}. Overall, results for test-retest repeatability were comparable to LTA, although the latter appeared marginally superior in terms of the between-day CV. With respect to agreement, ADP and PAR4-AP peak aggregation measured consistently higher with 96-WPA whereas the EC_{50} was consistently lower. No significant measurement bias was observed with AA.

Dependence of PAR4 on other agonist-receptor pathways for the activation and aggregation of human platelets

All studies were performed in vitro using blood from healthy volunteers with results pooled from a minimum of 6 subjects. Activation of platelets by PAR4-AP (25 μM) resulted in strong p-selectin expression (86.5 ± 2.6%), platelet-monocyte binding (85.8 ± 1.5%) and platelet aggregation (88.5 ± 3.4%). Platelet responses to PAR4-AP were

completely inhibited by the PAR4 antagonist BMS-986120 but were unaffected by SCH-79797 (PAR1 antagonist), apyrase (ADP scavenger) or indomethacin (cyclooxygenase inhibitor). In contrast, PAR1 platelet activation and aggregation were both partially inhibited by apyrase. BMS-986120 had no effect on platelet activation or aggregation in response to PAR1, ADP or AA stimulation.

Tolerability and safety of systemic PAR4 agonism in mice

PAR4-agonist peptide (PAR4-AP) AYPGKF-NH2 (target blood concentration 11.25 μM and 112.5 μM) or control (0.9% saline) was administered intravenously to mice (n=15 total, 5 mice per group). Mice were observed for 24 hours then sacrificed with blood drawn for laboratory analyses and tissue samples prepared for histological examination. AYPGKF-NH2 was well tolerated with no evidence of systemic perturbation, thrombosis or change in any of the haematological, inflammatory, hepatic and renal blood markers examined. No distortion of parenchymal architecture, necrosis or inflammatory cell infiltrate of the lungs, spleen, liver and kidney was observed in any of the samples.

Effect of oral PAR4 antagonism with BMS-986120 on platelets and thrombosis

Forty volunteers were enrolled into a phase 1 parallel-group prospective randomized open-label blinded endpoint trial. Ex vivo platelet activation, platelet aggregation and thrombus formation were measured at 0, 2 and 24 hours after (a) oral BMS-986120, or (b) oral aspirin followed at 18 hours with oral aspirin and oral clopidogrel. BMS-986120 demonstrated highly selective and reversible inhibition of PAR4-AP stimulated platelet activation and aggregation. Compared to pre-treatment, BMS-986120 reduced total thrombus area at high shear by 29.2% ($p<0.001$) at 2 hours and by 21.4%

(p=0.002) at 24 hours, driven by a decrease in platelet-rich thrombus formation. In contrast to aspirin and aspirin in combination with clopidogrel, BMS-986120 had no effect on thrombus formation at low shear (p=0.08). There were no serious adverse events.

Effect of exosite 1 thrombin inhibition with JNJ-9375 on coagulation, platelets and thrombosis

Fifteen healthy volunteers were enrolled into a double-blind randomized crossover study of JNJ-9375 (2.5, 25 and 250 μg/mL), bivalirudin (positive control) and matched placebo. JNJ-9375 caused concentration-dependent prolongation of blood coagulation and agonist-selective inhibition of platelet activation. Compared to placebo, JNJ-9375 (250 μg/mL) reduced mean total thrombus area by 41.1% ($p<0.001$) at low shear and 32.3% (p=0.025) at high shear. Under both shear conditions, there was a dose-dependent decrease in fibrin-rich thrombus ($p<0.001$ for both). In contrast to bivalirudin, JNJ-9375 had no effect on platelet-rich thrombus formation.

CONCLUSIONS

This thesis demonstrates that a) both 96-WPA and LTA demonstrate good and comparable within- and between-day repeatability but owing to a systematic measurement bias the techniques should not be considered interchangeable, b) under conditions designed to replicate clinically relevant levels of thrombin, platelet responses to PAR4 stimulation were not dependent on input from other major agonist-receptor pathways or vice-versa, c) even at suprathreshold blood concentrations, intravenous PAR4 agonism with AYPGKF-NH2 was not associated with adverse effects in mice, d) oral PAR4 antagonism with BMS-986120 inhibits ex vivo human thrombus formation

at high shear driven by a reduction in platelet deposition, and e) exosite 1 thrombin antagonism with JNJ-9375 inhibits ex vivo human thrombosis formation driven by a reduction in fibrin deposition. Results from this thesis suggest PAR4 antagonism and exosite 1 thrombin inhibition have major potential as novel antiplatelet and anticoagulant strategies and that further investigation in clinical trials is warranted.

LAY SUMMARY

Thrombosis refers to the development of a blood clot (thrombus) within a blood vessel. This can lead to obstruction of blood flow and is the most common cause of heart attacks, strokes, and clots within the deep veins (DVT) or lung (pulmonary embolus). Platelet aggregation and activation of the coagulation system are critical to blood clot formation. Consequently, antiplatelet agents (e.g. aspirin) and anticoagulants (e.g. warfarin) are the cornerstone of treatment and prevention of thrombotic events.

Although the current antiplatelet and anticoagulant drugs are of undoubted benefit in a broad range of patients, important limitations persist. Bleeding remains a major concern and despite treatment many patients continue to suffer from recurrent life-threating thrombotic events. This has driven the continued search for safer and more effective therapies. BMS-986120 is a new type of antiplatelet agent that works by blocking the platelet receptor protease-activated receptor 4 (PAR4). JNJ-9375 is new type of anticoagulant that targets exosite 1 of thrombin. Preclinical studies have indicated these drugs may be effective new classes of antiplatelet and anticoagulant agents, respectively. However, it remains to be determined if either can prevent thrombus development in humans. Thus, the principal aims of this thesis were to examine for the first time the effect of PAR4 inhibition with BMS-986120 and exosite 1 thrombin inhibition with JNJ-9375 on human platelets, coagulation and thrombosis.

One of the key methods in examining the effect on drugs on platelets and thrombus formation is platelet aggregometry. The current gold standard technique is light transmission aggregometry (LTA). However, LTA can be difficult and is limited in

terms of the number of tests that can be performed on each patient blood sample because of the volumes required. 96-well platelet aggregometry (96-WPA) is a newer technique of platelet aggregation that may address some of the issues of LTA. Prior to undertaking our main studies examining PAR4 and exosite 1 thrombin inhibition we undertook a series of experiments to determine if using 96-WPA could replace LTA for this thesis. Finally, there is evidence that PAR4 may have additional effects in humans beyond blood clot formation. Data from animal studies suggests PAR4 may be involved in blood pressure control or have other potentially important roles within the circulation. It would be very important to understand these effects in humans and there are a number of safe study methods that would allow this by injecting small doses of drugs that stimulate PAR4. These drugs are readily available but have never been given to humans and so it would be essential to first test them in animals to make sure they are not associated with any harmful effects. We therefore conducted a study in mice to determine if injection of AYPGKF-NH2, a drug that stimulates PAR4, was well tolerated and safe.

The major findings of this thesis were that inhibiting PAR4 and thrombin exosite 1 both substantially prevented ex vivo human thrombus formation. PAR4 inhibition predominantly reduced thrombus formation under conditions similar to those found in narrowed coronary arteries whereas exosite 1 thrombin inhibition predominantly reduced thrombus formation under conditions found in veins and large arteries. Interestingly, platelet responses to PAR4 stimulation were not dependent on other common pathways that are targeted by current antiplatelet drugs or vice-versa. We also demonstrated that both 96-WPA and LTA provide good and comparable levels of precision for measuring platelet aggregation, but the two techniques should not be

considered interchangeable. Overall, our results suggest PAR4 and exosite 1 thrombin inhibition have major potential as novel antiplatelet and anticoagulant strategies, respectively, and support future clinical trials to establish their effectiveness in patients.

CONTENTS

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ABBREVIATIONS

96-WPA	96-well plate aggregometry
AA	Arachidonic acid
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AE	Adverse event
ALP	Alkaline Phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
ATIII	Antithrombin III
BMI	Body mass index
BMS	Bristol-Myers Squibb
BT	Bleeding time
CI	Confidence interval
CV	Coefficient of variation
DAPT	Dual antiplatelet therapy
DOAC	Direct oral anticoagulant
ECG	Electrocardiogram
GAG	Glycosaminoglycan
GMFI	Geometric mean fluorescent intensity
GP	Glycoprotein
JNJ-9375	JNJ-64179375
LMWH	Low molecular weight heparin
LTA	Light transmission aggregometry
PAR	Protease-activated receptor
PAR-AP	Protease-activated receptor-activating peptide
PCI	Percutaneous coronary intervention
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC-β	Phospholipase C-β
PPP	Platelet-poor plasma
PROBE	Prospective randomized open-label blinded endpoint
PRP	Platelet-rich plasma

QMRI	Queen's Medical Research Institute
SAE	Serious adverse events
SD	Standard deviation
TAFI	Thrombin activatable fibrinolysis inhibitor
TF	Tissue factor
TI	Therapeutic index
UFH	Unfractionated heparin
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
WCC	White cell count

DECLARATION

This thesis represents research undertaken at the Clinical Research Facility, University of Edinburgh, Clinical Research Imaging Centre, University of Edinburgh, Queen's Medical Research Institute (QMRI), University of Edinburgh and the Animal Unit, University of Edinburgh between March 2015 and July 2019.

I was personally responsible for the setup, recruitment, conduct and analysis of all work in this thesis with the exception of the flow cytometry and blood laboratory analyses described in Chapter 6. This has been appropriately acknowledged.

This thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged. All studies were undertaken in accordance with the declaration of Helsinki of the World Medical Association and the regulations of the South East Scotland Ethics Committee.

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OVERVIEW

Cardiovascular disease remains among the primary causes of global mortality. One in four deaths worldwide are due to ischaemic heart disease or stroke, while venous thromboembolism (VTE) is the leading cause of disability-adjusted life-years lost in low- and middle-income countries [1,2]. The major pathological process common to all these conditions is thrombosis. Consequently, antithrombotic agents have been a cornerstone in the treatment and prevention of ischaemic cardiovascular events since the benefits of aspirin and warfarin were first established [3,4]. Over the past 3 decades, newer drugs have been developed in an effort to improve efficacy and reduce unwanted complications. However, despite progress, many individuals continue to experience recurrent thrombotic events and treatment-related bleeding remains a major concern [5–10].

Previously, the pathways involved in thrombosis and haemostasis were thought to be inexorably linked suggesting a ceiling of treatment benefit may have been reached. In recent years, however, de novo signaling mechanisms have been identified that in theory could facilitate an improved therapeutic index because of their apparent greater role in thrombosis as compared to haemostasis. Among novel targets under investigation, platelet protease-activated receptor 4 (PAR4) and thrombin exosite 1 have emerged as two of the most promising antiplatelet and anticoagulant candidates respectively. Accordingly, the primary purpose of this thesis was to examine the therapeutic potential of targeting these pathways in a series of in vitro and ex vivo human studies.

Chapter 1

Introduction

1 Introduction

1.1 HAEMOSTASIS AND THROMBOSIS OVERVIEW

Haemostasis and thrombosis are two distinct but intrinsically related processes. Haemostasis is the normal physiological response to vascular injury, acting to prevent blood loss and maintain circulation by forming a temporary clot to seal the vessel wall. Primary haemostatic responses largely involve platelets, whereas secondary haemostatic processes are characterised by activation of the coagulation cascade and fibrin formation [11]. While useful, in reality this distinction is somewhat arbitrary with both systems working in parallel to prevent blood loss. Following development of a haemostatic plug, local antithrombotic and anticoagulant mechanisms act to prevent unwanted thrombus growth and support wound healing.

Thrombosis is defined as inappropriate clot formation within a vascular compartment leading to obstruction and downstream infarction. It occurs when the balance between prothrombotic or procoagulant and antithrombotic or anticoagulant processes is disrupted leading to a hypercoagulable state. Thrombus consists largely of platelets, fibrin and entrapped red blood cells although the composition varies considerably depending on the vascular bed. In arteries, platelets predominate, whereas in veins and other low shear environments, fibrin has a much greater role [12,13]. Consequently, agents that inhibit platelets (antiplatelet agents) are the drugs of choice for the prevention and treatment of arterial thrombotic (atherothrombotic) events, whereas anticoagulants are of greatest efficacy in patients at risk from venous thromboembolism.

1.1.1 Platelets

Platelet accumulation at sites of vascular injury is a complex process regulated by multiple interactions. Immobilized von Willebrand factor plays a critical role in the early stages, anchoring circulating platelets to the vessel wall via the platelet receptor complex glycoprotein (GP) Ib-V-IX [14]. This interaction is further supported by platelets binding to any exposed collagen through their GPVI and α2β1 receptors [15]. Following attachment, platelets undergo shape change and degranulation with release of multiple prothrombotic agonists, including thromboxane A2, adenosine diphosphate (ADP) and thrombin. Secretion of these agonists serves as an important positive feedback loop, leading to recruitment and activation of more platelets [16]. A cascade of intracellular signaling ensues, culminating in upregulation of activated GPIIb/IIIa receptors, irreversible aggregation and thrombus growth [17].

1.1.2 Coagulation cascade

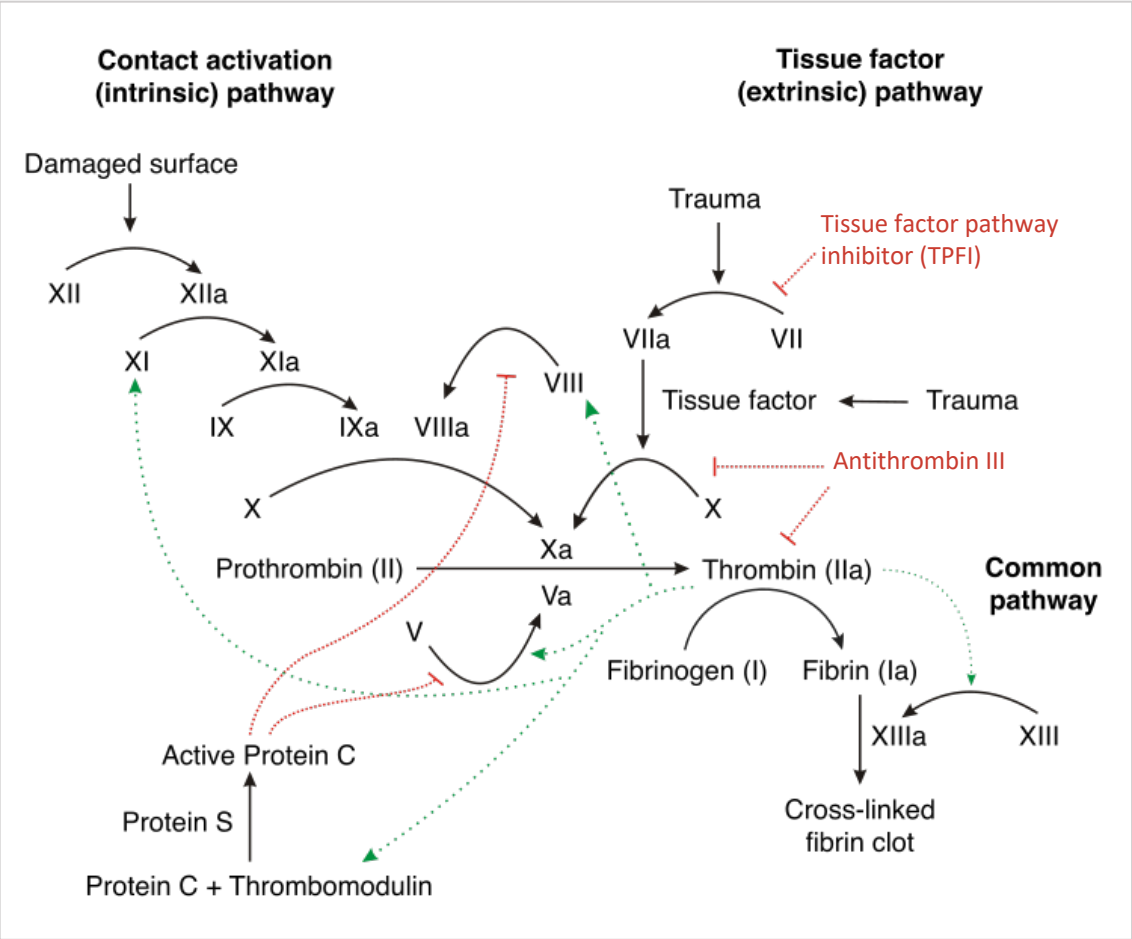


Figure 1.1. Coagulation cascade

The coagulation system acts in concert with platelet aggregation to maintain haemostasis, and under pathological conditions, promote thrombosis. Two pathways of coagulation are described, both of which converge to trigger activation of factor X, thrombin generation and fibrin formation (Figure 1.1).

Intrinsic pathway

The intrinsic (contact) pathway begins with activation of factor XII, a process that also involves plasma pre-kallikrein and high-molecular-weight kininogen [18]. Classically this occurs when blood is exposed to artificial surfaces, but other substrates include

polyphosphates (e.g. from platelets or bacteria) [19,20], misfolded protein aggregates [21,22], and collagen [22]. Activation of the intrinsic pathway leads to assembly of the IXa : VIIIa tenase-complex, which efficiently activates factor X [23]. Studies have shown that while deficiencies in the intrinsic pathway affect thrombus formation, they may not be associated with an increased bleeding tendency [18].

Extrinsic pathway

The extrinsic (tissue factor) pathway is triggered by exposure of tissue factor expressing cells to the circulation, typically in the setting of vascular injury. Tissue factor (TF) is a potent procoagulant, binding to and activating factor VII. The resulting TF : factor VIIa complex can then efficiently activate the final common pathway either directly or indirectly through factor IX [24]. In contrast to the intrinsic pathway, the extrinsic pathway appears essential for haemostasis [24,25].

1.2 THROMBIN

Thrombin is a multifunctional enzyme and one of the most important agonists in the cardiovascular system [26]. It exerts procoagulant and prothrombotic as well as anticoagulant and antithrombotic effects and is thus central to the regulation of both clot promotion and inhibition (Figure 1.1). Thrombin is also a potent modulator of vascular tone and endothelial function [27], mitogen [28] and effector of inflammation [25,29].

1.2.1 Structure and generation

Thrombin is a trypsin-like serine proteinase made up of 3 functionally important sites: the active (or catalytic) site, exosite 1 and exosite 2 [30]. Exosites 1 and 2 are anion-binding sites on the surface of thrombin that play a critical role in directing function of the protease [31–33]. Thrombin is generated from its inactive precursor, prothrombin, by activated factor X. In the setting of normal haemostasis, this largely occurs on the surface of activated platelets [32]. Thrombin generation is initially driven by the extrinsic or intrinsic pathways, but as levels of the protease rise, feed-back activation of factors V, VIII and XI leads to auto-amplification and a burst in production [34].

1.2.2 Procoagulant and prothrombotic activities

The major procoagulant activity of thrombin is cleavage of fibrinogen to produce fibrin monomers. The resulting monomers can then polymerise to produce an insoluble meshwork that encases and supports the developing clot [35]. Other procoagulant activities of thrombin include feedback activation of factors V, VIII, XI, activation of factor XIII, and activation of thrombin activatable fibrinolysis inhibitor (TAFI). Activated factor XIII promotes fibrin cross-linking [36] whereas TAFI inhibits fibrinolysis [37].

Thrombin is also a potent platelet agonist, stimulating platelets directly via the surface receptors GP1b, protease-activated receptor 1 (PAR1) and PAR4 [38]. GP1b appears to principally act as a cofactor for PAR1 activation, although may directly contribute to platelet signaling [39]. PAR1 and PAR4 belong to the same family of G-protein coupled receptors but have distinct activation kinetics and downstream signaling pathways [40].

1.2.3 Anticoagulant / antithrombotic activities

Thrombin binds to and activates protein C resulting in inhibition of the coagulation cascade and down-regulation of thrombin generation (Figure 1.1) [41]. Activation of protein C by thrombin is slow in the absence of thrombomodulin, a membrane protein present on the surface of intact endothelium [42]. Binding of thrombomodulin to thrombin also inhibits fibrinogen and PAR1 cleavage [43,44].

1.2.4 Cofactors and inhibitors

Table 1.1. Thrombin cofactors and inhibitors

Cofactor / Inhibitor	*Enhances cleavage / activation*	*Inhibits cleavage / activation*
Procoagulant cofactor		
Na^+	Increased protease activity	
GP1b	PAR1; GPV; Factor XI	
Fibrin	Factor XIII	
Anticoagulant cofactor		
Thrombomodulin	Protein C; TAFI	Fibrinogen; PAR1
Inhibitor		
Antithrombin III		Inactivates thrombin
Heparin cofactor II		Inactivates thrombin

Abbreviations used: TAFI, thrombin activatable fibrinolysis inhibitor; PAR1, protease-activated receptor 1; and GP, glycoprotein.

It is essential the many and opposing functions of thrombin are tightly regulated in order to ensure efficient haemostasis while avoiding widespread thrombosis. Here, cofactors play a critical role. Cofactors bind to and modulate thrombin substrate specificity thereby enabling direction of protease function appropriate to the local environment. They can be broadly classified into either procoagulant or anticoagulant depending on their actions [33,42,43,45–48].

The principal inhibitors of thrombin are antithrombin III and heparin cofactor II, acting by binding to and irreversibly neutralising the active site of the protease. Antithrombin III also inhibits factors IXa, Xa, XIa, and XIIa [49]. Both antithrombin III and heparin cofactor II require glycosaminoglycan cofactors (GAGs; e.g. heparan sulfates) to function effectively [49,50]. Table 1.1 outlines the main effects of the most important thrombin cofactors and inhibitors described to date.

1.3 CURRENT ORAL ANTIPLATELET AGENTS

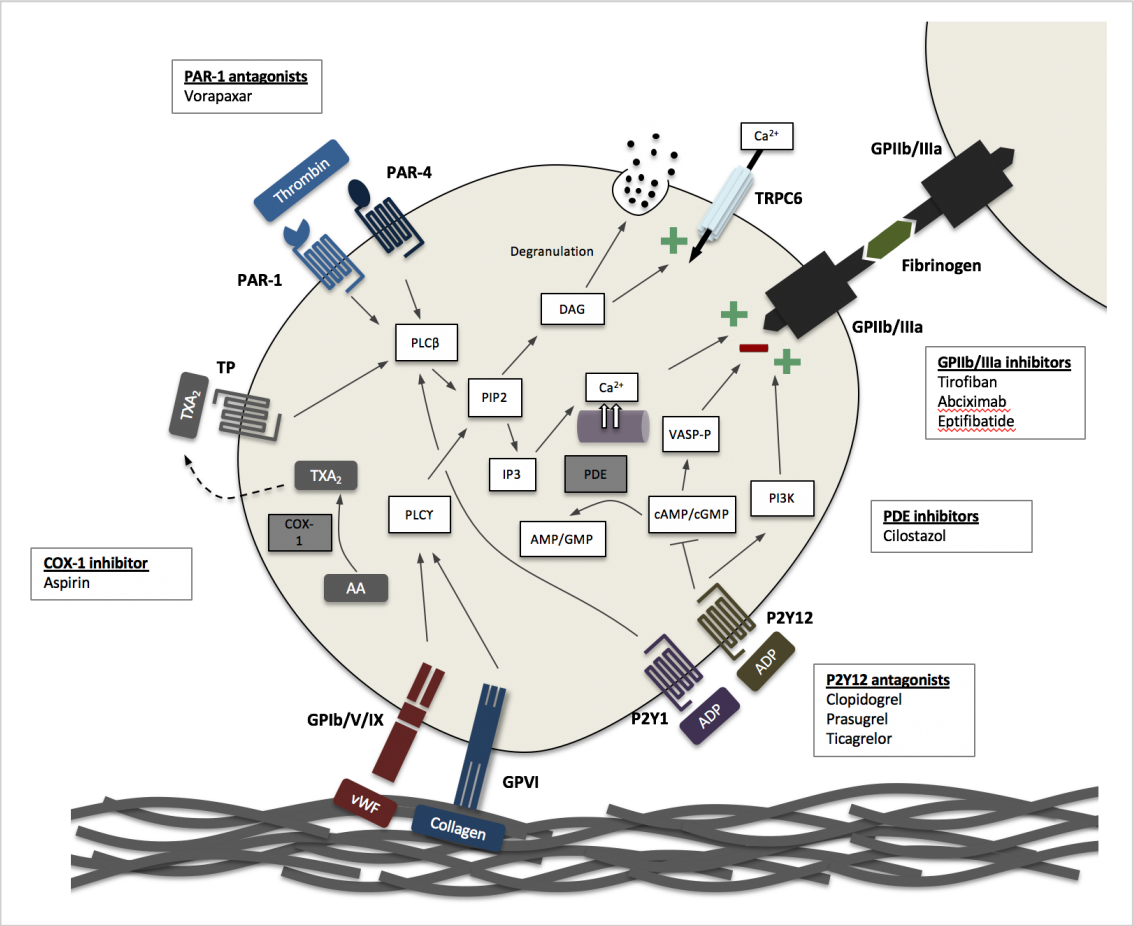


Figure 1.2. Platelet activation pathways and sites targeted by the most widely used current antiplatelet agents.
Abbreviations used: AA, Arachidonic acid; ADP, adenosine diphosphate; c, cyclic; Ca2+, calcium; AMP, adenosinemonophosphate; COX-1, cyclo-oxygenase-1; DAG, diacylglycerol; GMP, guanosine monophosphate; GP, glycoprotein; IP3: inositol trisphosphate; PAR, protease-activated receptor; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PIP2: phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TP, thromboxane receptor; TRPC, transient receptor potential channel; TXA2, thromboxane A2; vWF, von Willebrand factor.

Currently there are 3 main classes of oral antiplatelet agents used in clinical practice. Aspirin, a cyclo-oxygenase-1 inhibitor, $P2Y_{12}$ receptor antagonists, and vorapaxar, a PAR1 antagonist (Figure 1.2). Aspirin and $P2Y_{12}$ receptor antagonists prevent thromboxane A2 and adenosine diphosphate (ADP) platelet activation, respectively [51,52]. Vorapaxar inhibits thrombin and PAR1-mediated platelet activation [53].

GPIIb/IIIa inhibitors are a fourth major class of antiplatelet agents but are all administered intravenously. They inhibit the final common pathway of platelet aggregation and are therefore potent platelet antagonists. Due to the incidence of adverse events, principally bleeding [54–56], their use is restricted to selected patients and for a limited duration (< 108 hours) [57]. Cilostazol is licensed for the symptomatic relief of claudication in patients with peripheral vascular disease. It inhibits phosphodiesterase-III with antiplatelet effects but does not appear to reduce the incidence of thrombotic events [58].

1.3.1 Benefits and limitations

Aspirin

Aspirin is the first and most studied antiplatelet therapy. In acute coronary syndrome (ACS), aspirin has been shown to reduce the risk of mortality by 23% after 5 weeks [59] and the risk of myocardial infarction or death by 51 to 74% at 3 months [60,61]. Similar benefits were found in patients with acute ischaemic stroke [62]. The Antithrombotic Trialists' Collaboration meta-analysis established longer-term use of aspirin in patients at high-risk of events reduced the incidence of death, myocardial infarction or stoke by approximately 20 to 25% [63]. However, this came at the cost of a 50% increased risk of major bleeding. This increased risk of bleeding cannot be separated from efficacy. As such, the net clinical benefit becomes progressively less favourable in lower risk populations. Consequently, while aspirin for primary prevention may reduce the incidence of cardiovascular events in selected groups, the absolute increase in risk of major bleeding generally outweighs the benefits [64,65].

$P2Y_{12}$ receptor antagonists

Intensification of aspirin monotherapy with a $P2Y_{12}$ receptor antagonist (dual antiplatelet therapy; DAPT) has proven effective in selected high-risk populations. Compared to aspirin alone, combined treatment with aspirin and clopidogrel reduces the 1-year incidence of cardiovascular events in patients with ACS and following percutaneous coronary intervention (PCI) by approximately 20% [66–72]. Even greater benefits have been demonstrated with the more potent and consistent $P2Y_{12}$ receptor antagonists, prasugrel and ticagrelor [69,70]. DAPT is the current standard of care in these patients and may also improve outcomes in acute stroke when given for short periods (< 90 days) [73,74].

As with aspirin, the major limitation of $P2Y_{12}$ receptor antagonists is increased bleeding. This risk is incremental to aspirin and dependent on the strength of $P2Y_{12}$ inhibition [67,69,70,72]. Thus, while patients with ACS remain at higher risk of events for up to 3 years and perhaps longer, use of DAPT is generally limited to ≤ 6-12 months and the greater antithrombotic efficacy of ticagrelor or prasugrel reserved for selected patients [57,75]. Treatment-related bleeding also explains why DAPT has failed to deliver net clinical benefit in many other patient groups. This includes patients with chronic atherothrombotic disease [76,77] and those with acute ischaemic stroke beyond 90 days [77–79].

Vorapaxar

Two large phase 3 clinical trials have examined the benefit of vorapaxar added to standard care in patients with cardiovascular disease [9,80]. Vorapaxar proved effective in patients with established atherothrombotic disease but failed to meet the primary

efficacy endpoint in ACS. In both studies, there was a substantial increase in bleeding including intracranial haemorrhage, especially in patients with a history of stroke. On the basis of these trials, PAR1 antagonism with vorapaxar is only approved for the secondary prevention of events in patients with a history of myocardial infarction or peripheral vascular disease (Food and Drug Administration). It is not licensed in ACS and is contraindicated in patients with a history of stroke.

1.4 CURRENT ANTICOAGULANT AGENTS

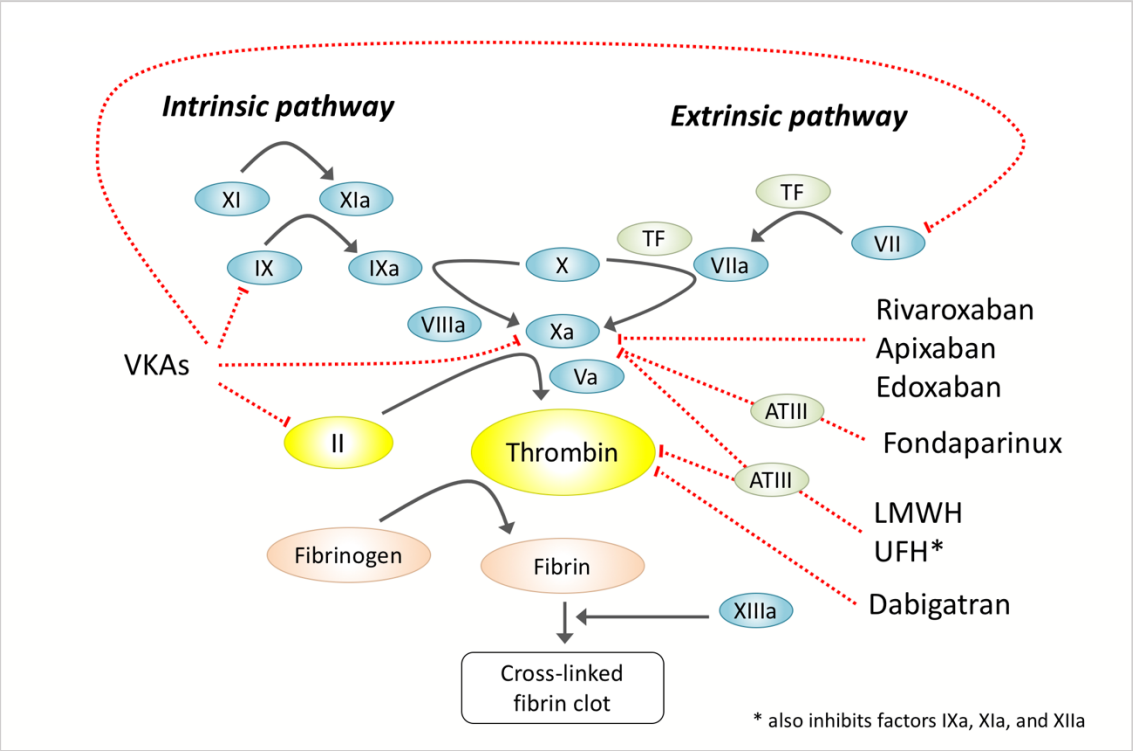


Figure 1.3. Mechanism of action of the most common anticoagulants in clinical use
Abbreviations used: TF, tissue factor; ATIII, antithrombin III; VKAs, vitamin K antagonists (i.e. warfarin); LMWH, low molecular weight heparin; and UFH, unfractionated heparin.

The major classes of anticoagulants used in clinical practice are unfractionated heparin, low molecular weight heparins (LMWHs), fondaparinux, vitamin K antagonists and direct oral anticoagulants (DOACs). Unfractionated and low molecular weight heparins bind to and increase the activity of antithrombin III, thereby potentiating factor Xa and thrombin inhibition [81]. Unfractionated heparin also inhibits factors IXa, XIa, and XIIa [82]. Fondaparinux potentiates the activity of antithrombin III against factor Xa [83], whereas vitamin K antagonists (e.g. warfarin) inhibit synthesis of clotting factors II, VII, IX, and X [84]. DOACs either directly inhibit thrombin (dabigatran) or factor Xa (rivaroxaban, apixaban and edoxaban; Figure 1.3) [84].

1.4.1 Benefits

Anticoagulants are of proven benefit in a wide range of thromboembolic disorders. In patients with or at risk of venous thromboembolism, their use is associated with a significant reduction in the incidence of recurrent non-fatal and fatal thromboembolic events [85–91]. Anticoagulants are also a mainstay in the treatment of inpatients with acute coronary syndrome, decreasing the risk of recurrent myocardial infarction and death [92–95]. Vitamin K antagonists reduce the incidence of thromboembolic complications in patients with mechanical heart valves and together with DOACs have proven effective in patients with atrial fibrillation at risk of embolic events [96–99].

1.4.2 Limitations

All current anticoagulants share the same major limitation as antiplatelet drugs, namely increased bleeding. Estimates of risk depend on the indication and population studied but in general warfarin and LMWHs are associated with a several fold increased risk of major bleeding as compared to placebo [97,100–103]. Warfarin-related bleeding or a high INR are among the most common iatrogenic causes of unplanned hospital admissions [104]. While DOACs have addressed many of the practical issues surrounding warfarin, reductions in bleeding have largely been limited to the treatment of patients with acute venous thromboembolism [98,105] and to a lesser extent atrial fibrillation (AF) [98]. Indeed, DOACs are not licensed for thromboprophylaxis in non-surgical patients due to an excess of bleeding [106] and are contraindicated in patients with mechanical heart valves owing to an increased incidence of ischaemic stroke and haemorrhagic events [107]. Thus, despite newer medications, treatment-related bleeding remains a significant problem and for many patients this leads to sub-optimal dosing or exclusion from anticoagulation altogether [102,105,108–112]. In AF, for

example, over one third of patients with a clinical indication for anticoagulation (CHADSVASC score ≥ 1) are not on any treatment and up to 50% prescribed a DOAC are inappropriately given a low dose because of bleeding concerns [108,109].

SUMMARY

Thrombotic disease remains one of the leading causes of global morbidity and mortality. Antithrombotic drugs have proven effective in the treatment and prevention of thrombotic and thromboembolic events in a wide range of clinical settings. However, despite progress, many at-risk individuals continue to experience life-threatening events. All the current antithrombotic agents are associated with an increased risk of bleeding that restricts their potential for greater efficacy and limits their net clinical benefit. A ceiling of treatment potential would appear to have been reached suggesting that if more effective antithrombotic protection is to be afforded to a wide range of patients, novel antiplatelet and anticoagulant strategies are likely to be required. Among those of greatest interest are targeting the platelet receptor PAR4 and exosite 1 of thrombin.

1.5 PROTEASE-ACTIVATED RECEPTOR 4

PAR4 belongs to a family of four specialised G-protein coupled receptors (PAR1 to 4) expressed on the surface of cells [113]. PAR4 is found on a number of cell types within humans and is activated by several proteases. These include thrombin, plasmin, trypsin and cathepsin G.

1.5.1 Platelet PAR4

Human platelets express both PAR4 and PAR1. Together, these receptors are the principal target for thrombin-mediated platelet activation [114]. PAR1 was the first PAR discovered, but to date, trials of PAR1 antagonists have generally disappointed with limited reductions in ischaemic events largely off-set by increased bleeding [9,80]. PAR4 was initially viewed as a weak platelet agonist [115,116]. However, it is now clear PAR4 is active at lower thrombin concentrations than first suggested with distinct signaling kinetics that may offer advantages over existing antiplatelet strategies [117–119].

1.5.1.1 PAR4 platelet signaling

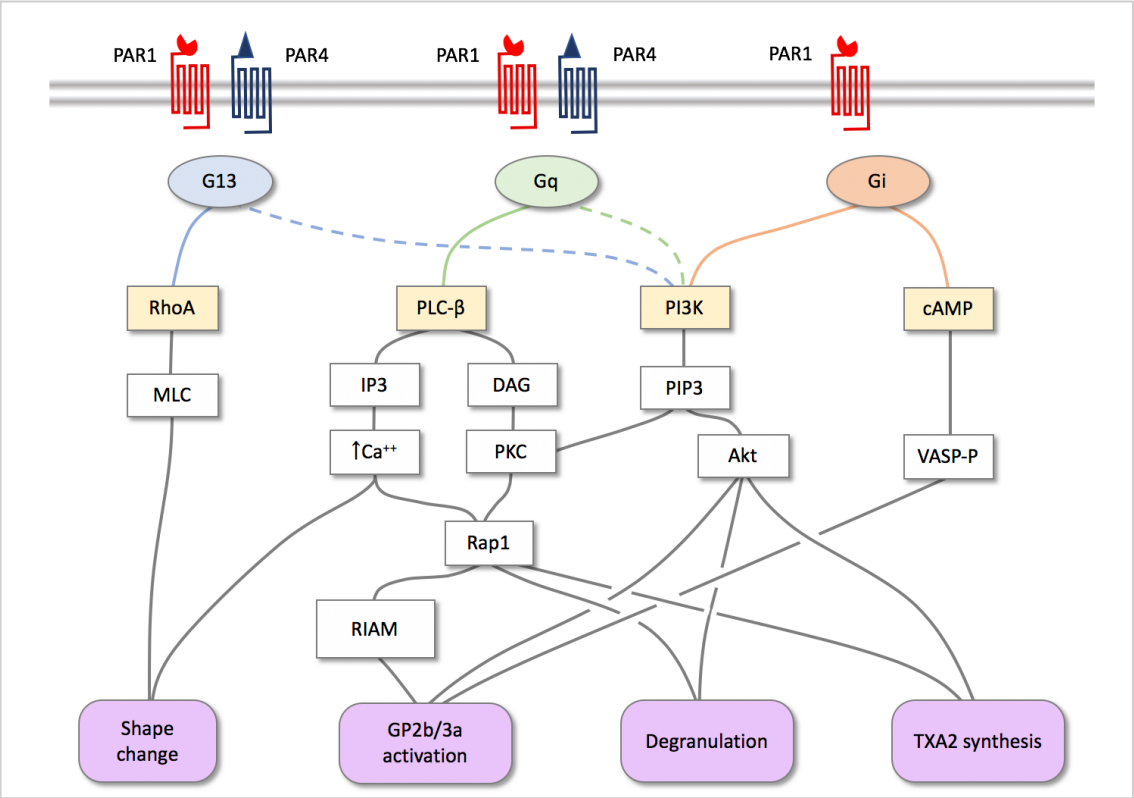


Figure 1.4. Simplified illustration of the major platelet PAR4 (and PAR1) intracellular signaling pathways.
Abbreviations used: Ca^{++}, calcium; DAG, diacylglycerol; IP3: inositol trisphosphate; PI3K, phosphatidylinositol 3-kinase; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C; Rap 1, ras-related protein 1; RIAM, rap1–GTP-interacting adapter molecule; RhoA, ras homolog gene family, member A; MLC, myosin light chain; akt, protein kinase B; cAMP, cyclic adenosine monophosphate; VASP-P, vasodilator-stimulated phosphoprotein-phosphorylated; and TXA2, thromboxane A2.

PAR receptors are activated in a unique manner. Proteolytic cleavage of the extracellular domain exposes a tethered ligand, which then binds intramolecularly to effect intracellular signal conduction [120]. PAR4 signaling is largely mediated by the G-protein subunits Gq and G13 [113,121]. Coupling to Gq induces platelet secretion, shape change and GPIIb/IIIa upregulation, mainly through activation of phospholipase C-β (PLC-β) [122]. G13 signaling triggers shape change via the Rho/Rho-kinase pathway (Figure 1.4) [123]. Phosphatidylinositol 3-kinase (PI3K) plays a major role in

inside-out platelet signaling [124]. Although PAR1 and to a lesser extent PAR4 may activate PI3K directly, the principal mechanism appears to through released ADP acting on Gi coupled $P2Y_{12}$ receptors [121,125].

1.5.1.2 Potential advantages of targeting PAR4

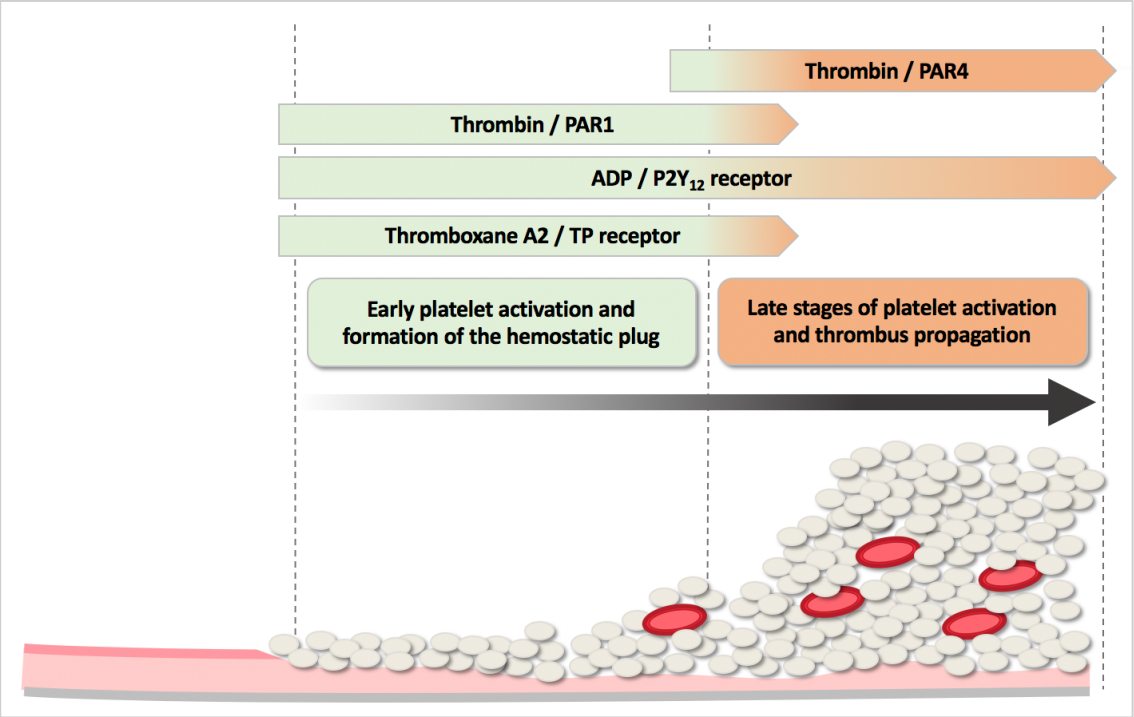


Figure 1.5. Illustration of the theoretical predominant roles of various agonists and receptors in platelet responses.

In response to thrombin, PAR1 and PAR4 signal with markedly different kinetics. PAR1 contains a hirudin-like sequence that interacts with exosite 1 [126] to facilitate rapid association and activation [127]. PAR4 does not contain this sequence but has optimized its cleavage sequence to allow high affinity interactions and contains an anionic cluster to slow dissociation [128]. Activation-dependent receptor phosphorylation and internalization are also significantly slower for PAR4 than for PAR1 [1].

PAR1 is therefore the high-affinity receptor for thrombin, resulting in a rapid intracellular calcium spike and platelet aggregation to low levels of agonist. However, activation is transient with PAR1 dependent on input from the $P2Y_{12}$ / PI3K pathway to sustain protein kinase C (PKC) signaling and platelet aggregation [40,129]. In contrast, PAR4 induces a slow but prolonged response to thrombin that, independent of additional support, acts to maintain the high late Ca^{2+} and PKC signals essential for irreversible aggregation [114,129,130]. Thus, whereas PAR1 and other agonist-platelet pathways appear to be important for initiating platelet activity, PAR4 appears to function primarily to sustain platelet aggregation and support thrombus growth (Figure 1.5). These data suggest that targeting PAR4 may protect against thrombosis, while at the same time, avoid interfering with early haemostatic platelet responses to the same extent as PAR1 antagonists and other antiplatelet agents [131].

1.5.1.3 Existing PAR4 antagonists

Pepducins

Pepducins are cell-penetrating peptides that bind to G proteins, modulating receptor and G-protein interactions to effect downstream signaling [132]. The anti-PAR4 pepducin, P4pal-10 (N-pal-SGRRYGHALR-NH2), has been shown to inhibit PAR4-activating peptide (PAR4-AP) and thrombin stimulated human platelet aggregation [132]. P4pal-i1 (N-pal-ATGAPRLPST-NH2) is an alternative anti-PAR4 pepducin that targets the first rather than the third intracellular loop. In a guinea pig carotid artery injury model, P4pal-i1 was found to prolong the time to occlusion [117]. There remains concern over the lack of specificity of anti-PAR4 pepducins [133,134] although in the study by Leger and colleagues, P4pal-i1 demonstrated little cross-inhibition [117].

Peptidomimetics

Peptidomimetics are based on the sequence of a naturally occurring protein, interacting with the target to either produce the same biological effect or competitively block function. tc-YPGKF-NH2 is a peptide analogue of the PAR4 tethered ligand sequence with the addition of a trans-cinnamoyl (tc) group. It has been shown to block thrombin-induced platelet aggregation in rodents [135] but has limited capacity against human PAR4 [136,137].

Low molecular weight compounds

Low molecular weight PAR4 antagonists include YD-3 (1-benzyl-3 (ethoxycarbonyl-phenyl)-indazole) [138], an indazole derivative, and more recently ML354 (1-methyl-5-nitro-3-phenyl-1H-indole-2-methanol) [139], a substituted indole-based derivative. These compounds inhibit PAR4-AP stimulated human platelet aggregation [140,141]. However, YD-3 is hampered by a lack of efficacy [142] and both suffer from a lack of specificity [141].

Function-blocking antibodies

Function-blocking antibodies selective for PAR4 have been developed [143]. They have been shown to prevent human platelet aggregation [144] and in animal models inhibit thrombosis with a much wider safety profile compared to clopidogrel [131]. Multiple new function-blocking anti-PAR4 antibodies are under investigation but to date no human studies have been reported [145].

1.5.1.4 BMS-986120

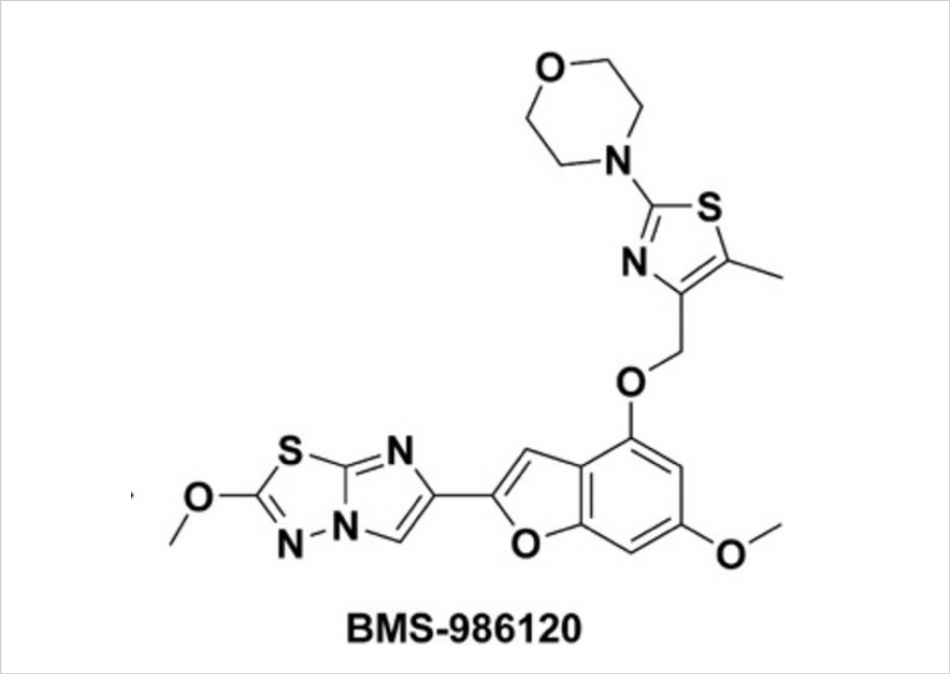


Figure 1.6. Chemical structure of BMS-986120

BMS-986120 is a high-affinity, potent, selective and reversible small-molecule PAR4 antagonist (Bristol-Myers Squibb; Figure 1.6). It is the first orally bioavailable PAR4 antagonist.

In vitro studies

In vitro studies have demonstrated the potency and selectivity of BMS-986120 for inhibiting human PAR4 [131]. BMS-986120 abolished PAR4-AP induced calcium mobilization in HEK cells expressing recombinant human PAR4 but was ineffective against PAR1-AP or PAR2-AP. Moreover, BMS-986120 strongly inhibited aggregation of human platelets to gamma-thrombin (a derivative of thrombin that is selective for PAR4) and PAR4-AP, but had no effect against PAR1-AP, collagen, ADP or the thromboxane A2 receptor agonist, U46619 [131].

In vivo animal studies

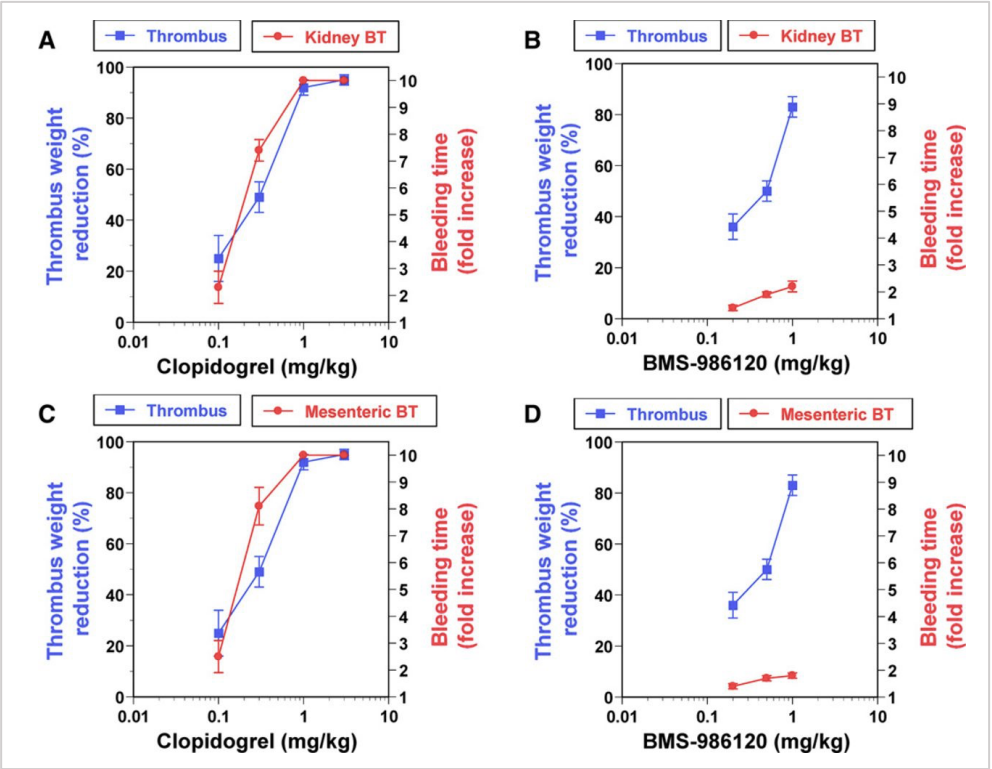


Figure 1.7. Dose-dependent effect of clopidogrel and BMS-986120 on thrombus reduction and bleeding times (BTs) in cynomolgus monkeys.
(A) Effects of clopidogrel on thrombus reduction and kidney BT. (B) Effects of BMS-986120 on thrombus reduction and kidney BT. (C) Effects of clopidogrel on thrombus reduction and mesenteric BT. (D) Effects of BMS-986120 on thrombus reduction and mesenteric BT. Thrombus reduction was expressed as a percent reduction of drug-treated relative to the mean vehicle-treated thrombus weight. BT effect was expressed as a ratio of drug-treated versus the mean vehicle-treated value. Data are means ± SEM. Figure reproduced from Wong et al., Sci Transl Med. 2017.

Wong and colleagues examined the pharmacodynamic effects of BMS-986120 in animal models of thrombosis and bleeding [131]. In a cynomolgus monkey model of carotid injury, BMS-986120 (1 mg/kg) maintained blood flow close to baseline and reduced thrombus formation by 82%. PAR4 antagonism with BMS-986120 also demonstrated substantially less impact on bleeding times relative to clopidogrel resulting in a much wider therapeutic index (Figure 1.7).

Human studies

The safety and tolerability of BMS-986120 has been assessed in a double-blind, placebo-controlled, single- and multiple-ascending dose study [146]. BMS-986120 inhibited PAR4-AP stimulated ex vivo platelet aggregation and p-selectin expression in a dose-dependent manner but had no clinically relevant effect on routine laboratory assessments including coagulation tests or template bleeding times. There were no drug-related discontinuations or bleeding-related clinical findings, and the frequency of treatment-related adverse events was similar to placebo.

1.5.2 Wider role of PAR4

Acting via PAR1, thrombin and other proteases elicit a number of important vascular effects beyond platelet activation and aggregation. These include endothelium-dependent arterial vasodilatation, endothelium-independent venoconstriction, release of tissue plasminogen activator, smooth muscle cell hypertrophy, proliferation and migration, and extracellular matrix synthesis [27,147,148]. Activation of PAR1 also plays an important role in the maintenance of normal endothelial integrity [149] and it is proposed interference of this protective function may have contributed to the increased bleeding seen with PAR1 antagonists [150]

PAR4 receptors are also expressed on a number of vascular cell types, including smooth muscle and endothelial cells [151–153]. In humans, functional PAR4 has been demonstrated in isolated coronary artery segments [151] with elevated expression reported in the tunica media of atherectomy and saphenous vein tissue from patients with diabetes mellitus [153]. Data from animal studies suggests PAR4 may be involved in the control of vascular tone, ischaemia reperfusion injury, and the exaggerated

intimal hyperplasia seen in diabetic conditions [137,154–156]. However, inter-species differences in PAR function and activation may limit extrapolation of these results to man [157,158] and to date there have been no studies examining the in vivo vascular effects of PAR4 in humans.

Like PAR1, the vascular functions of human PAR4 may be diverse but are essentially unknown. Determining such effects would not only deepen our understanding of vascular biology but may have important implications regarding the safety and effectiveness of PAR4 inhibitors as future antiplatelet agents. We have previously characterised the in vivo vasomotor and endothelial effects of PAR1 agonism in healthy volunteers through local venous and arterial infusion of a PAR1-AP [27,148]. Similar studies with a PAR4 agonist can be expected to provide major insights and a number of highly specific PAR4-AP compounds are readily available. However, to date, there have been no reports of any PAR4 agonist being administered to man. This is an issue, particularly because in addition to vascular cells, PAR4 is found on neutrophils [159], alveolar macrophages [160], neurons [161], synovial cells [162], and within the kidneys [163,164], airways [165] and liver [166]. Indeed, topical administration of a PAR4 agonist to mice mesenteric venules increased leucocyte rolling and adhesion, while intraperitoneal injection promoted leukocyte migration [159]. In rodents, intra-plantar and intra-articular injection of a PAR4 agonist resulted in localized oedema and granulocyte infiltration [137,167–169], whereas intranasal PAR4 agonism induced the recruitment of a small number of neutrophils into the airways of mice [160]. Studies have also suggested PAR4 may play a role in modulating nociceptive responses, which are both tissue and dose specific [167,169–171].

Given the multi-tissue and multi-organ distribution of PAR4, it would be crucial to examine the safety and tolerability of systemic PAR4 agonism in animals prior to undertaking in vivo studies in man. Such work would provide the platform for essential research into the wider actions of PAR4 in the human vasculature and future of PAR4 antagonists.

SUMMARY

PAR4 is a G-protein coupled receptor on the surface of human platelets as well as a number of other cell types within the vasculature and beyond. Activated by several proteases including thrombin, mechanistic studies suggest the principal role of platelet PAR4 appears to be in the later stages of platelet activation, primarily acting to sustain thrombus growth and propagation. Therefore, it has been hypothesised that targeting PAR4 may provide effective antithrombotic protection while at the same avoid interfering with haemostatic platelet responses to the same extent as current agents. BMS-986120 is a first-in-class, highly selective oral PAR4 antagonist that in animal models of thrombosis and bleeding demonstrated a much wider therapeutic index as compared to clopidogrel. However, it remains to be determined what effect BMS-986120 might have on thrombus formation when administered to humans and how this compares to the current standard of care antiplatelet agents.

Like PAR1, the vascular functions of PAR4 may extend beyond platelets but remain largely unknown. Defining these effects would not only add to our understanding of vascular biology but may have important implications regarding

the effectiveness and safety of PAR4 inhibitors as future antiplatelet agents. Our group has previously examined the in vivo effects of PAR1 agonism on vascular tone and endothelial function but to date no PAR4 agonist has ever been given to humans. Because of the potential for unwanted haemodynamic, inflammatory and end-organ effects, it would be essential to first examine the safety and tolerability of PAR4 agonism in animals.

1.6 THROMBIN EXOSITE 1

Exosite 1 of thrombin (hereafter exosite 1) is an electropositive region on the surface of thrombin that together with exosite 2 plays an important role in regulating catalytic function of the protease. In general, exosite 1 binds thrombin to substrates, cofactors and inhibitors to promote access to the catalytic site, whereas exosite 2 localises thrombin to the surface of cells or proteins [31–33,172]. Both exosites promote procoagulant, prothrombotic and anticoagulant effects depending on the interaction.

Exosite 1 is the predominant fibrinogen recognition site for thrombin, greatly enhancing fibrin production [173]. Exosite 1 also binds to PAR1 to support thrombin-mediated platelet activation [127,174], thrombomodulin [42], and ADAMTS13, a plasma protease responsible for cleaving anchored large multimers of von Willebrand factor [175]. Exosite 2 anchors thrombin to platelet GP1b, thereby facilitating activation of PAR1, GPV and factor XI [176], and GAGs [42]. Both exosites assist feedback activation of clotting factors V and VIII [177].

1.6.1 Potential advantages of exosite 1 thrombin inhibition

All the currently licensed anticoagulants act to either inhibit thrombin generation, promote antithrombin III activity or block the active site of the protease directly [178]. Consequently, they result in broad inhibition of thrombin function that invariably fails to discriminate between protease interactions relating to thrombosis and those essential to haemostasis. Targeting exosite 1 allows a more selective approach to thrombin inhibition, preventing fibrinogen binding while still maintaining catalytic function of the protease and exosite 2 substrate recognition. This capacity to avoid overly

interfering with other thrombin activities relating to haemostasis may offer advantages in terms of affording a wider therapeutic index [131,179].

Early evidence

Functional blocking aptamers specific to exosite 1 and exosite 2 have been used to probe thrombin exosite function and examine the potential of exosite 1 thrombin inhibition. Using aptamers, Derszniak and colleagues demonstrated exosite 1 but not exosite 2 thrombin antagonism potently inhibited fibrin generation and ex vivo thrombus formation under flow conditions [180]. Similar outcomes were observed in a cone and plate chamber model [181]. In the same study, exosite 1 thrombin inhibition was found to have no effect on platelet adhesion. This potentially haemostatic sparing effect is consistent with reports exosite 1 thrombin inhibition does not affect collagen-induced platelet responses [182] or the ability to form haemostatic plugs [183].

Further insights into the possible safety of exosite 1 thrombin inhibition come from a unique case report [184]. A patient presenting with a large traumatic subdural haematoma was found to have markedly prolonged coagulation times (prothrombin time, 40 s; activated partial thromboplastin time, 240 s; thrombin time, 173 s) owing to the presence of an anti-exosite 1 thrombin IgA antibody. Despite persistence of the antibody and continued evidence of intense anticoagulation, the patient made a full recovery without surgical intervention and had no abnormal bleeding events during 8 years of follow up.

1.6.2 JNJ-64179375

JNJ-64179375 (hereafter JNJ-9375; Janssen Research and Development) is a first-in-class, recombinant, fully human, IgG4 monoclonal antibody synthesized to mimic the anticoagulant effects of the anti-exosite 1 thrombin IgA antibody described above. It binds reversibly and with high affinity and specificity to the exosite 1 region of thrombin [179]. Animal studies of thrombosis and bleeding have raised considerable interest that exosite 1 thrombin inhibition with JNJ-9375 may represent a paradigm shift in anticoagulant therapy [179]

JNJ-9375 has been shown to inhibit fibrinogen cleavage in a dose-dependent manner and reduce thrombomodulin-dependent activation of protein C approximately 7-fold [185]. Confirming selectively of action, JNJ-9375 had no effect on the interaction of thrombin with heparin and both unfractionated heparin and LMWH continued to dramatically accelerate antithrombin III protease inactivation [185]. In both rat and cynomolgus monkey models, JNJ-9375 potently inhibited thrombus formation with a much wider therapeutic index (TI) when compared to apixaban (TI 33 vs. 3 in rats; 40 vs. 10 in monkeys).

SUMMARY

Exosite 1 thrombin inhibition is an attractive novel mechanism of anticoagulation because of the potential for antithrombotic efficacy without the same bleeding risk as current agents. JNJ-9375 is a first in class exosite 1 thrombin inhibitor that in animal models demonstrated potent antithrombotic effects with a substantially wider therapeutic index when compared to apixaban. However, inter-species differences in procoagulant pathways limit extrapolation of these results to

humans [186] **and while unique insights into the low bleeding potential of JNJ-9375 have been afforded by the antibody it was synthesized to mimic, it remains to be determined what effects exosite 1 thrombin inhibition with JNJ-9375 has on human thrombosis.**

1.7 ASSESSMENT OF PLATELET AGGREGATION

Platelet aggregometry is critical to the evaluation of patients with suspected platelet dysfunction and plays a central role in the development of novel antithrombotic agents. By determining the effect of a drug on platelet responses to a range of known agonists, aggregometry provides crucial information on the mode of action as well as the potential for in vivo efficacy. Consequently, platelet aggregation is essential for the characterisation and therapeutic assessment of platelet PAR4 and exosite 1 thrombin inhibition.

The current 'gold-standard' technique for platelet function testing is light transmission aggregometry (LTA) [187]. Established in the 1960's, LTA utilises changes in sample turbidity that occur when platelets aggregates [188,189]. However, because of the volume required for each test (typically >200 μL) and the limited number of channels available (typically between 4 and 8), LTA can be restrictive in terms of material costs and the range of test conditions examinable per blood draw [190]. LTA is also considered labour intensive and technically challenging.

Ninety-six-well plate aggregometry (96-WPA) is a newer method of assessing platelet aggregation developed to address some of the limitations of LTA. Like LTA, 96-WPA measures changes in turbidity, but requires less than half the sample volume per test and allows for simultaneous examination of a much wider range of study conditions owing to the large number of channels available (n=96) [190,191]. 96-WPA does not provide as much secondary information compared to LTA (e.g. occurrence of the secondary wave of aggregation) but would seem ideally suited for many research and clinical applications that require probing of platelet function [190–192]. Platelet

aggregation is essential to this thesis and the technical advantages of 96-WPA could provide additional opportunities. However, before one test can replace another, it is essential to establish the test-retest repeatability and agreement between the two techniques. This is currently lacking.

1.8 AIMS

Following preliminary work to validate a new method of platelet aggregation, the principal aims of this thesis were to evaluate the effects of PAR4 and exosite 1 thrombin inhibition on human platelet responses, coagulation and ex vivo thrombus formation. We also sought to examine the safety and tolerability of systemic PAR4 agonism in mice in order to facilitate future research into the wider vascular role of human PAR4.

1.9 HYPOTHESES

The individual hypotheses of this thesis were:

1. 96-WPA will demonstrate similar test-retest repeatability to LTA with good agreement (Chapter 3).
2. Human platelet responses to PAR4 activation will be independent of signaling from other major agonist-receptor pathways (Chapter 4).
3. Intravenous administration of the PAR4 agonist AYPGKF-NH2 to mice will be well tolerated with no signs of systemic or organ toxicity (Chapter 5).
4. Oral PAR4 antagonism with BMS-986120 will selectively inhibit human PAR4-mediated platelet responses with no effect on coagulation (Chapter 6).
5. Oral PAR4 antagonism with BMS-986120 will inhibit ex vivo human thrombus formation with greater effect at high shear (Chapter 6).
6. Exosite 1 thrombin antagonism with JNJ-9375 will prolong human coagulation but have limited effect on platelet aggregation (Chapter 7).
7. Exosite 1 thrombin antagonism with JNJ-9375 will inhibit ex vivo human thrombus with greater effect at low shear (Chapter 7).

Chapter 2

Methods

2 METHODS

2.1 HUMAN STUDIES

2.1.1 Study subjects

Study populations are described in detail in their respective chapters. Only healthy male and female volunteers were recruited. Exclusion criteria included any clinically significant coexisting condition including hypertension, hyperlipidaemia, diabetes mellitus, gastrointestinal disease, coagulopathy, known liver disease, and recent infective or inflammatory condition. Volunteers were not permitted to take any prescription or non-prescription medication (including acetylsalicylic acid, paracetamol, vitamins and herbal supplements) within at least 7 days (study specific) of an experimental visit. Prior to each visit, volunteers must have abstained from alcohol for at least 24 hours (study specific) or food including caffeine-containing products for at least 8 hours (study specific).

Volunteers were not permitted to be involved in other clinical trials at the time of the study.

2.1.2 General ethical considerations and consent

Each study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki of the World Medical Association. All volunteers were given adequate time and opportunity to discuss their potential involvement and provided written informed consent prior to any study procedure.

2.1.3 Study specific ethical considerations

2.1.3.1 Effects of PAR4 antagonism in healthy volunteers

This was a phase I parallel group prospective randomized open-label blinded endpoint (PROBE) trial of a novel oral PAR4 antagonist. The study was conducted in accordance with Good Clinical Practice, as defined by the International Conference on Harmonisation, and the ethical principles underlying European Union Directive 2001/20/EC and the United States Code of Federal Regulations, Title 21, Part 50 (21CFR50). The study is registered on the clinicaltrials.gov website (NCT02439190). Clinical Trial Authorization was provided by the Medicines and Healthcare products Regulatory Agency of the United Kingdom.

2.1.4 Recruitment

Potential participants were recruited through paper and digital advertisement restricted to University of Edinburgh public places or from the existing research database of volunteers who had agreed to be contacted regarding inclusion in future studies. An email address was provided for potential participants to register their interest in taking part. A member of the study team then contacted the participant and provided an information sheet. If the participant was interested in taking part, a screening and study appointment was arranged.

2.1.5 Study sites and conditions

All study visits were conducted at the Queen's Medical Research Institute (QMRI) of Edinburgh or the Clinical Research Facility at the Royal Infirmary of Edinburgh. Studies were performed in a quiet, temperature-controlled room (22-24 °C) with the participants semi-recumbent.

2.1.6 Determination of drug concentrations

Plasma concentrations of BMS-986120 were determined from blood samples drawn uncuffed through a 17-G cannula in the ante-cubital fossa into 3 mL K_2EDTA vacutainers (Becton-Dickinson, Cowley, UK) and placed on wet ice. Within 1 hour of collection, samples were centrifuged at 1200 *g* (2-8 °C) for 10 minutes. Plasma was decanted and stored at -20 °C before analysis. From these samples, concentrations of BMS-986120 were determined using a validated liquid chromatography tandem-mass spectrometry method with a lower limit of quantification of 0.250 ng/mL, accuracy coefficient of variation of <5 % and precision (intra- and inter-assay) coefficients of variation of <10 %.

Serum JNJ-9375 and plasma bivalirudin concentrations were determined from blood samples taken immediately distal to the perfusion chamber into 3.5 mL serum gel and 2.7 mL sodium citrate (3.2%) tubes (Becton-Dickinson, Cowley, UK), respectively. JNJ-9375 samples were allowed to clot for 30 minutes then centrifuged at 1500 *g* (20 °C) for 20 minutes. Bivalirudin samples were centrifuged at 1500 *g* (15 °C) for 15 minutes within 1 hour of collection. Samples were then aliquoted and stored immediately at -70 °C before analysis. Concentrations of JNJ-9375 were determined by electro-chemiluminescence using the Meso Scale Discovery platform and plate reader (Rockville, Maryland, USA). Values were then regressed from the standard curve in Watson LIMS (version 7.4.1, Thermo, PA, USA) using a five-parameter logistic regression model with $1/Y^2$ weighting.

2.1.7 Platelet studies

2.1.7.1 Blood sampling

All blood samples were drawn uncuffed through a 17-G cannula in the ante-cubital fossa. For each sample, the first 2.5 to 5 mL of blood was discarded so as to avoid including platelets potentially activated from cannulation.

2.1.7.2 Assessment of platelet activation

Platelet p-selectin expression and platelet-monocyte aggregates are sensitive markers of in vivo platelet activation. They are routinely used as markers of platelet activation in response to clinical conditions, stimuli or drugs [193–195]. In all experiments, platelet p-selectin expression and platelet-monocyte aggregates were determined by flow cytometry.

Whole blood was collected into either D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Enzo Life Sciences, Exeter, UK; final concentration 750 nM) or sodium citrate (final concentration 0.38%) then aliquoted into micro-centrifuge tubes pre-filled with or without agonist and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. For studies involving thrombin, Pefabloc FG (final concentration 1.5 mg/mL; Quadratech Diagnostics, Surrey, UK) was added prior to the addition of agonists. Thrombin stimulation can provoke extensive fibrin formation that precludes flow cytometry. Pefabloc FG prevents this but does not interfere with platelet activation. Protocol specific details are provided in each chapter.

Samples were incubated for 15-20 minutes at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within 24 hours using a FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

2.1.7.3 Assessment of platelet aggregation

Blood samples were drawn uncuffed from the antecubital fossa through a 17-G needle into sodium citrate (final concentration 0.38%). To obtain platelet-rich plasma (PRP), samples were immediately centrifuged at 250-300 *g* (room temperature) for 15 minutes. Platelet-poor plasma (PPP) was obtained by centrifugation of 5 mL of PRP at 5000 g for 6 minutes. PRP and PPP were then stored in a water bath at 37 °C prior to use.

Light transmission aggregometry (LTA) was performed with the 8-channel PAP-8E aggregometer (Bio/Data Corp, Horsham, PA, USA). Prior to each test, each channel was blanked for reference with 250 μL of PPP. Two hundred and twenty-five mL of PRP was added to a channel maintained at 37 °C followed by 25 μL of agonist. Peak aggregation was recorded at the end of 12-15 minutes.

Ninety-six-well plate aggregometry was performed using a Synergy HT plate reader (Biotek). Immediately prior to each test, 11.1 μL of agonist was added to a well. Using a multi-pipette, 100 μL of PRP was added by reverse pipetting and the plate then immediately placed in the reader. Absorbance was determined at 595 nm every 15 s for 12 minutes between vigorous shaking at 37 °C. Changes in absorbance were converted

to percentage aggregation by reference to the absorbances of PRP and PPP. Protocol specific details are provided in each chapter.

2.1.8 Ex-vivo thrombus formation

2.1.8.1 Badimon perfusion chamber

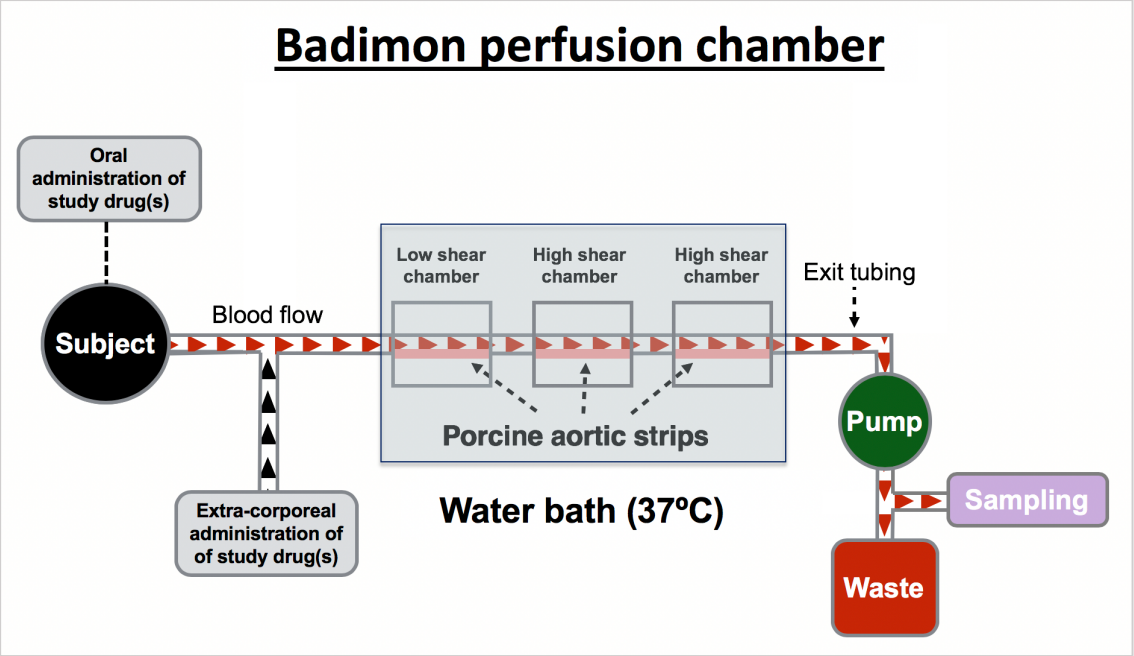


Figure 2.1. Schematic of the Badimon perfusion chamber

Vessel wall injury and examination of the effects of antithrombotic agents on thrombus formation cannot be undertaken safely in vivo in humans. While such agents may alter biomarker measures of platelet activity (e.g. platelet aggregation) or coagulation, ex vivo thrombosis assays provide more detailed assessment of the potential effects on thrombus formation.

For all experiments in this thesis, thrombus formation was assessed using the Badimon chamber (Figure 2.1). This is one of the most validated perfusion models for measuring the effect of study drugs on ex vivo human thrombus formation [196–203]. A major

advantage of the Badimon chamber is that it permits assessment using whole blood without the need for anticoagulants. Study drugs can be administered orally or through an extracorporeal circuit thereby avoiding exposure of the volunteer to the study compound if desired or indicated.

The Badimon chamber has been extensively described [196–203]. In brief, a pump is used to draw native blood from the antecubital vein directly through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. In all our experiments, each chamber contained a strip of porcine aorta from which the intima and a thin layer of media has been removed. Porcine aorta closely resembles the structure of a human artery and by removing the intima and a thin layer of media, the model represents one of vascular and deep arterial injury (e.g. plaque rupture). After the porcine strips are sited, warm saline (37°C) is drawn through each chamber. This serves to ensure each strip is at body temperature and that any loose surface material is removed. Next, blood is drawn through the chambers and as it flows across the strips, thrombus forms. At the end of the study, the strips are again washed with warm saline (37°C) for precisely 1 minute to remove any blood and unbound thrombus. The strips are then removed, sectioned and stained for thrombus quantification.

Preparation of aortic strips

The porcine aortic strips were prepared from whole untouched aorta (Pel-Freez Biologicals). First, the arch and distal aorta were removed along with any branches, excess perivascular adipose or other tissue. The prepared aorta was then opened out by incising rostral to caudal along the mid-anterior surface and strips cut using a template to ensure a standard size. Finally, each strip was carefully denuded by making a small

incision and removing the intima and a thin layer of media from rostral to caudal. Each strip was checked under a microscope to ensure there were no tears, flaps or loose tissue as this may lead to exaggerated thrombus formation.

Rheological conditions

Table 2.1. Shear rate

Lumen diameter (mm)	Blood flow (mL/min)	Wall shear rate (s^{-1})
1.0	5	840
	10	1680
	20	3360
	30	5040
	40	6720
2.0	5	105
	10	212
	20	425
	30	640
	40	850

Rheological conditions in the Badimon chamber can be altered to mimic different vascular compartments. In each study, flow conditions in the first chamber were set to simulate those of patent medium-sized arteries (inner lumen diameter, 2.0 mm; vessel wall shear rate, 212 s^{-1}; mean blood velocity, 5.3 cm/s; Reynolds number: 30), whereas those in the second and third chambers were set to simulate those of mild to moderately stenosed coronary arteries (inner lumen diameter, 1.0 mm; vessel wall shear rate: 1690 s^{-1}; mean blood velocity, 21.2 cm/s; Reynolds number: 60). Shear conditions at the vessel wall were calculated from the theoretical expression for shear rate given for a Newtonian fluid in tube flow (Table 2.1) [204,205]. Each study lasted for exactly 5 minutes during which flow was maintained at a constant rate of 10 mL/min. All studies were performed using the same perfusion chamber and by the same operator.

2.1.8.2 Histomorphometric analysis

As thrombus forms along the entire length of the exposed porcine aortic strip, the mean transverse cross-sectional area gives a reliable reflection of total thrombus [206]. Following fixation, the proximal and distal 1 mm of the exposed substrate were discarded, and the remainder cut into eight segments. Individual segments were then embedded in paraffin wax from which 4-μm sections were prepared for histomorphometric analysis.

Total thrombus area

To detect total thrombus area, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (Leica Microsystems GmbH, Wetzlar, Germany) for 5 minutes. Sections were then incubated at room temperature for 1 hour with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL, Dako, Glostrup, Denmark) and monoclonal mouse anti-human CD61 antibody (1.28 μg/ml, Dako). Antigen visualization was performed using a Bond Polymer refine detection kit (Leica Microsystems GmbH) and treatment with 3,3'-diaminobenzidine substrate chromogen (66 mM, Dako). Finally, sections were counterstained with a modified Masson's trichrome (hematoxylin and sirius red 0.1 %).

A semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany) and image analysis software (Definiens, Munich, Germany) were used to quantify total thrombus area. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established, allowing automated detection of total thrombus area with a high level of precision.

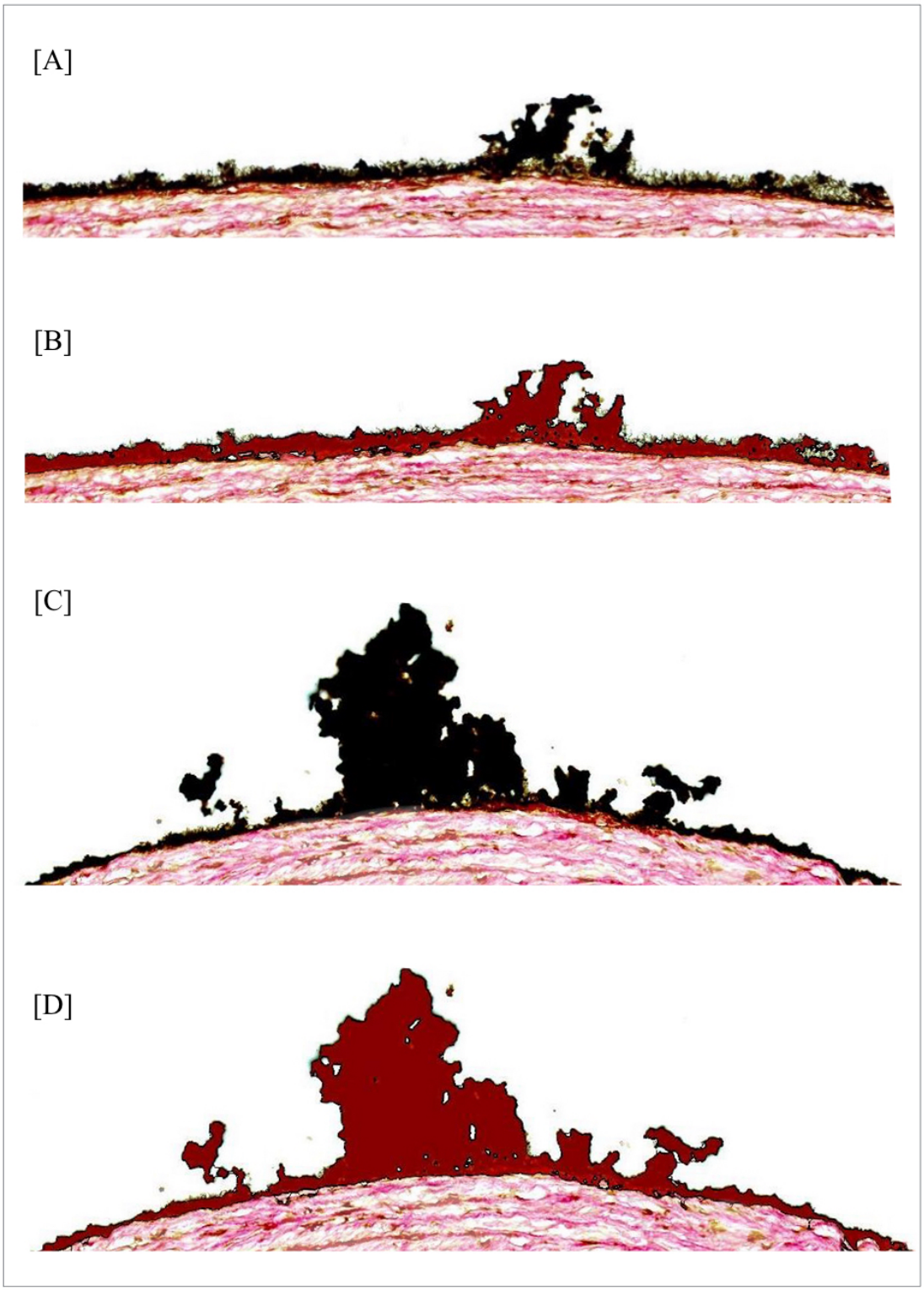


Figure 2.2. Representative cross-section examples (x20 magnification) of thrombus formed at [A] low shear and [C] high shear in the Badimon perfusion chamber. High-resolution classifiers based on colour were established allowing automated detection of total thrombus area [B+D] with a high level of precision.

Fibrin- and platelet-rich thrombus area

To examine the effect of study drug(s) on fibrin-rich and platelet-rich thrombus formation, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (VWR, Radnor, PA, USA) for 10 minutes and non-specific binding blocked using 20 % normal goat serum (Biosera, Nuaille, France) in Tris-Buffered Saline with 0.01% Tween (TBST)). Sections were then incubated with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL) to detect fibrin and CD61 monoclonal mouse anti-human antibody (0.32 μg/mL) to detect platelets. Following TBST washes, goat anti-rabbit peroxidase (1:500; Abcam, Cambridge, UK) was applied and the presence of antigen visualized with Tyramide Cy3 (1:50; Perkin Elmer, Boston, MA, USA) and FITC (1:50; Perkin Elmer, Waltham, MA, USA) before nuclear counterstaining with DAPI (5 μg/mL; Sigma-Aldrich).

As for detection of total thrombus formation, a semi-automated slide scanner (Axioscan Z1) and image analysis software (Definiens) were used to quantify thrombus composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established, allowing automated detection of fibrin-rich and platelet-rich thrombus formation with a high level of precision.

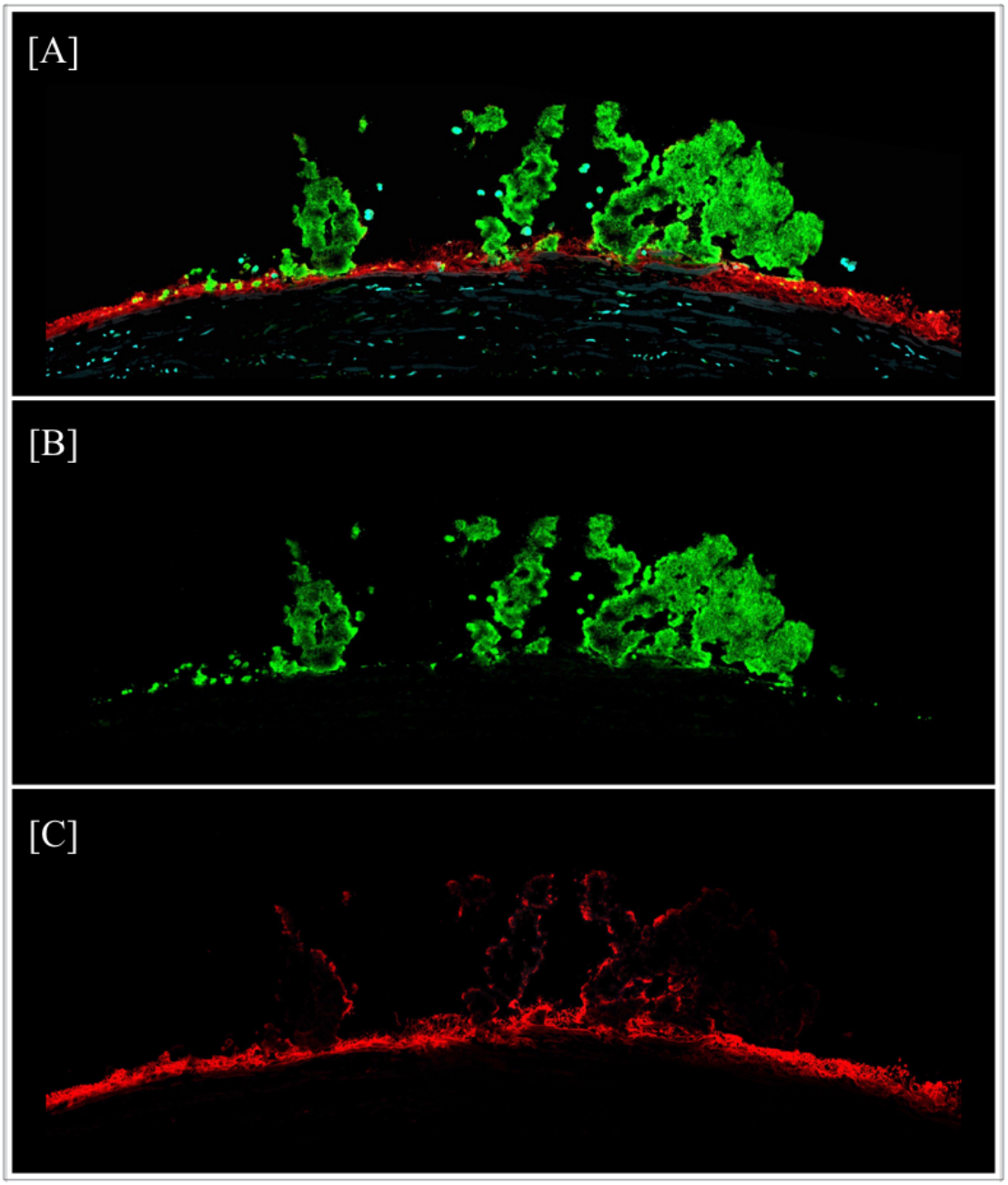


Figure 2.3. Representative images of immunofluorescent staining of thrombus formed at high shear in the Badimon chamber.
[A] Sections were stained with CD61 monoclonal mouse anti-human antibody to detect platelets (green) and polyclonal goat antihuman fibrin(ogen) antibody to detect fibrin (red) before nuclear counterstaining with DAPI (light blue. Channels were then split to allow quantification of [B] platelet-rich thrombus and [C] fibrin-rich thrombus. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established, allowing automated detection of fibrin-rich and platelet-rich thrombus formation with a high level of precision.

Controls

Prior to the first experimental sample, non-specific binding of the primary antibodies was excluded using tissue negative controls (perfusion chamber porcine sections exposed to saline rather than blood). To ensure staining for platelets and fibrin(ogen) was the result of detection of the intended antigen, secondary antibody controls (with the primary antibody absent) were run in parallel for each volunteer. No labelling was observed.

2.2 ANIMAL STUDIES

2.2.1 Animals

Male Rodentia mice aged between 10 and 12 weeks were purchased from Charles River (UK).

2.2.2 Housing

Mice were housed in the animal unit of the University of Edinburgh and fed a diet of normal chow.

2.2.3 Ethics

All experiments were conducted in accordance with the UK Scientific Procedures Act 1986 and had local institutional ethics approval.

2.2.4 Dosing

Fifteen mice exhibiting normal behaviour in the observation period were selected for dosing. Study drug (AYPGKF-NH2) or control (0.9% saline) was administered by intravenous tail vein injection.

2.2.5 Observation and fate of mice

Following study drug administration, mice were observed for 24 hours for signs of distress including pain, fever or abnormal behaviour. All mice were then sacrificed by intraperitoneal injection of Euthatal followed by withdrawal of the maximum volume of blood possible (via the abdominal vena cava or heart) directly into sodium citrate (final concentration 0.32% sodium citrate) and dissection.

2.2.6 Laboratory analyses

Standard clinical chemistry assays for bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), albumin, alkaline phosphatase, urea and creatinine were performed on whole blood.

Creatinine

Creatinine was determined using the creatininase / creatinase specific enzymatic method described by Börner and colleagues [207] utilising a commercial kit (Alpha Laboratories Ltd., UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., UK). Within run coefficient of variation (CV) was < 3% while intra-batch CV was < 5%.

Urea

Urea was determined by hydrolysis and measurement of NAD production (monitored at 340 nm) in the presence of glutamate-dehydrogenase. The enzymatic method employed was a commercial kit from Alpha Laboratories Ltd. adapted for use on a Cobas Fara centrifugal analyser. Within run CV was < 3% while intra-batch CV was < 5%.

Albumin

Serum albumin measurements were determined using a commercial serum albumin kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara centrifugal analyser. The measurement of serum albumin is based on its quantitative binding to bromocresol green (BCG). The albumin-BCG-complex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration in the sample. Within run CV was < 2.5% while intra-batch CV was < 4%.

Bilirubin

Total bilirubin was determined by the acid diazo method described by Pearlman and Lee [208] using a commercial kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara centrifugal analyser. In this method a surfactant is used as a solubiliser. Conjugated and solubilised unconjugated bilirubin react with diazotised sulphanilic acid to produce an acid azobilirubin, the absorbance of which (measured at 550 nm) is proportional to the concentration of bilirubin in the sample. Within run CV was < 4% while intra-batch precision was CV < 5%.

Alanine Aminotransferase

Alanine Aminotransferase (ALT) was measured using the method described by Bergmeyer and colleagues [209] utilising a commercial kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara analyser. Within run CV was < 4% while intra-batch CV was < 8%.

Aspartate Aminotransferase

Aspartate Aminotransferase (AST) was determined by a commercial kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara analyser. Alpha-oxogluterate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction utilises the oxaloacetate for a kinetic determination of NADH consumption. Within run CV was < 4% while intra-batch precision CV was < 5%.

Alkaline Phosphatase

Alkaline Phosphatase (ALP) was determined by a commercial kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara analyser. In the presence of magnesium ions, the

substrate p-nitriphenyl phosphate is hydrolysed by ALP from the sample to form p-nitophenol which is yellow in colour and can be monitored at 405 nm. Within run CV was < 4% while intra-batch CV was < 5%.

2.2.7 Flow cytometry

Quantification of total white cell count (WCC), monocyte count, neutrophil count, and platelet count was performed on whole blood using flow cytometry. Twenty μL samples were mixed with either 1 μL of each of the following antibodies CD45-AF488, CD11b-BV421, Ly6g-APC (BioLegend, San Diego, CA, US) for the detection of leucocytes, monocytes, and neutrophils respectively or 1 μL of CD42a-FITC (Becton-Dickinson) for the detection of platelets. Samples were then fixed with FACS-Lyse and washed twice in Dulbecco's phosphate-buffered saline.

White blood cells and platelets were identified based on forward and side scatter characteristics and the expression of CD45 and CD42a respectively. CD45+ cells were further gated with those positive for CD11b identified as monocytes and those positive for Ly6g identified as neutrophils. All samples were processed within 24 hours using the Accuri C6 flow cytometer (Becton-Dickinson) and analysed using FlowJo v10. platelets gated by characteristic forward and side scatter properties and CD42a expression using appropriate isotype controls.

2.2.8 Histological analyses

The lungs, liver, spleen and a single kidney were removed intact and fixed in 4 % paraformaldehyde for 24 hours at room temperature prior to being transferred into 70% ethanol for a further 24 hours. Following fixation, segments from each organ were

embedded in paraffin wax and 4-μm sections prepared. Sections were stained with haematoxylin and eosin for histological analysis. Digital images of each section were acquired at ×200 magnification using a semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany).

Chapter 3

Reproducibility and agreement between two methods of platelet aggregation

3 Repeatability and agreement between two methods of platelet aggregation

3.1 SUMMARY

Background

Light transmission aggregometry is the 'gold standard' for assessing platelet function but suffers from a number of technical limitations. Ninety-six-well plate aggregometry (96-WPA) offers practical advantages over LTA but to date there is limited data regarding test-retest repeatability and agreement between the two methods.

Objective

To determine the within-day (intra-assay) and between-day (inter-assay) repeatability of 96-WPA and LTA as well as agreement between the two methods.

Method and Results

Repeatability was determined by pooling results from dose-response curves constructed for each agonist (adenosine diphosphate (ADP), arachidonic acid (AA), protease-activated receptor 4-activating peptide (PAR4-AP)) repeated a) 7 times (within-day) or b) on 7 consecutive weekdays (between-day) in two volunteers. To examine agreement, dose-response curves for each agonist were compared in 10 volunteers. 96-WPA demonstrated good within- and between-day repeatability for the assessment of peak aggregation and EC_{50} in each of the agonists studied. Results were comparable to LTA, although the latter appeared marginally superior in terms of the between-day coefficient of variation. With respect to agreement, ADP and PAR4-AP peak aggregation

measured consistently higher with 96-WPA compared to LTA while the EC_{50} was consistently lower. No significant bias was observed with AA.

Conclusion

96-WPA demonstrated good within- and between-day repeatability for the assessment of peak aggregation and EC_{50} to various agonists with results comparable to LTA. However, there was a systematic measurement bias with two of the three agonists studied and the two methods should not be considered interchangeable.

3.2 INTRODUCTION

Platelet aggregometry plays a central role in the investigation and development of novel antithrombotic agents. The current 'gold-standard' technique for assessing platelet aggregation is light transmission aggregometry (LTA) [187]. However, LTA is considered technically challenging and can be restrictive in terms of the number of test conditions permitted per blood draw [190]. Ninety-six-well plate aggregometry (96-WPA) is a newer method of platelet aggregation that may offer advantages over LTA, but there remain little data regarding the test-retest repeatability and agreement between the two techniques [190,191]. Accordingly, we sought to determine the within-day (intra-assay) and between-day (inter-assay) repeatability of 96-WPA and LTA as well as the agreement between the two methods.

3.3 METHODS

3.3.1 Study population

Ten healthy non-smoking male and female volunteers between the ages of 18 and 40 years (inclusive) were recruited. Exclusion criteria were any clinically significant coexisting condition including hypertension, hyperlipidaemia, diabetes mellitus, gastrointestinal disease, coagulopathy, known liver disease, and recent infective or inflammatory condition. Volunteers must not have been taking any prescription medications for 4 weeks, over-the-counter medications, herbal supplements and vitamins for 1 week, and alcohol or caffeine containing products for 24 hours prior to blood sampling.

All patients provided written informed consent prior to any study procedure. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

3.3.2 Agonists

Adenosine diphosphate (ADP) and the protease-activated receptor 4-activating peptide (PAR4-AP) AYPGKF-NH2 were purchased from Sigma-Aldrich (Gillingham, UK). Arachidonic acid (AA) was purchased from Alpha Laboratories (Eastleigh, UK). Each agonist was prepared at 7 different concentrations then aliquoted and frozen (-40°C) prior to the start of the study.

3.3.3 Study protocol

For each agonist, repeatability and agreement were determined for peak aggregation and EC_{50}. The EC_{50} is the concentration of a drug that gives half-maximal response and is commonly used in research and clinical practice. Moreover, we hypothesised that because most agonist dose-response relationships are sigmoidal, the EC_{50} may be more sensitive than peak aggregation for the detection of variability.

Blood sampling and preparation

Blood samples (60 mL) were drawn uncuffed from the antecubital fossa through a 17-G needle into tri-sodium citrate (0.32% final concentration). For each sample, the first 5 mL of blood was discarded. To obtain PRP, samples were immediately centrifuged at 300 g (room temperature) for 20 minutes. PPP was obtained by centrifugation of 5 mL of PRP at 5000 g for 6 minutes. PRP and PPP were then stored in a water bath at 37°C prior to use.

Within-day repeatability

Dose-response curves for each agonist were repeated 7 times in two different volunteers. Replicates for both instruments were performed at the same time and from the same preparation of PRP and platelet-poor plasma (PPP). All sample preparation and tests were undertaken by the same operator. All testing was completed within 115 minutes of blood withdrawal.

Time since sampling is known to affect platelet function beyond a certain limit [194,195]. To examine whether this had influenced our results, within-day changes in peak aggregation and EC_{50} for replicate 7 were compared to replicate 1.

Between-day repeatability

Dose-response curves for each agonist were repeated on 7 consecutive weekdays at the same time of day (± 1 hour) in two different volunteers. Each replicate for both instruments was performed at the same time and from the same preparation of PRP and PPP. All sample preparation and tests were undertaken by the same operator. All testing was completed within 115 minutes of blood withdrawal.

Agreement

To examine the agreement between the two techniques, dose-response curves for each agonist were determined in both instruments at the same time and from the same preparation of PRP and PPP in 10 volunteers. All testing was completed within 115 minutes of blood withdrawal.

3.3.4 Platelet aggregation

Light transmission aggregometry

LTA was performed with the 8 channel PAP-8E aggregometer (Bio/Data Corp, Horsham, PA, USA). Prior to each test, each channel was blanked for reference with 250 μL of PPP. Two hundred and twenty-five μL of PRP was added to a channel maintained at 37°C followed by 25 μL of agonist. The 7 different concentrations of each agonist were studied simultaneously. Peak aggregation was recorded at the end of 12 minutes.

96-well plate aggregometry

Immediately prior to each test, 11.1 μL of agonist was added to a well. The 7 different concentrations of each agonist were studied simultaneously. Using a multi-pipette, 100 μL of PRP was added by reverse pipetting and the plate then immediately placed in the reader (Biotek, Synergy HT plate reader). Absorbance was determined at 595 nm every 15 s for 12 minutes between vigorous shaking at 37 °C. Changes in absorbance were converted to percentage aggregation by reference to the absorbances of PRP and PPP.

3.3.5 Statistical analysis

Peak aggregation from each test was used to plot agonist dose-response curves using a four-parameter logistic model (GraphPad v7.0e; GraphPad Software Inc, CA, USA) from which the EC_{50} was determined. To assess repeatability, the standard deviation (SD), coefficient of variation (CV) and coefficient of repeatability (CR) were examined. CR quantifies absolute measurement error in the same units as the test result and was calculated by multiplying the standard error of measurement ($\sqrt{\Sigma\sigma}/n$) by 2.77. Agreement between methods was determined by plotting the difference in peak

aggregation or EC_{50} for each agonist against the mean according to the method of Bland and Altman [210]. A paired *t*-test was used to determine if the estimated biases were significant. Two-sided p-values of ≤ 0.05 were considered statistically significant.

3.4 RESULTS

3.4.1 Within-day temporal changes in peak aggregation and EC_{50}

96-WPA

Mean peak aggregation with ADP (20 μM), AA (2 mM) and PAR4-AP (400 μM) were 75.8%, 85.4%, and 89.1% for replicate 1 and 77.4%, 83.1% and 87.4% for replicate 7 respectively. The corresponding mean EC_{50} were 0.74 μM, 242.8 mM, and 81.3 μM for replicate 1 and 0.84 μM, 242.4 mM, and 85.3 μM for replicate 7 respectively.

LTA

Mean peak aggregation with ADP (20 μM), AA (2 mM) and PAR4-AP (400 μM) was 67.5%, 80.0%, and 80.5% for replicate 1 and 68.5%, 76.0% and 76.0% for replicate 7 respectively. The corresponding mean EC_{50} were 1.18 μM, 247.0 mM, and 99.0 μM for replicate 1 and 1.10 μM, 247.4 mM, and 94.1 μM for replicate 7 respectively.

3.4.2 Within-day repeatability

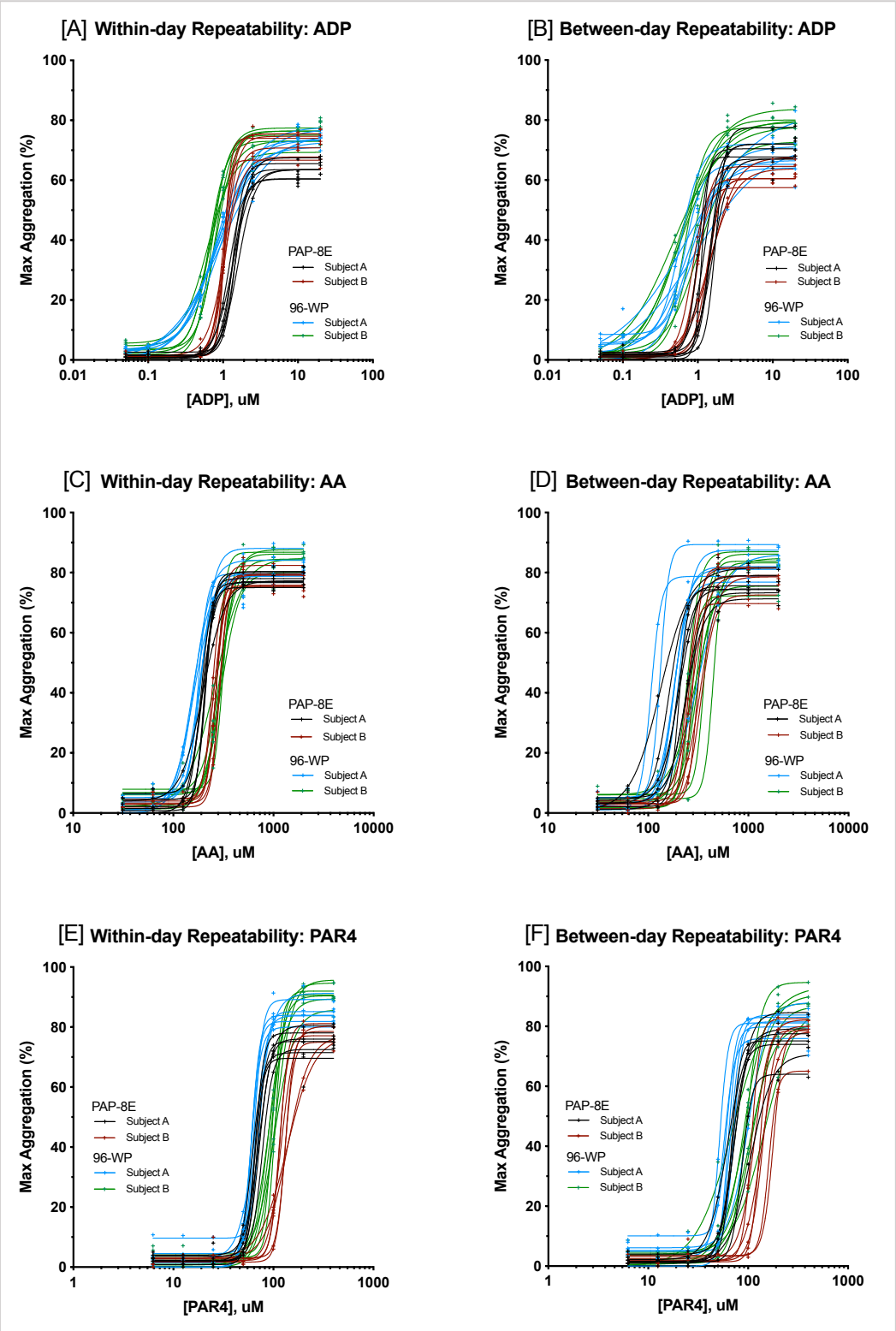


Figure 3.1. Within- and between-day dose-response curves for each replicate (n=7) by agonist studied.

Dose-response curves for each replicate and within-day measures of test-retest repeatability are presented in Figure 3.1 and Table 3.1, respectively. LTA and 96-WPA demonstrated good and comparable within-day measures of repeatability with each agonist for both peak aggregation and EC_{50}. The mean EC_{50} CRs for ADP, AA, and PAR4-AP were 0.19 μM, 32.8 μM, and 19.8 μM for LTA and 0.19 μM, 40.7 μM, and 14.1 μM for 96-WPA, indicating that a change above these values would be sufficient to be 95% confident that a true change had occurred (Table 3.1).

Table 3.1. Within-day measures of repeatability

	Light transmission aggregometry				96-well plate aggregometry			
Peak Aggregation	**Mean, %**	**SD**	**CV**	**CR**	**Mean, %**	**SD**	**CV**	**CR**
ADP	68.8	3.8	5.4	9.7	76.1	2.6	3.4	6.6
AA	77.8	3.0	3.8	7.6	84.1	3.0	3.6	8.4
PAR4-AP	76.5	2.9	3.8	7.5	87.0	3.0	3.6	7.8
EC_{50}	**Mean, μM**	**SD**	**CV**	**CR**	**Mean, μM**	**SD**	**CV**	**CR**
ADP	1.22	0.07	5.8	0.19	0.77	0.07	9.6	0.19
AA	234.2	12.8	5.3	32.8	232.3	15.9	7.1	40.7
PAR4-AP	97.8	7.7	7.9	19.8	81.1	5.5	6.9	14.1

SD indicates standard deviation; CV, coefficient of variation (%); CR, coefficient of repeatability; ADP, adenosine diphosphate; AA, arachidonic acid; and PAR4 AP, protease-activated receptor 4 activating peptide.

3.4.3 Between-day repeatability

Table 3.2. Between-day measures of repeatability

	Light transmission aggregometry				96-well plate aggregometry			
Peak Aggregation	**Mean, %**	**SD**	**CV**	**CR**	**Mean, %**	**SD**	**CV**	**CR**
ADP	67.9	3.5	5.1	8.9	72.7	6.2	8.7	17.3
AA	75.1	3.7	4.9	9.4	82.2	4.4	5.4	11.3
PAR4-AP	76.1	6.1	8.0	15.6	83.4	5.5	6.6	14.0
EC_{50}	**Mean, μM**	**SD**	**CV**	**CR**	**Mean, μM**	**SD**	**CV**	**CR**
ADP	1.29	0.26	20.1	0.66	0.73	0.17	23.4	0.43
AA	242.9	37.6	16.1	96.6	252.2	67.1	28.5	172.1
PAR4-AP	107.8	19.7	18.6	50.6	84.7	19.5	23.1	49.9

SD indicates standard deviation; CV, coefficient of variation (%); CR, coefficient of repeatability; ADP, adenosine diphosphate; AA, arachidonic acid; and PAR4 AP, protease-activated receptor 4 activating peptide.

Dose-response curves for each replicate are presented in Figure 3.1. Compared to LTA, 96-WPA was generally associated with a numerically less favourable coefficient of variation but overall the results were broadly similar (Table 3.2).

3.4.4 Agreement

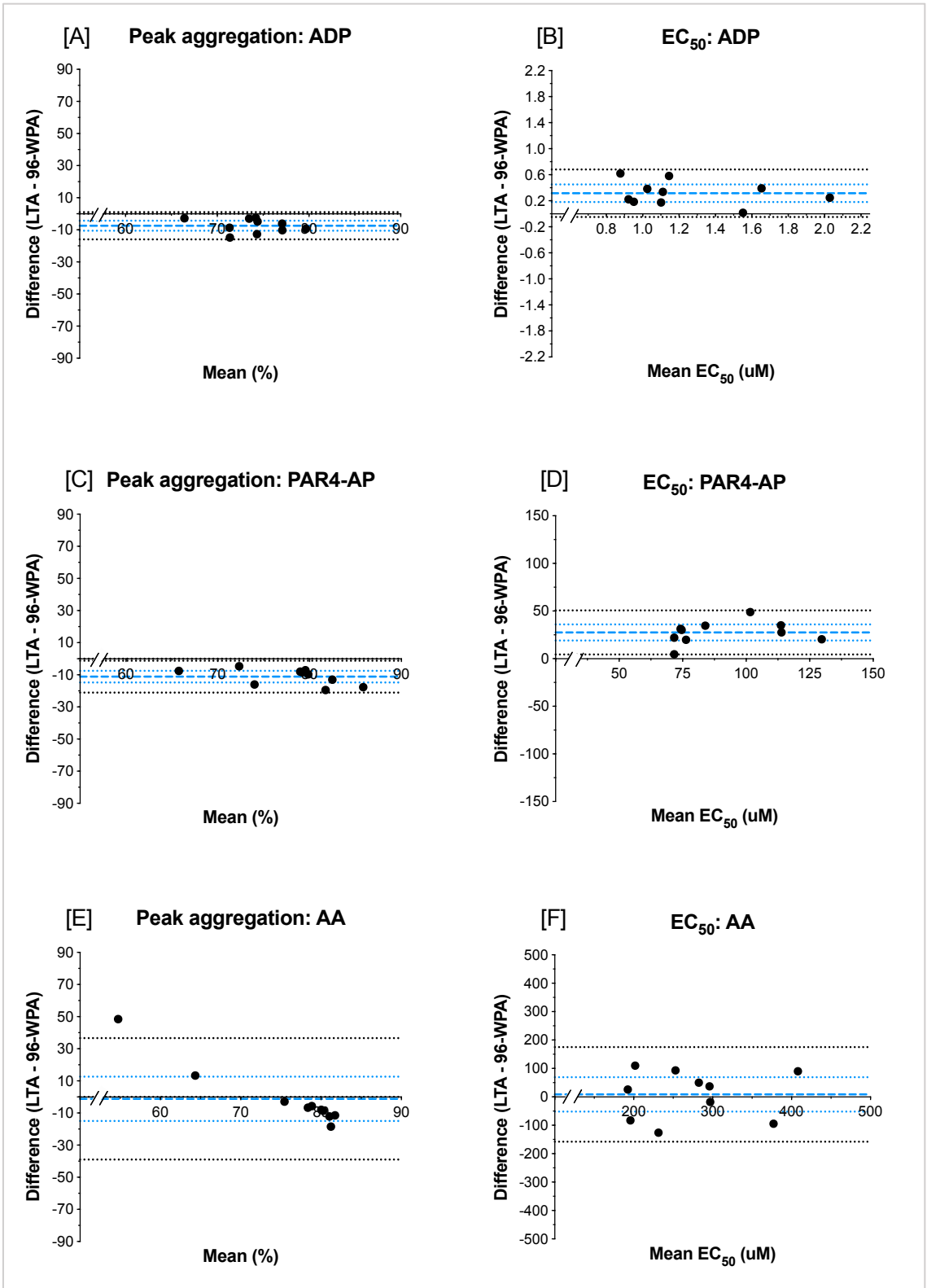


Figure 3.2. Bland-Altman plots
Bland-Altman plots by agonist examining agreement between LTA and 96-WPA for peak aggregation [A, C, E] and EC_{50} [B, D, F].

Table 3.3. Agreement between LTA and 96-WPA

	Bias*	*95% CI*	**LLOA**	*95% CI*	**ULOA**	*95% CI*
Peak Aggregation						
ADP	-7.5	*-10.6, -4.4*	-16.0	*-21.3, -10.6*	1.0	*-4.4, 6.4*
AA	-1.2	*-15.0, 12.6*	-39.1	*-63.0, 15.2*	36.6	*12.7, 60.6*
PAR4-AP	-11.2	*-14.8, -7.5*	-21.1	*-27.4, -14.8*	-1.24	*-7.5, 5.0*
EC_{50}						
ADP	0.32	*0.18, 0.45*	-0.05	*-0.28, 0.18*	0.68	*0.45, 0.91*
AA	8.6	*-52.2, 69.3*	-157.9	*-263.2, -52.7*	175.1	*69.8, 280.3*
PAR4-AP	27.5	*19.0, 35.9*	4.3	*-10.3, 19.0*	50.6	*36.0, 65.2*

*LTA minus 96-WPA. CI indicates confidence interval; LLOA, lower limit of agreement; ULOA, upper limit of agreement; ADP, adenosine diphosphate; AA, arachidonic acid; and PAR4 AP, protease-activated receptor 4 activating peptide.

Bland-Altman plots for measurement agreement between LTA and 96-WPA are presented in Figure 3.2 with bias and limits of agreement in Table 3.3. Compared to LTA, peak aggregation measured consistently higher with 96-WPA for ADP (bias 7.5%, $p < 0.001$) and PAR4-AP (bias 11.2%, $p < 0.001$), and the EC_{50} measured consistently lower (ADP bias 0.32 μM, $p < 0.001$; PAR-AP bias 27.5 μM, $p < 0.001$). The bias appeared constant for both ADP and PAR4-AP over the range of peak aggregation and EC_{50} measured.

Individual point differences between LTA and 96-WPA for EC_{50} with AA were spread around the line of equality indicating an absence of a systematic difference (bias 8.6 μM, $p = 0.76$). For peak aggregation, the bias was small and not significant (-1.2%, $p = 0.85$) but may not have been constant across the range of peak aggregations studied.

3.5 DISCUSSION

We examined the test-retest repeatability and agreement of 96-WPA and LTA for the determination of peak platelet aggregation and EC_{50} with 3 common agonists. The main findings were a) 96-WPA demonstrated good within- and between-day repeatability with comparable results to LTA, but that b) there was a systematic measurement bias for 2 out of 3 of the agonists studied and the two methods should not be considered interchangeable.

96-WPA offers practical advantages over LTA but to date there are limited data regarding test-retest repeatability. With respect to within-day precision of 96-WPA, we found an absolute change in peak aggregation of 6.6%, 7.8% and 8.7% in response to ADP, AA and PAR4-AP, respectively, would be sufficient to be 95% certain a true change had occurred. For EC_{50}, the corresponding values were 0.14 μM, 40.7 μM, and 14.1 μM. These results along with other measures of repeatability were similar to those obtained with LTA. Changes in peak platelet aggregation and EC_{50} induced by clinically relevant compounds are typically more than 50% or one order of magnitude [191,211–213]. Thus, under the experimental conditions of the study, we conclude that both 96-WPA and LTA demonstrated good and equivocal intra-assay repeatability.

Outcome measures for between-day test-retest repeatability were less favourable for both 96-WPA and LTA, especially for EC_{50}. One possible explanation is greater between-day analytical imprecision. However, as between-day repeatability also takes into account pre-analytical and biological factors, other sources are likely to contribute. These include blood sampling and centrifugation as well as temporal changes in an individual's platelet reactivity. Irrespective of the cause, between-day repeatability

remained good with respect to the magnitude of change that can be detected. Comparing methods, results for 96-WPA were largely similar to LTA, although LTA appeared marginally superior in terms of the coefficient of variation.

An interesting finding was that both ADP and PAR4-AP peak aggregation measured consistently higher with 96-WPA compared to LTA, while the EC_{50} was consistently lower. No significant differences occurred with AA, although for peak aggregation examining a greater range of values is recommended as the bias may not have been constant. 96-WPA uses polystyrene or polypropylene wells with shaking to promote mixing and platelet aggregation. Effects are determined by alterations in light absorbance. In contrast, LTA uses a glass cuvette and stir bar with aggregation measured by changes in light transmission. Our observations suggest these, or other technical differences, may have differential and agonist-specific effects on platelet reactivity. This requires further evaluation but ultimately results from this study indicate 96-WPA and LTA should not be considered interchangeable.

3.6 LIMITATIONS

Inclusion of a greater number of replicates or broader range of agonists would have been desirable and feasible if assessment of precision had been limited to peak aggregation only. However, we felt it was important to include EC_{50} as this is a central parameter and as hypothesised proved more sensitive to the detection of variability. Platelet reactivity is known to decline after a period of time, potentially affecting our results. To mitigate this, all processing was completed within the pre-determined 115-minute time limit (based on in-house data) and importantly there were no notable differences in platelet responses between first and last replicates. The intensive study

protocol imposed limitations in terms of the number of volunteers that could be included. In order to provide greater certainty of the intra- and inter-assay precision of 96-WPA, data should be pooled from a larger study population.

3.7 CONCLUSION

96-WPA demonstrated good within- and between-day repeatability for the assessment of peak aggregation and EC_{50} with results comparable to LTA. However, there was a systematic measurement bias with two of the three agonists studied and the two methods should not be considered interchangeable. Given the lack of published reference data for 96-WPA and absence of superiority in terms of precision we intend to use LTA for the remainder of this thesis. We do though support use of 96-WPA, especially when examining a large number of test conditions is paramount or compound availability and costs are an issue.

Chapter 4

Dependence of PAR4 on other agonist-receptor pathways for the activation and aggregation of platelets

4 Dependence of PAR4 on other agonist-receptor pathways for the activation and aggregation of platelets

4.1 SUMMARY

Background

Protease-activated receptor 4 (PAR4) antagonists have shown promise in animal studies for the prevention of atherothrombotic events. However, whether platelet responses coupled to PAR4 are dependent on signaling from other agonist-receptor pathways or vice-versa remains uncertain. This may have important therapeutic implications.

Objective

To examine if platelet activation and peak aggregation coupled to PAR4 stimulation is conditional on reinforcement from other major agonist-receptor pathways and vice-versa.

Method and Results

All studies were performed in vitro using blood from healthy volunteers not taking any medication. Platelet activation was determined by p-selectin expression and platelet-monocyte binding using flow cytometry. Peak platelet aggregation was determined by light transmission aggregometry. Results were pooled from a minimum of 6 volunteers. Activation of platelets by PAR4-AP (25 μM) resulted in strong p-selectin expression (86.5% ± 2.6), platelet-monocyte binding (85.8% ± 1.5) and platelet aggregation (88.5% ± 3.4). Platelet responses to PAR4-AP were completely attenuated by the PAR4 antagonist BMS-986120 but were unaffected by SCH-79797 (PAR1 antagonist),

apyrase (ADP scavenger) or indomethacin (cyclooxygenase inhibitor). In contrast, PAR1 platelet activation and aggregation were both partially inhabited by apyrase. BMS-986120 had no effect on platelet activation or aggregation in response to PAR1, ADP or AA stimulation.

Conclusions

Our results demonstrate that under conditions designed to mimic thrombin levels found within the local environment of a developing clot, platelet responses to PAR4 stimulation are not dependent on input from PAR1, ADP or AA. Equally, platelet responses to PAR1, ADP or AA stimulation were not affected by PAR4 inhibition. This lack of functional overlap highlights the potential of targeting PAR4 as a promising novel antiplatelet strategy.

4.2 INTRODUCTION

Platelet aggregation is central to thrombus formation and a major contributor to atherothrombotic disease [11]. Protease-activated receptor 4 (PAR4) is a thrombin activated G-protein coupled receptor present on the surface of platelets [115]. PAR4 stimulation results in marked platelet activation and aggregation with recent evidence suggesting PAR4 inhibition may provide safer and more effective antithrombotic protection than current agents [114,129–131]. However, whether PAR4-coupled platelet responses are conditional on input from other major agonist-receptor pathways or vice-versa remains uncertain. This may have important therapeutic implications. Accordingly, we sought to examine the interaction between PAR4 and other major receptor-agonist pathways for the activation and aggregation of platelets.

4.3 METHODS

4.3.1 Study aims

The primary objectives were to determine a) if PAR4-coupled platelet responses are dependent on adenosine diphosphate (ADP), thromboxane A2 or PAR1 signaling, and compare this to PAR1, and b) if platelet responses coupled to ADP, thromboxane A2 or PAR1 are dependent on PAR4.

4.3.2 Study population

Healthy non-smoking male and female volunteers between the ages of 18 and 40 years (inclusive) were recruited. Exclusion criteria were any clinically significant coexisting condition including hypertension, hyperlipidaemia, diabetes mellitus, gastrointestinal disease, coagulopathy, known liver disease, and recent infective or inflammatory condition. Volunteers must not have been taking any prescription medications for 4

weeks, over-the-counter medications, herbal supplements and vitamins for 1 week, and alcohol or caffeine containing products for 24 hours prior to blood sampling.

All patients provided written informed consent prior to any study procedure. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

4.3.3 Materials

Adenosine diphosphate (ADP), indomethacin (a cyclooxygenase inhibitor) and apyrase (an ADP scavenger) were purchased from Sigma-Aldrich (Gillingham, UK), arachidonic acid (AA) from Alpha Laboratories (Eastleigh, UK), SCH-79797 (a selective PAR1 antagonist) from Tocris (Abingdon, UK) and tirofiban (a GPIIb/IIIa antagonist) from Beacon Pharmaceuticals (Swadlincote, UK). PAR4-activating peptide (PAR4-AP; A-Phe(4-F)-PGWLVKNG), PAR1-AP (SFLLRN) and BMS-986120 (PAR4 antagonist) were courtesy of Bristol-Myers Squibb (Princeton, USA). All compounds were dissolved in either phosphate-buffered saline (PBS; ADP, SCH-79797, tirofiban, PAR4-AP and PAR1-AP), de-ionised water (AA) or dimethyl sulphoxide (indomethacin and BMS-986120) and stored in frozen aliquots (-40°C) prior to the start of the study. On the day of the study, indomethacin and BMS-986120 were diluted to their target concentrations with phosphate-buffered saline and platelet-poor plasma, respectively.

4.3.4 Compound concentration selection

Concentrations of PAR4-AP and PAR1-AP were selected to reflect higher thrombin levels present during thrombosis with all agonist concentrations chosen to achieve near

maximum platelet responses while at the same time avoiding excessive dosing given this can mask the effects of antagonists on non-cognate pathways. A similar approach was applied to antagonists. Based on in-house data and consistent with other publications [214], the following compound concentrations were selected: PAR4-AP, 25 μM; PAR1-AP, 25 μM; ADP, 10 μM; AA 5 mM; BMS-986120, 200 nM; SCH-79797, 64 μM; tirofiban, 400 nM; apyrase, 2 U/mL; and indomethacin, 20 μM.

4.3.5 Blood sampling and preparation

Blood samples (60 mL) were drawn uncuffed from the antecubital fossa through a 17-G needle into tri-sodium citrate (0.32% final concentration). For each sample, the first 5 mL of blood was discarded. To obtain PRP, samples were immediately centrifuged at 300 g (room temperature) for 20 minutes. PPP was obtained by centrifugation of 5 mL of PRP at 5000 g for 6 minutes. PRP and PPP were then stored in a water bath at 37°C prior to use.

4.3.6 Aggregometry

Platelet aggregation was determined by aggregometry using an 8 channel PAP-8E aggregometer (Bio/Data Corp, Horsham, PA, USA). Prior to each test, each channel was blanked for reference with 250 μL of PPP. Two hundred and twenty-five mL of PRP and either 12.5 μL of antagonist or control (PBS) were added to a channel maintained at 37°C and allowed to incubate for 5 minutes before spiking with 12.5 μM of agonist or control (PBS). Peak aggregation was recorded at the end of 12 minutes. All testing was completed within 60 minutes of blood draw. Results were pooled from a minimum of 6 volunteers.

4.3.7 Flow cytometry

Platelet activation was assessed by p-selectin expression and platelet-monocyte aggregates using flow cytometry. These are sensitive measures of platelet degranulation and widely used markers of platelet activation [193–195]. Blood (5 mL) was collected into 50 μL of 75 mM D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Enzo Life Sciences, Exeter, UK) then immediately aliquoted into micro-centrifuge tubes pre-filled with control (PBS) or antagonist and allowed to incubate for 5 minutes before spiking with agonist or control (PBS) and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for a further 20 minutes at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). A FACSCalibur flow cytometer (Becton-Dickinson) was used with all samples processed within 24 hours. Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA). Results were pooled from a minimum of 6 volunteers.

4.3.8 Statistical analysis

Unless otherwise stated, data is presented as mean ± standard deviation (SD) for continuous variables and percentages with counts for categorical variables. A one-way ANOVA with Dunnett's or Sidak's multiple comparisons test were used to determine if the differences in mean platelet responses were significant. Two-sided p-values of ≤ 0.05 were considered statistically significant. Analyses were performed using R version 3.3.1 (R Project for Statistical Computing, Vienna, Austria).

4.4 RESULTS

4.4.1 PAR4-mediated platelet activation

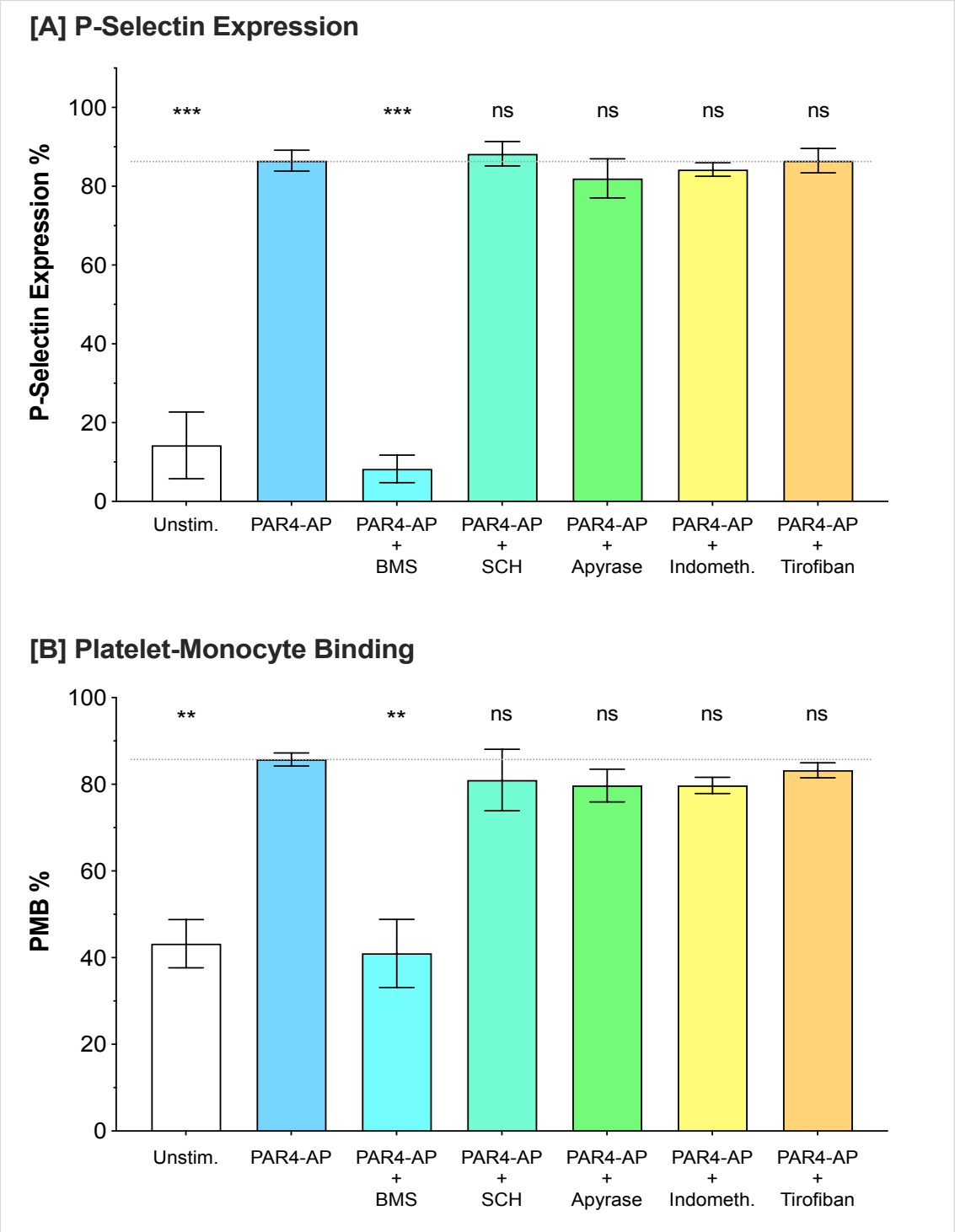


Figure 4.1. Plots of PAR4-AP stimulated platelet activation in the presence or absence of antagonists.
Data shown are the mean [A] p-selectin and [B] PMB responses with standard deviation (n=6). Statistical comparisons (Dunnett's multiple comparison test) versus PAR4-AP are shown above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: PMB, platelet monocyte binding; BMS, BMS-986120; SCH, SCH-79797; Indo, indomethacin; and Tiro, tirofiban.

Activation of platelets by PAR4-AP (25 μM) resulted in strong p-selectin expression (86.5 ± 2.6%) and platelet-monocyte binding (85.8 ± 1.5%). BMS-986120 potently inhibited PAR4-AP platelet activation (absolute difference (△) for p-selectin -78.3%, p<0.001; PMB △-44.8%, p<0.01). In contrast, SCH-79797 (p-selectin △1.8%, p=0.7; PMB △-4.8%, p=0.7), apyrase (p-selectin △-4.5, p=0.6; PMB △-6.0%, p=0.1), indomethacin (p-selectin △-2.3%, p=0.3; PMB △-6.0%, p=0.07) and tirofiban (p-selectin △0%, p=1.0; PMB △-2.5%, p=0.5) had no effect on PAR4-AP stimulated platelet activation (Figure 4.1).

4.4.2 PAR4-mediated platelet aggregation

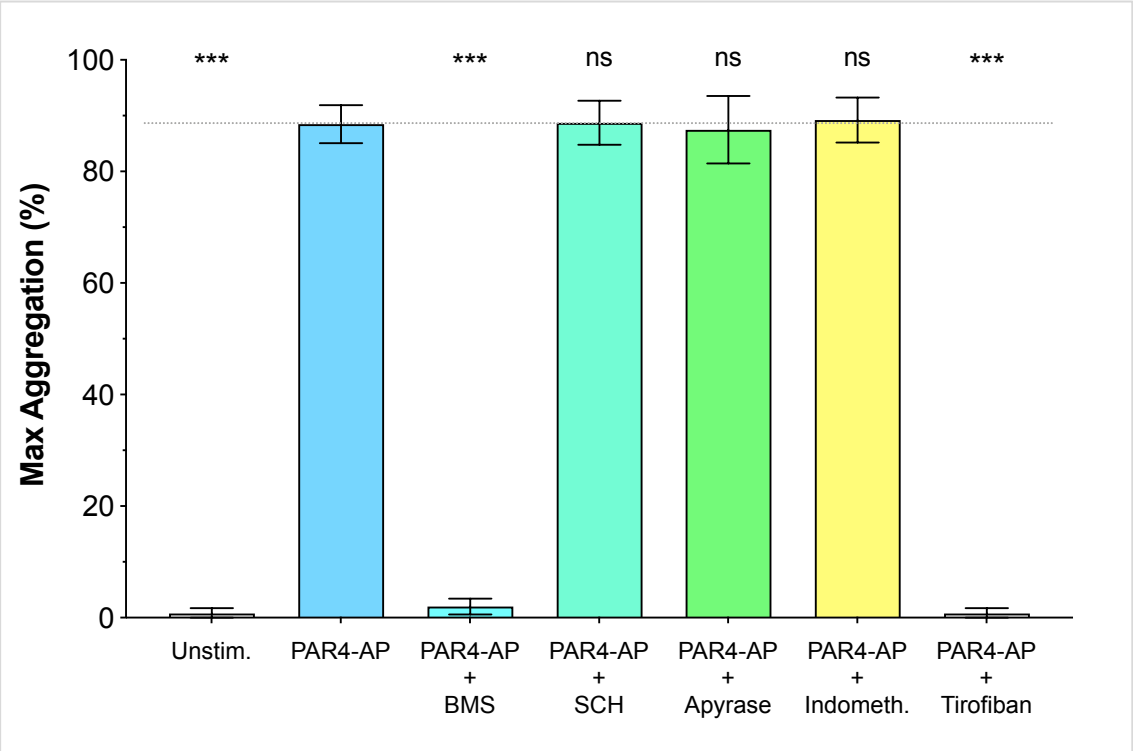


Figure 4.2. Plots of PAR4-AP stimulated platelet aggregation in the presence or absence of antagonists.
Data shown are the mean max platelet aggregation responses ± standard deviation (n=6). Statistical comparisons (Dunnett's multiple comparison test) versus PAR4-AP are shown above each plot: * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: BMS, BMS-986120; SCH, SCH-79797; and Indometh., indomethacin.

PAR4-AP (25 μM) resulted in strong platelet aggregation (88.5 ± 3.4%) and was potently inhibited by both BMS-986120 (absolute difference (△) -86.5%, p<0.001) and tirofiban (△-88.5%, p<0.001; Figure 4.2). SCH-79797 (△0.25%, p=1.0), apyrase (△-1.0%, p=0.97) and indomethacin (△0.8%, p=0.96) had no effect on PAR4-stimulated platelet aggregation.

4.4.3 PAR1-mediated platelet activation

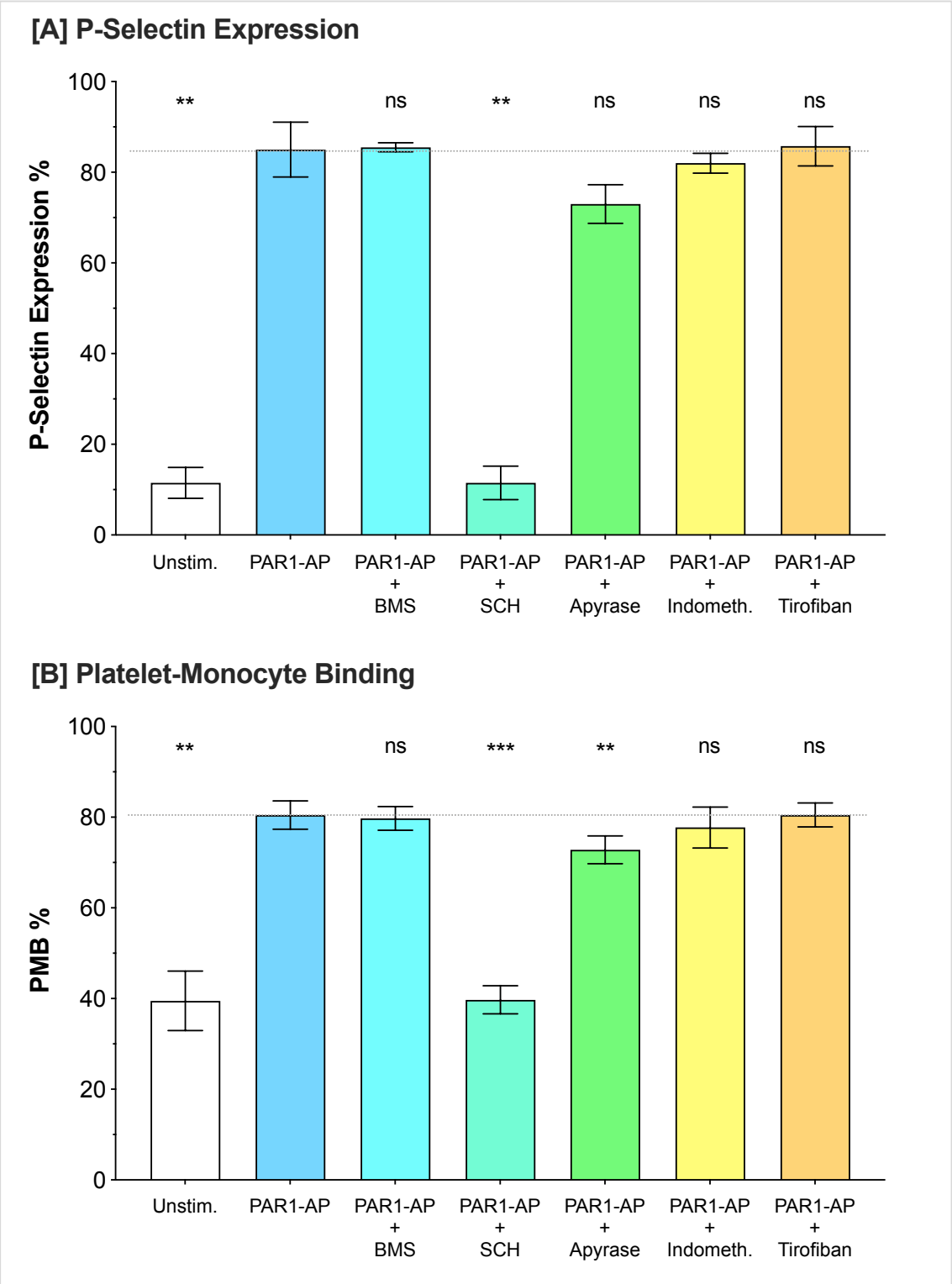


Figure 4.3. Plots of PAR1-AP stimulated platelet activation in the presence or absence of antagonists.
Data shown are the mean [A] p-selectin and [B] PMB responses with standard deviation (n=6). Statistical comparisons (Dunnett's multiple comparison test) versus PAR1-AP are shown above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: PMB, platelet monocyte binding; BMS, BMS-986120; SCH, SCH-79797; and Indo, indomethacin.

PAR1-AP (25 μM) resulted in strong p-selectin expression (85.0 ± 6.1%) and platelet-monocyte binding (80.5 ± 3.1%; Figure 4.3). Responses to PAR1-AP were potently inhibited by SCH-79797 (absolute difference (△) for p-selectin -73.5%, p=0.002; PMB △-40.8%, p<0.001) but not affected by coadministration of BMS-986120 (p-selectin △0.5%, p=1.0; PMB △-0.8%, p=1.0), apyrase (p-selectin △-3.0, p=0.6; PMB △-2.8%, p=0.3) or tirofiban (p-selectin △0.8%, p=1.0; PMB △0%, p=1.0). In contrast to PAR4 stimulation, the ADP scavenger apyrase appeared to attenuate platelet activation to PAR1-AP. This effect was not statistically significant for p-selectin expression (△-12.0%, p=0.11) but significant for platelet-monocyte binding (△-9.0, p<0.01).

4.4.4 PAR1-mediated platelet aggregation

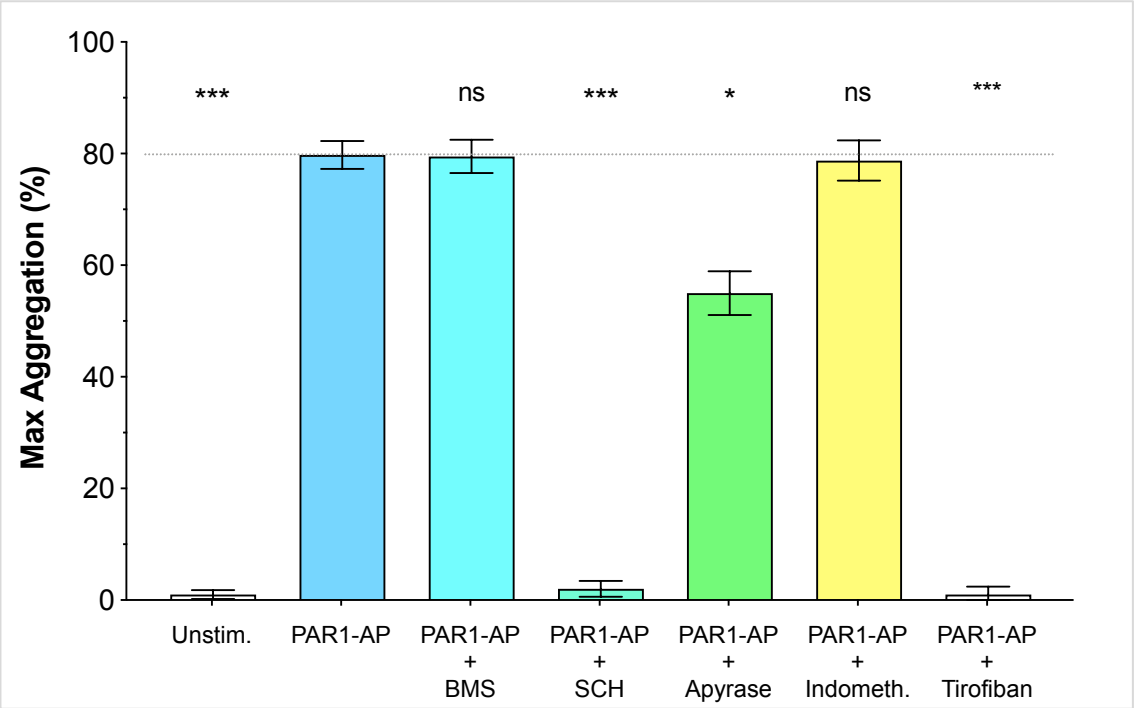
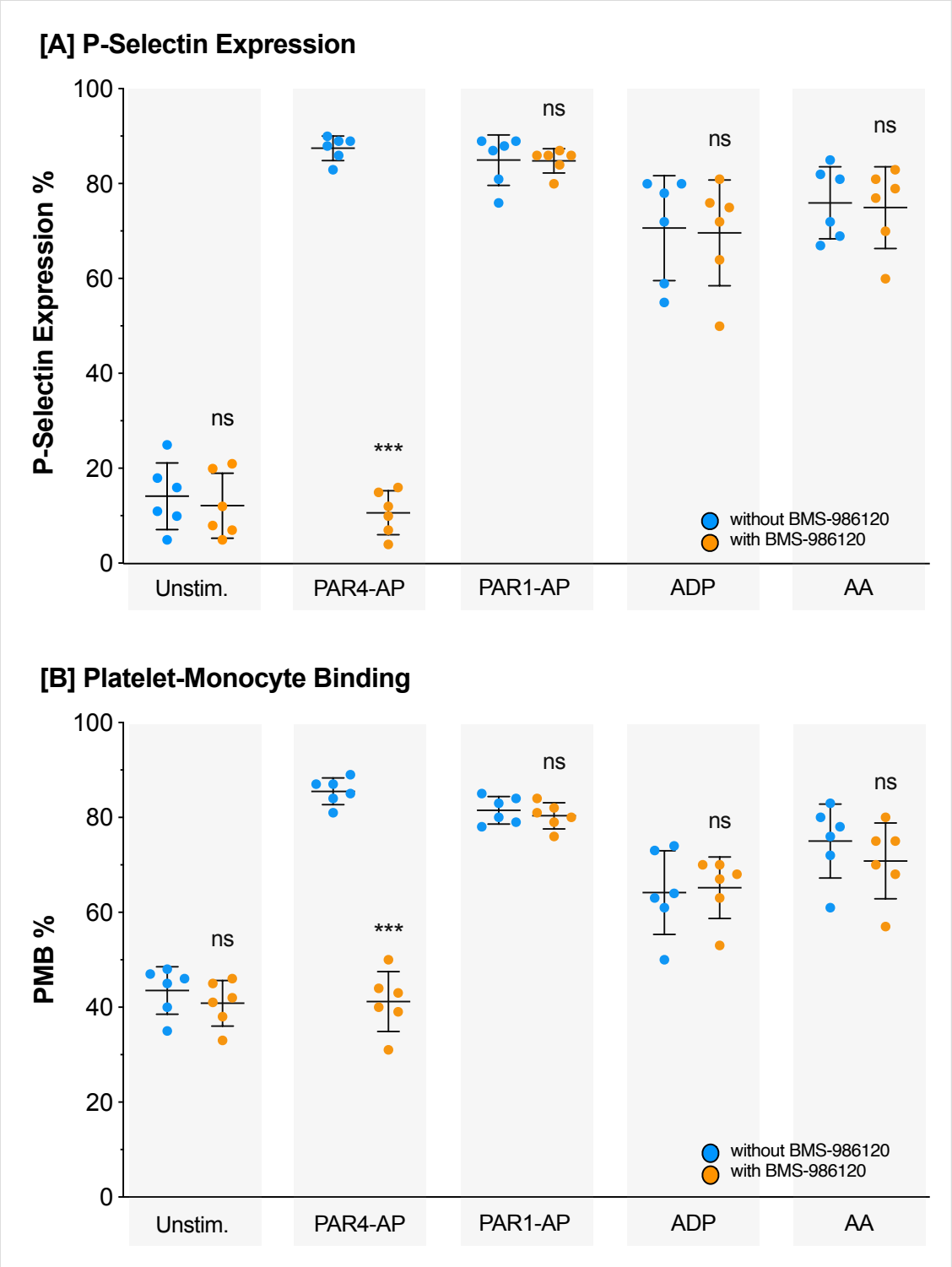


Figure 4.4. Plots of PAR4-AP stimulated platelet aggregation in the presence or absence of antagonists.
Data shown are the mean max platelet aggregation responses with standard deviation (n=6). Statistical comparisons (Dunnett's multiple comparison test) versus PAR1-AP are shown above each plot: * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: BMS, BMS-986120; SCH, SCH-79797; Indo, indomethacin; and Tiro, tirofiban.

PAR1-AP (25 μM) resulted in strong platelet aggregation (79.8 ± 2.5%) that was potently inhibited by both SCH-79797 (absolute difference (△) -77.8%, p<0.001) and tirofiban (△-78.8%, p<0.001; Figure 4.4). As with platelet activation, PAR1-AP platelet aggregation was not affected by BMS-986120 (△-0.25%, p=1.0) or indomethacin (△-1.0%, p=1.0) but was partially inhibited by apyrase (△-24.8%, p=0.01).

4.4.5 Role of PAR4-signaling in platelet responses to other major agonist-receptor pathways



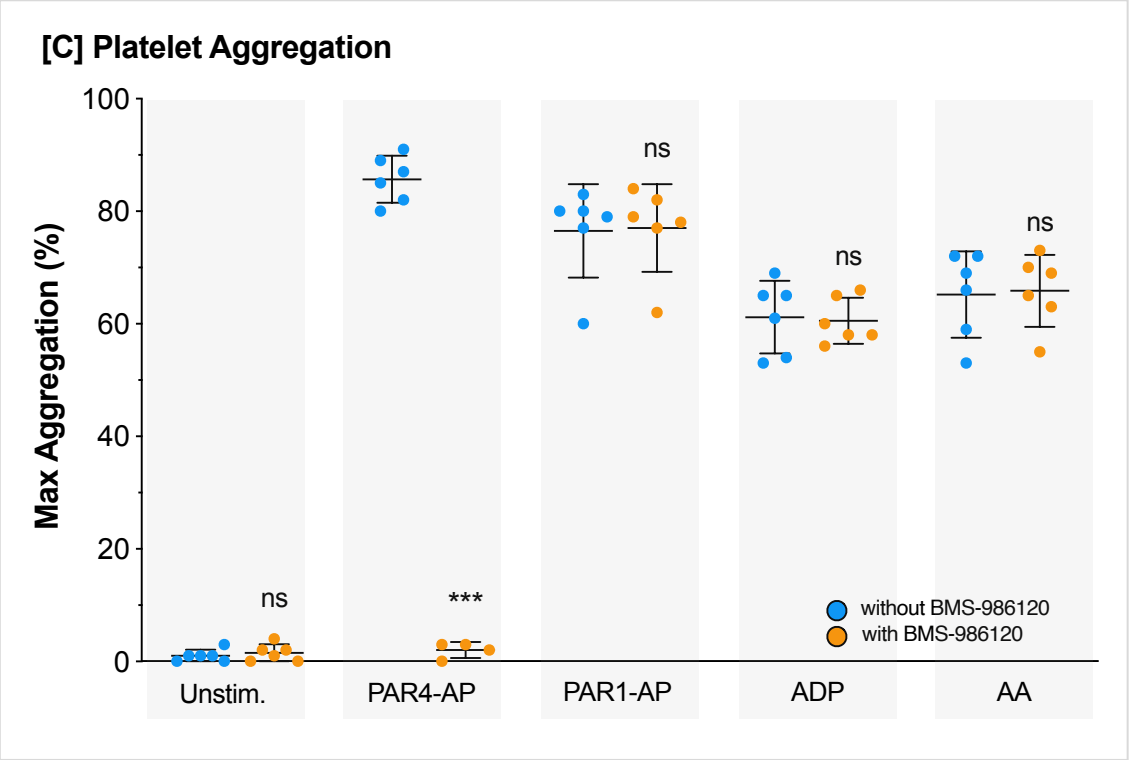


Figure 4.5. Plots of agonist-stimulated platelet responses in the presence or absence of PAR4 antagonism.
Data shown are the individual points for [A] p-selectin expression [B] PMB and [C] max platelet aggregation responses with mean and standard deviation (n=6). Statistical comparisons (Sidak's multiple comparison test) for BMS-986120 versus no BMS-986120 are shown for each agonist above the corresponding plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: ADP, adenosine diphosphate; AA, arachidonic acid; and PMB, platelet-monocyte binding.

PAR4 antagonism with BMS-986120 resulted in potent inhibition of PAR4-AP stimulated p-selectin expression (absolute difference (△) -76.8%, $p<0.001$), platelet-monocyte binding (△-44.3%, $p<0.001$) and platelet aggregation (△-83.2%, $p<0.001$) but had no effect on platelet responses to PAR1-AP, ADP or AA stimulation (p=ns for all; Figure 4.5).

4.5 DISCUSSION

We conducted a panel of studies to determine if platelet responses coupled to PAR4 activation are contingent on input from other major agonist-receptor pathways and vice versa. The principal findings were that observed platelet responses to a) PAR4 stimulation were not dependent on PAR1, ADP or thromboxane A2 signaling, b) PAR1, ADP and thromboxane A2 stimulation were not dependent on PAR4 signaling, and c) PAR1 stimulation were not dependent on PAR4 or thromboxane A2 signaling but were partially attenuated by ADP inhibition.

There is conflicting evidence as to whether platelet activation and aggregation responses to PAR signaling are conditional on additional input. Dawood and colleagues found that both PAR1- and PAR4-coupled platelet responses were dependent on thromboxane A2 and ADP signaling [214] with similar findings reported elsewhere [215]. Adam and colleagues observed that only PAR1-mediated platelet activation was reliant on feedback and from ADP alone [216], whereas Holinstat and colleagues demonstrated the reverse to be true [217].

We selected agonist concentrations of PAR4-AP to mimic thrombin levels within the local environment of a developing clot. Under these conditions, PAR4-stimulated platelet activation and peak aggregation were unaffected by inhibition of PAR1, ADP and thromboxane A2 signaling. This apparent lack of co-dependence on other major agonist-receptor pathways is consistent with evidence PAR4 activation occurs well after platelet degranulation [130] and induces sustained intracellular Ca^{2+} and protein kinase C signals independent of additional support [114,129,130]. PAR4 is thought to be an attractive target for antiplatelet therapy because of its more selective role toward

the later stages of platelet activation involved in thrombus stabilisation and propagation. Our results add to this, identifying PAR4 signaling as a major mechanism through which pathological platelet activation and aggregation can continue despite current standard of care antiplatelet agents.

In contrast to PAR4, PAR1-coupled platelet activation and aggregation were attenuated by the ADP scavenger apyrase. Previous studies have demonstrated ADP to be of major importance for platelet responses induced by low but not high thrombin concentrations [218–220]. Our results are consistent with this given PAR1 is cleaved by sub-nanomolar concentrations of thrombin whereas PAR4 is active at higher protease concentrations [114]. [218–220]Collectively these data strongly support that PAR1 platelet signaling is reinforced by ADP feedback and that the clinical effect of P2Y12 antagonists may partly relate to PAR1 inhibition. Indeed, in patients taking clopidogrel, platelet aggregation to PAR1 stimulation has been shown to be inversely correlated to the degree of P2Y12 inhibition [221].

Examination of the platelet effects of PAR4 inhibition has previously been limited by a lack of antagonist availability and specificity [117,141,222]. BMS-986120 is a first in class, potent and highly selective PAR4 antagonist [131,146]. Compared to earlier compounds, including P4pal-10, YD-3 and ML354, BMS-986120 has antiplatelet activity against alpha thrombin and demonstrated greater potency and selectivity of effect in preclinical studies [133,141,223]. PAR4 inhibition with BMS-986120 resulted in near complete inhibition of both PAR4-AP mediated platelet activation and aggregation but had no effect on platelet responses to other tested agonists. Thus, we add to existing evidence that BMS-986120 is PAR4 specific and demonstrate that

platelet activation and aggregation to ADP, thromboxane A2 and PAR1 stimulation is not dependent on PAR4 signaling.

4.6 LIMITATIONS

Agonist doses were selected to provide the most clinically relevant results. While inclusion of a broader range of agonist and antagonist concentrations would have been desirable, we believe it unlikely this would have altered our principal findings at the cost of a major impact on study feasibility. All tests were performed in vitro and in healthy volunteers. Future studies should examine the interaction between PAR4 and other agonist-receptor pathways ex vivo and in patients taking one or more of the current antiplatelet agents.

4.7 CONCLUSION

Under conditions designed to approximate clinically relevant levels of thrombin, platelet responses to PAR4 stimulation were not dependent on input from other major agonist-receptor pathways and vice-versa. This lack of functional overlap adds to existing evidence that PAR4 inhibition offers major promise as a novel antiplatelet strategy.

Chapter 5

Safety and tolerability of systemic PAR4 agonism in mice

5 Safety and tolerability of systemic PAR4 agonism in mice

5.1 SUMMARY

Background

Targeting protease-activated receptor 4 (PAR4) has emerged as a promising new antiplatelet strategy. PAR4 is also expressed on human vascular endothelium and smooth muscle cells but its function remains to be established.

Objective

To examine the safety and tolerability of the PAR4-activating peptide AYPGKF-NH2 given intravenously to mice so as to enable future studies into the in vivo vascular effects of PAR4 in man.

Method and Results

Male Rodentia mice aged 10 to 12 weeks were fed a diet of normal chow and observed for 1 week. Fifteen mice exhibiting normal behaviour were selected and administered low (systemic concentration 11.25 μM) or high dose (systemic concentration 112.5 μM) AYPGKF-NH2, or control (0.9% saline), via tail vein injection (5 mice per group). Mice were observed for 24 hours then sacrificed for necropsy. AYPGKF-NH2 was well tolerated with no notable adverse effects including fever, pain, distress or abnormal behaviour. Compared to control, AYPGKF-NH2 was not associated with change in any of the haematological, inflammatory, hepatic and renal blood markers examined. No distortion of parenchymal architecture, necrosis or inflammatory cell infiltrate was observed on histological analysis of the lungs, spleen, liver or kidney.

Conclusion

In mice, systemic administration of the PAR4 activating peptide AYPGKF-NH2 was well tolerated with no evidence of acute toxicity, inflammation or organ injury. Our results support the safety of AYPGKF-NH2 to investigate the vascular actions of PAR4 in humans.

5.2 INTRODUCTION

Previous studies have shown that PAR1 stimulation elicits a number of important vascular effects beyond platelet activation and aggregation. These include resistance vessel vasodilatation, venoconstriction, smooth muscle cell proliferation and maintenance of normal endothelial integrity [27,147,148]. PAR4 is also expressed on vascular smooth muscle cells and the endothelium but to date there have been no in vivo studies in man [151–153]. Data from animal studies suggests PAR4 may be involved in the control of vascular tone, ischaemia-reperfusion injury and the exaggerated intimal hyperplasia seen in diabetic conditions [137,154–156]. However, inter-species differences in PAR function and activation [157,158] may limit extrapolation of these results to man and the wider role of PAR4 within the human vasculature essentially remains unknown.

Like PAR1, the vascular actions of PAR4 may be diverse and such effects may mitigate or enhance the effectiveness of PAR4 inhibitors as future antiplatelet agents. While a number of highly specific PAR4 agonists are readily available, there have been no reports of such compounds being administered to man. Given the multi-tissue and multi-organ distribution of PAR4, including inflammatory cells, lung, kidneys and liver [159,160,163,164,166], it would be essential to first examine the effects of systemic PAR4 agonism in animals prior to undertaking in vivo studies in man. Accordingly, we set out to examine the safety and tolerability of the PAR4-activating peptide (PAR4-AP) AYPGKF-NH2 given intravenously to mice with the aim of enabling future research into the wider role of vascular human PAR4.

5.3 METHODS

5.3.1 Study objectives

To determine the safety and tolerability in mice of AYPGKF-NH2 administered intravenously at low and suprathreshold doses as compared to control. All experiments were conducted in accordance with the UK Scientific Procedures Act 1986 and had local institutional ethics approval.

5.3.2 AYPGKF-NH2

AYPGKF-NH2 (Ala-Tyr-Pro-Gly-Lys-Phe-NH2) is a PAR4-AP based on the native PAR4 tethered ligand sequence GYPGKF. An alanine substitution at position one increases selectivity and specificity for PAR4 [242]. High purity (95.85%) AYPGKF-NH2 was purchased from GL Biochem Ltd (Shanghai, China) and manufactured according to current Good Manufacturing Practice standards. On receival, product testing and analysis were repeated by the active pharmaceutical ingredient laboratory of the Queens Medical Research Institute, Edinburgh.

5.3.2.1 Preliminary human studies

Prior to the study, in vitro platelet testing with human blood was performed to confirm the specificity of our clinical-grade AYPGKF-NH2 for PAR4. These studies also served to establish the relevant dose range for future in vivo human vascular studies (see Appendix A) from which the appropriate systemic concentrations for this safety and tolerability study in mice could be agreed. On the basis of these data, 200 μM was determined to be the maximum local concentration of AYPGKF-NH2 of interest. Assuming a minimum total blood volume of 3.5 L and no metabolism, this would

equate to volunteers being exposed to a maximum systemic concentration of 7.87 μM over the entire proposed protocol.

5.3.3 Mice

Twenty adult male Rodentia mice (Charles River, UK) aged 8 to 10 weeks were housed in the animal unit of the University of Edinburgh and observed for 1 week. Animals were grouped 2 to 3 per cage and fed a diet of normal chow throughout the study.

5.3.4 Study protocol

5.3.4.1 Dosing

Fifteen mice exhibiting normal behaviour during the observation period were selected for dosing. AYPGKF-NH2 (low or high dose) or control (0.9% saline) was administered to mice via tail vein injection (n=15 total, 5 mice per group). Low dose refers to 100 μL of 164 μM AYPGKF-NH2, equating to a systemic concentration of 11.25 μM assuming a mice blood volume of 1.46 ml. High dose refers to 100 μL injection of 1.64 mM AYPGKF-NH2, equating to a systemic concentration of 112.5 μM. This is 14-fold greater than the maximum theoretical accumulated systemic dose volunteers would be exposed to in our proposed future studies. Following dosing, mice were observed for 24 hours with signs of pain, distress or abnormal behaviour recorded.

5.3.4.2 Necropsy

At 24 hours mice were culled by intraperitoneal injection of Euthatal followed by withdrawal of the maximum volume of blood possible (by abdominal vena cava or the heart) directly into 3.2% sodium citrate (final concentration 0.32%) and dissection.

5.3.5 Laboratory analyses

Standard clinical chemistry assays for bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), albumin, alkaline phosphatase, urea and creatinine were performed on whole blood by Dr F Howie (MRC Centre of Inflammation Research, Queen's Medical Research Institute, Edinburgh). A detailed description of the methodology for each test is described in Chapter 2: Methods.

5.3.6 Flow cytometry

Quantification of total white cell count, monocyte count, neutrophil count and platelet count was performed on whole blood by flow cytometry by Dr J Raftis (MRC Centre of Inflammation Research, Queen's Medical Research Institute, Edinburgh). A detailed description of the methodology is described in Chapter 2: Methods.

5.3.7 Histopathology

The lungs, liver, spleen and a single kidney were removed intact and fixed in 4% paraformaldehyde for 24 hours at room temperature prior to being transferred into 70% ethanol for a further 24 hours. Following fixation, segments from each organ were embedded in paraffin wax and 4 μm sections prepared. Sections were stained with haematoxylin and eosin. Digital images of each section were acquired at ×200 magnification for analysis using a semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany).

5.3.8 Statistical analysis

Unless otherwise stated categorical data are presented as percentages and continuous data as means ± standard deviation (SD). The effects of study agents on laboratory

endpoints were assessed by one-way analysis of variance with Tukeys test for multiple comparisons. Two-sided p-values of ≤0.05 were considered statistically significant. All statistical calculations were performed using PRISM version 7.0a (GraphPad Software, Inc., La Jolla, CA, USA).

5.4 RESULTS

5.4.1 Population

Tail vein injections were performed on 15 mice in the animal unit of the University of Edinburgh.

5.4.2 Tolerability

Intravenous injection of AYPGKF-NH2 (or control) was well tolerated with no notable adverse effects including fever, pain, distress or abnormal behaviour in any of the mice. There were no deaths prior to planned necropsy at 24 hours.

5.4.3 Effects on haematological and inflammatory markers

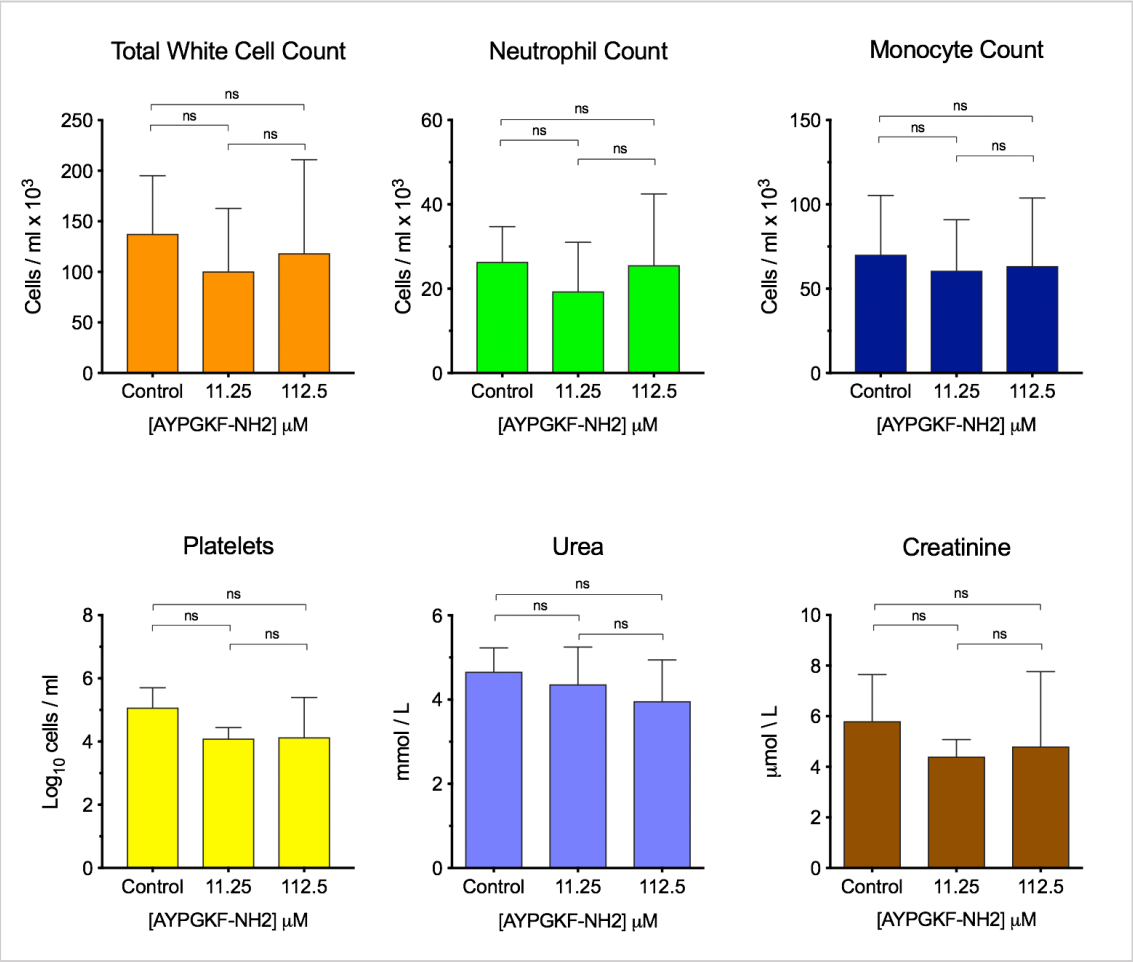


Figure 5.1. Effect of systemic PAR4 agonism with AYPGKF-NH2 versus control on haematological and renal laboratory indices
Data shown are the mean values ± 95% confidence intervals at 24 hours following exposure to control (0.9% NaCl, n=5), AYPGKF-NH2 at a systemic concentration of 11.25 uM (n=5), and AYPGKF-NH2 at a systemic concentration of 112.5 M (n=5). Statistical comparisons are

Systemic administration of AYPGKF-NH2 (low or high dose) was not associated with a significant rise or fall in any of the haematological or inflammatory markers examined (Figure 5.1). Total white cell count was 138 ± 46, 101 ± 50 and 119 ± 74 cells/μL, neutrophil count 26 ± 11, 19 ± 14 and 26 ± 22 cells/μL, monocyte count 70 ± 28, 61 ± 24 and 63 ±33 cells/μL, and platelet count 4.18 ± 3.96, 3.13 ± 3.89 and 3.72 ± 3.97 log_{10} cells/mL for control, low, and high dose AYPGKF-NH2, respectively.

5.4.4 Effects on lung

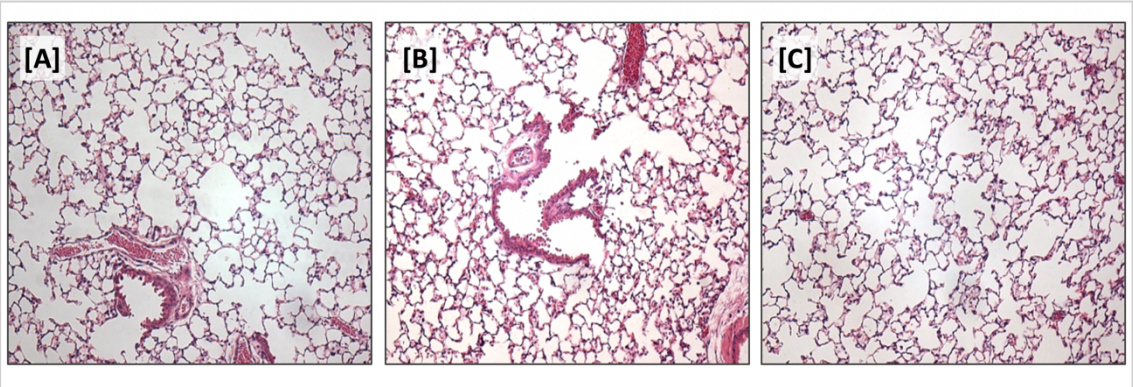


Figure 5.2. Effect of systemic PAR4 agonism with AYPGKF-NH2 on lung parenchymal tissue
Representative sections (x200 magnification) of lung parenchymal tissue from mice exposed to [A] control (n=5), [B] AYPGKF-NH2 at a systemic concentration of 11.25 μM (n=5), and [C] AYPGKF-NH2 at a systemic concentration of 112.5 μM (n=5). Sections are stained with

AYPGKF-NH2 was not associated with evidence of respiratory distress (e.g. rasping) throughout the observation period. Compared to control, there was no distortion of lung parenchymal architecture, necrosis or inflammatory cell infiltrate at either low or high dose (Figure 5.2).

5.4.5 Effects on kidneys

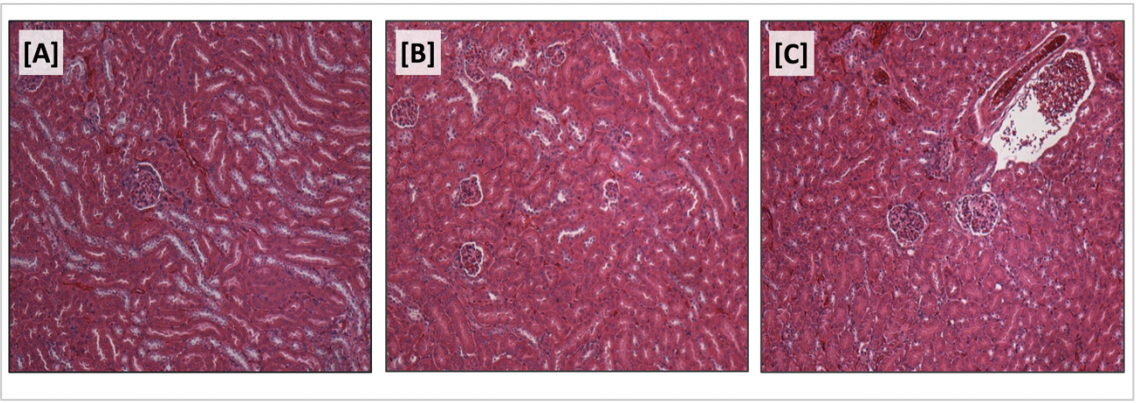


Figure 5.3. Effect of systemic PAR4 agonism with AYPGKF-NH2 on renal parenchymal tissue
Representative sections (x200 magnification) of renal parenchymal tissue from mice exposed to [A] control (n=5), [B] AYPGKF-NH2 at a systemic concentration of 11.25 μM (n=5), and [C] AYPGKF-NH2 at a systemic concentration of 112.5 μM (n=5). Sections are stained with

AYPGKF-NH2 was not associated with a change in blood markers of renal function as compared to control (Figure 5.1). Urea was 4.7 ± 0.5, 4.4 ± 0.7 and 4.0 ± 0.8 mmol/L, and creatinine 5.8 ± 1.5, 4.4 ± 0.5 and 4.8 ± 2.4 mmol/L for control, low dose and high dose AYPGKF-NH2, respectively. No distortion of renal parenchymal architecture, necrosis or inflammatory cell infiltrate was observed at either low or high dose (Figure 5.3).

5.4.6 Effects on liver

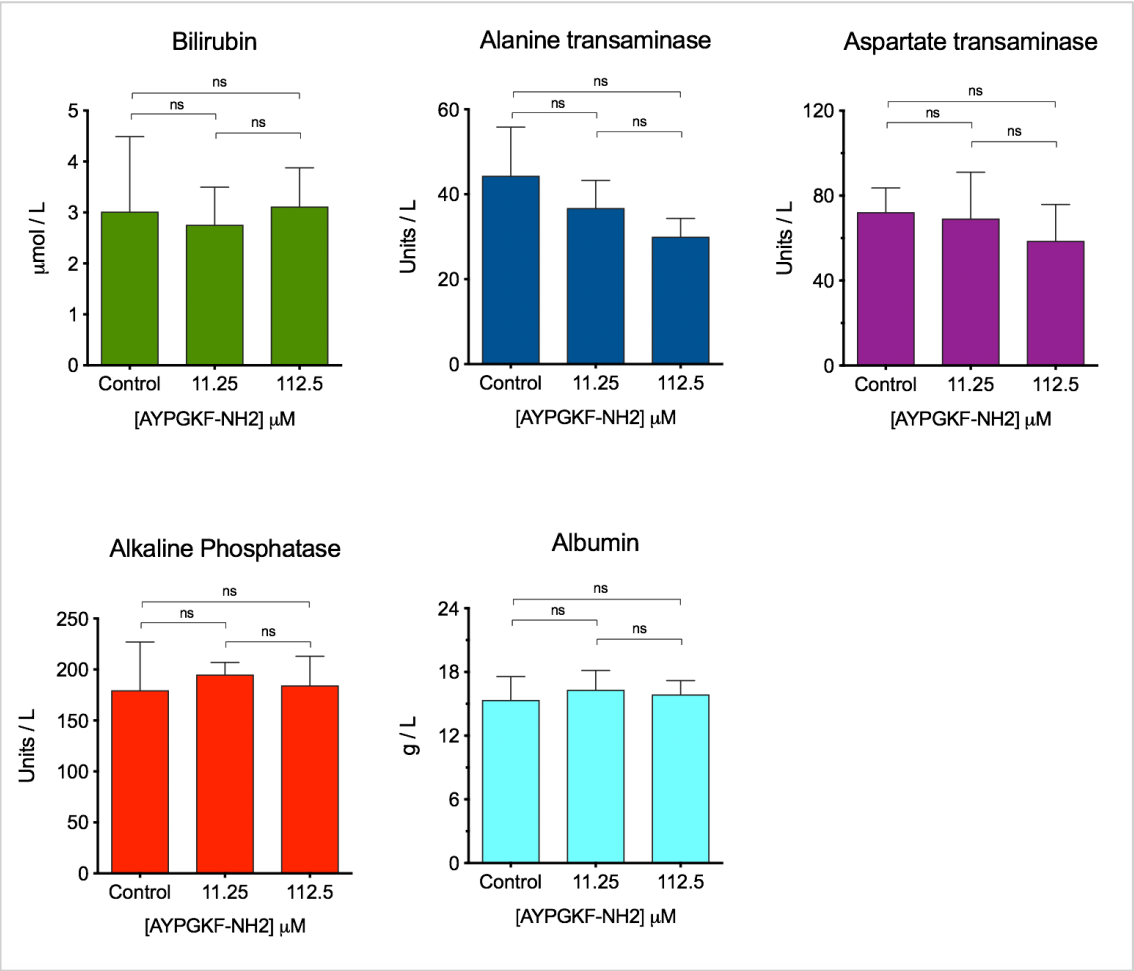


Figure 5.4. Effect of systemic PAR4 agonism with AYPGKF-NH2 versus control on hepatic laboratory indices

Data shown are the mean values ± 95% confidence intervals at 24 hours following exposure to control (0.9% NaCl, n=5), AYPGKF-NH2 at a systemic concentration of 11.25 uM (n=5), and AYPGKF-NH2 at a systemic concentration of 112.5 M (n=5). Statistical comparisons

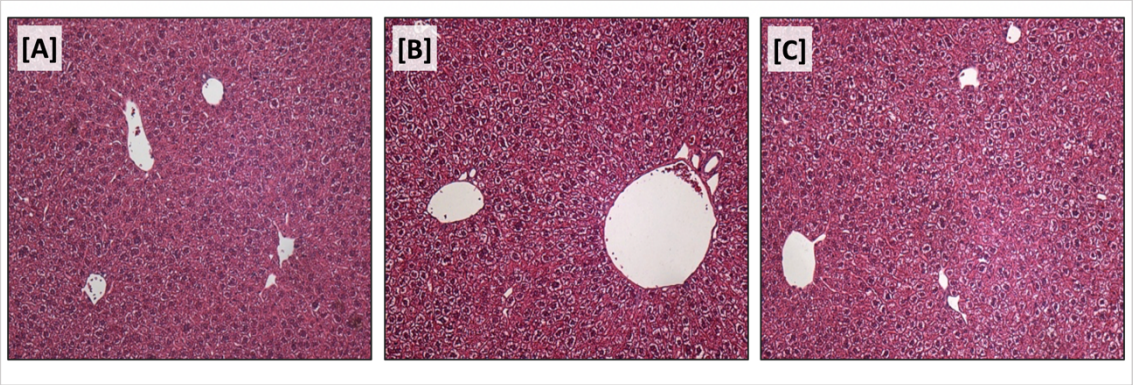


Figure 5.5. Effect of systemic PAR4 agonism with AYPGKF-NH2 on hepatic parenchymal tissue
Representative sections (x200 magnification) of hepatic parenchymal tissue from mice exposed to [A] control (n=5), [B] AYPGKF-NH2 at a systemic concentration of 11.25 μM (n=5), and [C] AYPGKF-NH2 at a systemic concentration of 112.5 μM (n=5). Sections are

AYPGKF-NH2 was not associated with a change in blood markers of hepatic injury or dysfunction as compared to control (Figure 5.4). Bilirubin was 3.02 ± 1.2, 2.76 ±0.6 and 3.12 ±0.6 μmol/L, ALT was 44.4 ± 9.2, 36.8 ± 5.2 and 30.0 ± 3.5 units/L, AST 72.3 ± 7.1, 69.2 ± 17.6 and 58.8 ± 13.7 units/L, alkaline phosphatase 179.6 ± 38.1, 195.0 ± 9.6 and 184.4 ± 23.1 units/L, and albumin 15.4 ± 1.8, 16.3 ±1.5 and 15.9 ±1.0 g/L for control, low dose AYPGKF-NH2, and high dose AYPGKF-NH2, respectively (Figure 5.4). No distortion of hepatic parenchymal architecture, necrosis or inflammatory cell infiltrate was observed at either low or high dose (Figure 5.5).

5.4.7 Effects on spleen

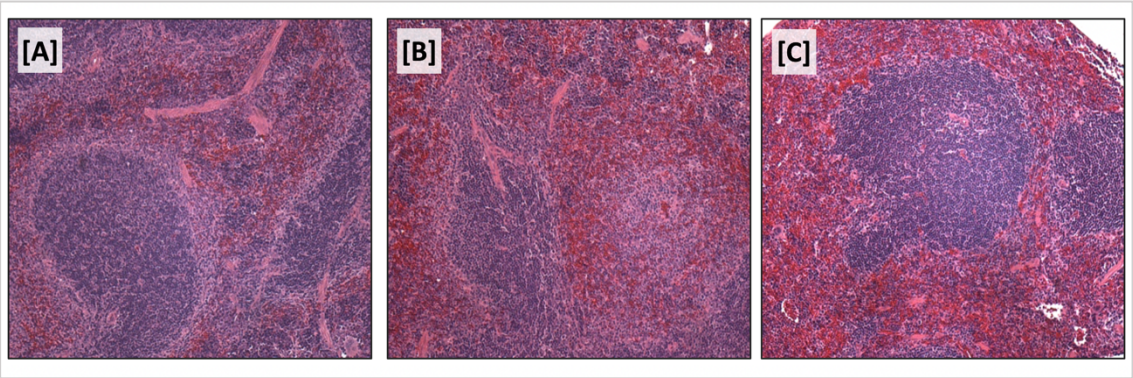


Figure 5.6. Effect of systemic PAR4 agonism with AYPGKF-NH2 on splenic parenchymal tissue
Representative sections (x200 magnification) of splenic parenchymal tissue from mice exposed to [A] control (n=5), [B] AYPGKF-NH2 at a systemic concentration of 11.25 µM (n=5), and [C] AYPGKF-NH2 at a systemic concentration of 112.5 µM (n=5). Sections are

AYPGKF-NH2 was not associated with distortion of splenic parenchymal architecture, necrosis or inflammatory cell infiltrate at either low or high dose, as compared to control (Figure 5.6).

5.5 DISCUSSION

The main findings of this study are that in mice, intravenous administration of the PAR4 agonist AYPGKF-NH2 was well tolerated with no signs of distress, systemic inflammation, thrombosis or organ-specific dysfunction, even at suprathreshold concentrations. Our results provide support for the safety of intravascular AYPGKF-NH2 to examine the in vivo vascular actions of PAR4 in humans.

One of the major concerns for in vivo studies of PAR4 agonism in man is the potential for harmful thrombus formation given PAR4's role in platelet activation and aggregation. In the present study, AYPGKF-NH2 was given intravenously to mice at very high doses. Despite this, no signs of end-organ infarction or necrosis were observed. There were also no significant changes in platelet numbers or adverse splenic pathology. Our findings are even more reassuring given that, in contrast to humans, mice platelets do not express PAR1 [114,224–228]. Thus, our data indicates that at the intended doses of AYPGKF-NH2 for future in vivo studies in man, the risk of occlusive thrombosis is likely to be very low.

It has been suggested PAR4 may have an important role in inflammation with PAR4 identified on the surface of neutrophils [159], macrophages [160] and B cells [229]. Vergnolle and colleagues demonstrated topical administration of AYPGKF-NH2 to rat mesenteric venules increased leucocyte rolling and adhesion, while intraperitoneal injection caused an increase in leukocyte migration [159]. In rodents, high-dose intraplantar and intraarticular injection of AYPGKF-NH2 resulted in localized oedema and granulocyte infiltration [137,167–169] PAR4 inhibition or deficiency in mice has been shown to block neutrophil migration to injury [230], attenuate systemic and

localised inflammatory responses to endotoxaemia [231], and reduce paw swelling to tissue factor-initiated inflammation. In the present study, intravenous AYPGKF-NH2 was not associated with fever, leucocytosis or organ-specific inflammation at either low or high dose. Consequently, while PAR4 would appear to act as a chemoattractant and promote proinflammatory responses to local or systemic stress, our data indicates PAR4 stimulation in isolation is not sufficient to trigger clinically relevant inflammation.

PAR4 is found in the lungs, including alveolar macrophages [160] and airway epithelial cells [165]. Moffat and colleagues demonstrated intranasal administration of a PAR4 agonist in mice induced the recruitment of a small number of neutrophils into the airways. By contrast, trypsin (200–2000 U /kg) caused profound inflammation and lung damage [160]. We did not observe any sign of respiratory distress following systemic PAR4 stimulation, either clinically or pathologically. Our results therefore add to current evidence that PAR4 signaling is not a powerful mediator of inflammation in the lungs of mice, even at supra-physiological levels.

Studies in mice have revealed a possible role for PAR4 signaling in the progression of acetaminophen-induced [166] and experimental cholestatic liver injury [232]. However, in healthy mice, we found that PAR4 stimulation was not associated with hepatic injury or dysfunction. PAR4 has been detected in renal parenchyma but unlike PAR1 and PAR 2 there is little evidence PAR4 has any significant function [163,164,233]. Our results support these findings with intravenous AYPGKF-NH2 not associated with any sign of acute renal impairment or injury.

PAR4 is expressed on peripheral nerves and plexus bodies [161]. Animal data has suggested PAR4 may modulate local nociceptive responses that are both tissue and dose specific. For example, in rodents, intraarticular injection of a PAR4 agonist was pronociceptive [169,171] whereas intracolonic and intraplantar injection was proanalgesic [170]. In the current study, systemic PAR4 agonism with AYPGKF-NH2 was not associated with visible pain or distress. However, in the absence of exploring responses to noxious stimuli, modulation of nociception cannot be discounted.

5.6 LIMITATIONS

Inter-species differences in PAR expression could limit the applicability of our results. Platelet expression of PAR1 and PAR3 varies considerably and this includes important distinctions between mice and humans [114,224–228]. Fortunately, PAR4 appears much more conserved, with mice, rats, guinea pigs, rabbits, dogs, monkeys and humans all sharing similar PAR4 expression throughout the vasculature [158]. However, it remains unclear whether species expression of PAR4 is conserved across non-vascular cells or indeed if PAR4 functions the same. Mice received a single dose and were culled after 24 hours. It is therefore conceivable harmful effects may not have manifested and the effects of chronic PAR4 stimulation remain unknown. However, AYPGKF-NH2 can be expected to have a short half-life based on other similar small peptides [24,260] and we believe our study protocol was appropriate with respect to our proposed future studies in man. This study provides evidence of the safety and tolerability of systemic PAR4 agonism with AYPGKF-NH2. It was not designed to determine the organ-specific functions of PAR4 and our results do not provide any evidence to this effect.

5.7 CONCLUSION

In mice, intravenous administration of the PAR4 activating peptide AYPGKF-NH2 was well tolerated with no evidence of acute systemic toxicity, inflammation, thrombosis or organ injury, even at suprathreshold concentrations. Our results support the safety of AYPGKF-NH2 to investigate the vasomotor and vascular endothelial actions of PAR4 in humans in vivo.

5.8 APPENDIX A

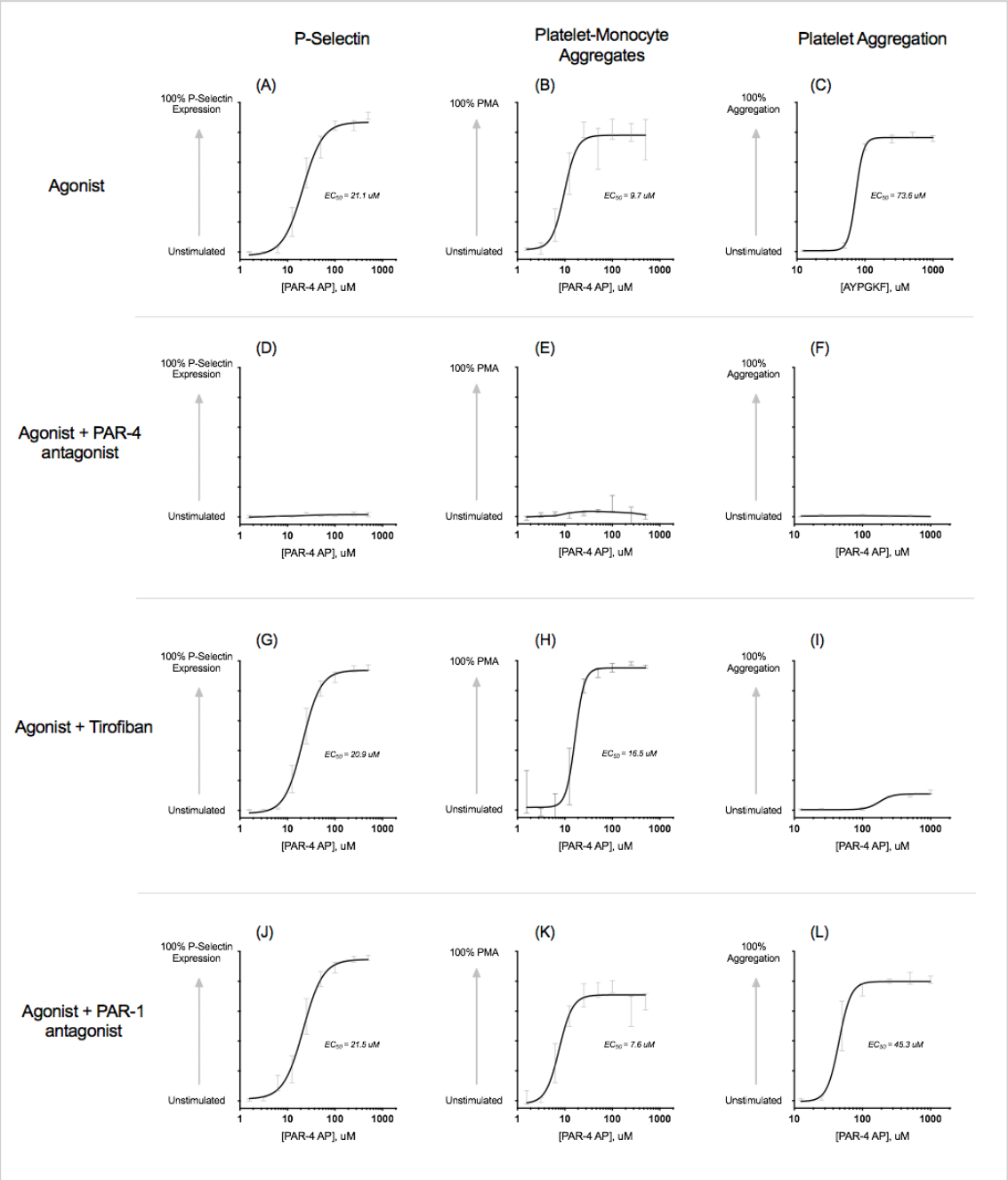


Figure 5.7. Dose response curves for platelet responses to AYPGKF-NH2 in the presence or absence of selected antagonists

Dose response curves for in vitro human platelet activation (P-selectin expression and platelet-monocyte aggregates) and platelet aggregation with AYPGKF-NH2 (A-C) alone, (D-G) in the presence of the PAR4 antagonist YD-3 (1000 nM), (G-I) in the presence of the GPIIb/IIIa inhibitor tirofiban (400 nM), and (J-L) in the presence of the PAR1 antagonist

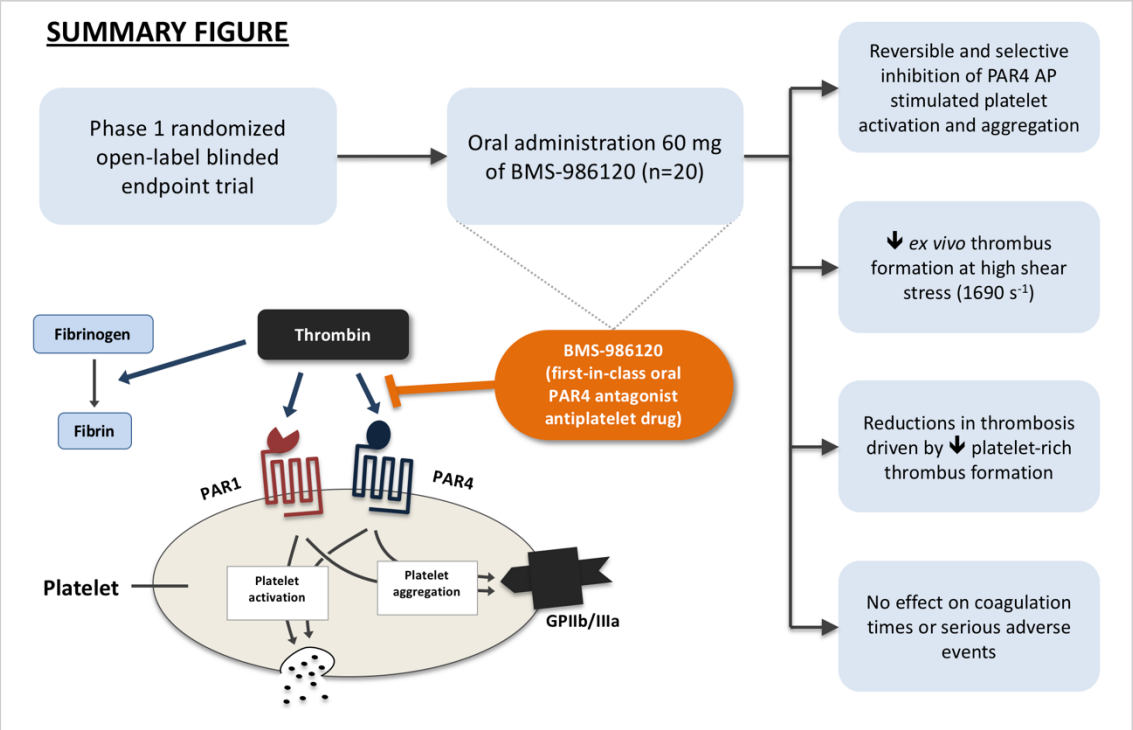
Prior to our toxicology studies, preliminary studies with blood from healthy volunteers (n=6) were conducted to confirm the specificity of our clinical grade AYPGKF-NH2 and to establish the dose range of interest [234–236]. AYPGKF-NH2 platelet activation and aggregation were fully inhibited by the PAR4 antagonist YD-3 but were not affected by the PAR1 antagonist SCH-79797. At a concentration of 200 μM, AYPGKF-NH2 induced full platelet activation and aggregation but remained almost completely inhibited by tirofiban (Figure 5.7).

Chapter 6

Effects of PAR4 Antagonism on Ex Vivo Human Platelets and Thrombosis

6 Effects of PAR4 Antagonism on Ex-Vivo Human Platelets and Thrombosis

6.1 SUMMARY



Background

There is an unmet clinical need for novel antiplatelet agents that can provide equivalent (or superior) antithrombotic efficacy to existing drugs but with a reduced bleeding risk. BMS-986120 is a novel first-in-class oral protease-activated receptor 4 (PAR4) antagonist that in animal models demonstrated potent antithrombotic effects with a substantially wider therapeutic index when compared to clopidogrel.

Objective

To determine the effect of oral PAR4 antagonism with BMS-986120 on human ex vivo platelet aggregation and thrombus formation.

Method and Results

Forty healthy volunteers completed a phase 1 parallel-group prospective randomized open-label blinded endpoint trial. Ex vivo platelet activation, platelet aggregation and thrombus formation were measured at 0, 2 and 24 hours after (a) oral BMS-986120 (60 mg), or (b) oral aspirin (600 mg) followed at 18 hours with oral aspirin (600 mg) and oral clopidogrel (600 mg). BMS-986120 demonstrated highly selective and reversible inhibition of PAR4-activating peptide (PAR4-AP; 100 μM) stimulated p-selectin expression, platelet-monocyte aggregates and platelet aggregation ($p<0.001$ for all). Compared to pre-treatment, total thrombus area ($\mu m^2/mm$) at high shear was reduced by 29.2% (95% CI, 18.3-38.7%; $p<0.001$) at 2 hours and by 21.4% (9.3-32.0%; $p=0.002$) at 24 hours. Reductions in thrombus formation were driven by a decrease in platelet-rich thrombus deposition: 34.8% (19.3-47.3%; $p<0.001$) at 2 hours and 23.3% (5.1-38.0%; $p=0.016$) at 24 hours. In contrast to aspirin alone, or in combination with clopidogrel, BMS-986120 had no effect on thrombus formation at low shear (p=ns). BMS-986120 administration was not associated with an increase in coagulation times or serious adverse events.

Conclusions

BMS-986120 is a highly selective and reversible oral PAR4 antagonist that substantially reduces platelet-rich thrombus formation under conditions of high shear stress. Our results suggest PAR4 antagonism with BMS-986120 has major therapeutic potential as a novel antiplatelet strategy.

6.2 INTRODUCTION

Having examined the impact of PAR4 agonism and antagonism on platelet activation and aggregation in vitro, we sought to determine the effect of oral PAR4 antagonism on platelets and thrombus formation in healthy volunteers. A phase 1 study was undertaken with subjects randomised to either PAR4 inhibition with BMS-986120 or the current standard of care antiplatelet agents, aspirin and clopidogrel. Compound effects were determined using the Badimon chamber, a validated translational model of human ex-vivo thrombosis.

6.3 METHODS

6.3.1 Study design

This was a phase I parallel group (n=20 per treatment arm) prospective randomized open-label blinded endpoint (PROBE) trial conducted at a single site (Clinical Research Facility, Royal Infirmary of Edinburgh, Scotland) between the 23rd September 2015 and 1st March 2016. Subjects were admitted to the Clinical Research Facility on Day 1. A total of 3 perfusion runs were performed per subject in each treatment arm. At each perfusion run, ex vivo platelet aggregation, platelet activation (treatment arm A only) and thrombus formation were measured. Aspirin ± clopidogrel were included as a positive control and assay validation tool.

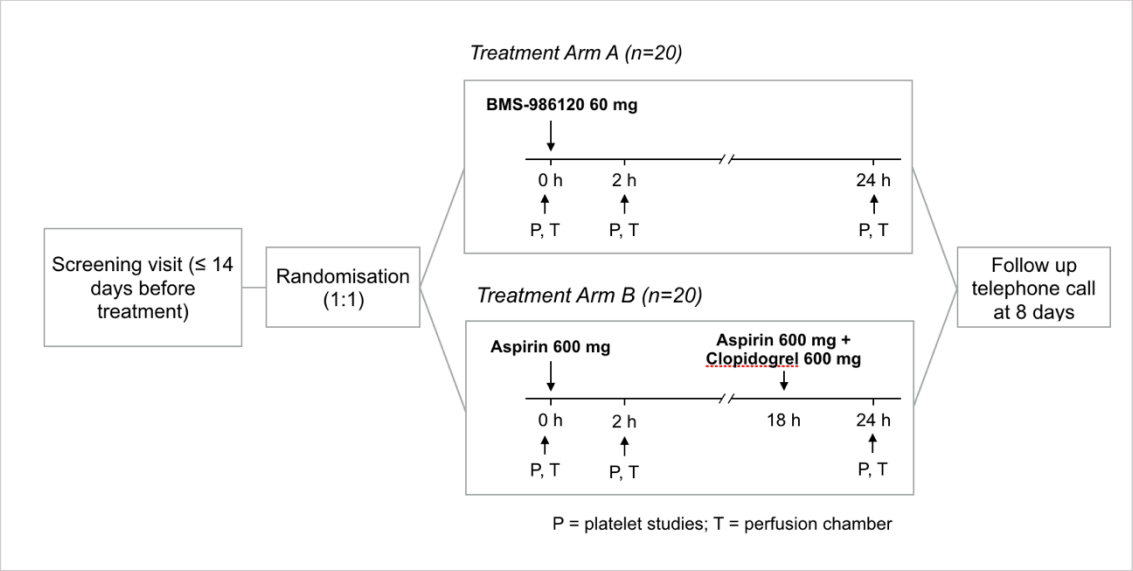


Figure 6.1. Schematic overview of study design

For treatment arm A, a baseline chamber run was performed immediately prior to BMS-986120 administration. A second run was performed 2 hours following oral administration of 60 mg BMS-986120. This second chamber run was designed to be conducted at the predicted Tmax. Subjects remained in the research facility overnight and a final chamber run was performed on day 2, approximately 24 hours post-dose. Subjects were discharged from the research facility after the conclusion of this run, with final discharge from the study following telephone follow-up on day 8.

For treatment arm B, a baseline chamber run was performed immediately prior to aspirin administration. A second run was performed on day 1, 2 hours following oral administration of 600 mg aspirin. Subjects remained in the research facility overnight. On day 2, subjects received oral administration of 600 mg aspirin and 600 mg clopidogrel. Four hours following oral administration of aspirin and clopidogrel, a final chamber run was performed. Subjects were discharged from the research facility after

the conclusion of this run, with final discharge from the study following telephone follow-up on Day 8. The study design schematic is presented in Figure 6.1.

The trial was sponsored by Bristol-Myers Squibb (BMS) and was designed collaboratively with the host academic center. The study was approved by the local research ethics committee, conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Clinical Trial Authorization was provided by the Medicines and Healthcare products Regulatory Authority of the United Kingdom.

6.3.2 Treatment assignment

Subjects meeting inclusion and exclusion criteria were eligible to be randomized. Subjects were randomized to receive either BMS-986120 (treatment arm A) or aspirin and clopidogrel (treatment arm B) according to a computer-generated randomization scheme prepared by a Randomization Coordinator within the Drug Supply Management Department of BMS Research and Development.

6.3.3 Study objectives

The primary study endpoint was the effect of BMS-986120 on total thrombus area as compared to pre-treatment. Secondary and exploratory endpoints included the effect of study drug (BMS-986120 or control) on platelet activation, platelet aggregation, platelet-rich and fibrin-rich thrombus formation, and blood coagulation.

The primary safety endpoint was the incidence of serious adverse events (SAEs) or death during and for up to 30 days post dosing. Additional safety endpoints included

changes in haematological and biochemical indices, haematuria (including microhaematuria), alteration in the 12-lead electrocardiogram (ECG), or abnormal findings on physical examination performed at baseline, 2 and 24 hours post dosing.

6.3.4 Study population

Healthy non-smoking male and female volunteers between the ages of 18 and 65 years (inclusive) and with a body mass index (BMI) of 18 to 32 kg/m^2 underwent screening including detailed medical history, physical examination, laboratory blood tests, urinalysis and 12-lead ECG. Exclusion criteria were women of child-bearing potential and any clinically significant coexisting condition including hypertension, hyperlipidemia, diabetes mellitus, gastrointestinal disease that could affect drug absorption, coagulopathy, recent infective or inflammatory condition, known liver disease or screening blood tests indicative of renal, liver, clotting, thyroid or hematological abnormality. Volunteers must not have been taking any prescription medications for 4 weeks, over-the-counter medications, herbal supplements and vitamins for 1 week, and alcohol or caffeine containing products for 72 hours prior to and for the duration of the study.

6.3.5 Sample size

Based on previous data, a sample size of 18 volunteers per study arm was determined to provide at least 80% power to reject the null hypothesis that a single 60 mg dose of BMS-986120 will reduce thrombus formation in the Badimon model of ex vivo human thrombosis, when the true mean change is a 20% decrease at the two-sided 5% level of significance [201–203,237]. This calculation assumes that thrombus area is log-

normally distributed and the differences on the log scale have a standard deviation of 0.31.

6.3.6 Dose selection

BMS-986120 is a competitive, reversible inhibitor of PAR4-activating peptide (PAR4-AP) induced platelet aggregation (K_{on} = 0.12 ± 0.043 $nM^{-1}min^{-1}$, K_{off} = 0.0082 ± 0.0016 min^{-1}, K_d = 0.098 ± 0.016 nM). In cynomolgus monkeys, BMS-986120 demonstrated dose-dependent (0.2-1.0 mg/kg) preservation of carotid arterial flow following electrolytic injury at the expense of a slight increase in mesenteric and kidney bleeding times [131]. In a single ascending (0.5-180 mg) and multiple ascending dose study (2.5-100 mg daily for up to 14 days) in healthy volunteers, BMS-986120 was found to be safe and well tolerated with complete and reversible inhibition of PAR4-AP stimulated platelet aggregation at ≥ 10 mg daily [146]. On the basis of these studies, a 60 mg dose was selected for the present phase 1 trial as this was calculated to be sufficient to inhibit platelet aggregation 2 hours post dose and would be at the edge of a potential pharmacodynamic effect at 24 hours. This would allow for "dose ranging" with a single dose of BMS-986120 whilst remaining well within the safety experience.

Doses of aspirin (600 mg) and clopidogrel (600 mg) were selected to reflect the maximal antithrombotic efficacy that might reasonably be expected in clinical practice following initiation of these antiplatelet agents in an acute setting.

6.3.7 Study outcome measures

6.3.7.1 Blood sampling and agonists

All blood samples for pharmacodynamic and pharmacokinetic assessments were drawn uncuffed through a 17-G cannula in the ante-cubital fossa. For each time point, the first 2.5 mL of blood was discarded. PAR1- and PAR4-APs (SFLLRN and A-Phe(4-F)-PGWLVKNG respectively) were provided by Bristol-Myers Squibb (Princeton, USA), adenosine diphosphate (ADP) by Sigma-Aldrich (Gillingham, UK) and arachidonic acid (AA) by Alpha Laboratories (Eastleigh, UK).

6.3.7.2 Pharmacokinetic assessment

Plasma concentrations of BMS-986120 were determined at 0, 1, 2, 3, 4, 5, 6, 9 and 24 hours post treatment using a validated liquid chromatography tandem-mass spectrometry method with a lower limit of quantification of 0.250 ng/mL, with an accuracy coefficient of variation of < 5%, and precision (intra- and inter-assay) coefficient of variation of < 10%. Blood samples were collected into 3 mL K_2EDTA vacutainers (Becton-Dickinson, Cowley, UK) and placed on wet ice. Within 1 hour of collection, samples were centrifuged at 1200 *g* (2-8 °C) for 10 minutes. Plasma was decanted and stored at -20 °C before analysis.

6.3.7.3 Platelet activation

Platelet p-selectin expression and platelet-monocyte aggregates were determined by flow cytometry. Blood (5 mL) was collected into 50 μL of 75 mM D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Enzo Life Sciences, Exeter, UK) then immediately aliquoted into micro-centrifuge tubes pre-filled with or without agonist and the following conjugated monoclonal antibodies: allophycocyanin (APC)-

conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for 20 minutes at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within 24 h using a FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

6.3.7.4 Platelet aggregation

Platelet aggregation was assessed by optical aggregometry (PAP-8E; Bio/Data Corp, Horsham, PA, USA) of platelet-rich plasma (PRP). To obtain PRP, 18 mL of blood was collected, mixed immediately with 2 mL of 3.8 % sodium citrate, and then centrifuged at 300 *g* (room temperature) for 15 minutes. For reference, 2 mL of PRP was centrifuged at 5500 *g* for 6 minutes to obtain platelet-poor plasma (PPP). All samples were allowed to equilibrate for 10 minutes (37 °C) after the addition of agonist and the peak aggregation recorded.

6.3.7.5 Ex vivo perfusion model of thrombosis

The effect of study compound on ex vivo thrombus formation was assessed using the Badimon perfusion chamber as described in detail in Chapter 2: Methods.

6.3.7.6 Histomorphometric analysis

Total thrombus formation, fibrin-rich thrombus formation and platelet-rich thrombus formation were assessed as described in detail in Chapter 2: Methods.

6.3.7.7 Safety and tolerability

The incidence of SAEs or death during and for up to 30 days post dosing were recorded. Adverse events (AEs) not meeting the SAE threshold were also recorded. All volunteers received telephone follow up on day 8. Reports of SAEs and AEs could originate from the volunteer, investigator or study personnel.

6.3.8 Statistical analysis

Following study completion, the database was locked and all statistical analyses carried out independent of the sponsor. The demographic and baseline characteristics of volunteers are expressed as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. The effect of study drug(s) on endpoints was assessed by general linear mixed effects models, with perfusion procedure (baseline, 2 and 24 hours) as fixed effects and subjects as random effects. Mean within-subject differences for the change from baseline were generated and analysed using the Least Significance Difference test. Prior to model fitting, total thrombus area, platelet area and fibrin area were log-transformed. Associations between plasma concentrations of BMS-986120 and study end-points were determined by Spearman's rank-order correlation (ρ). Two-sided p-values of ≤ 0.05 were considered statistically significant. Analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, New York) and R version 3.3.1 (R Project for Statistical Computing, Vienna, Austria).

6.4 RESULTS

6.4.1 Study population characteristics

Table 6.1. Baseline characteristics of study volunteers

Test variable	BMS-986120 (n=20)	Aspirin ± Clopidogrel (n=20)
Male (%)	20 (100)	20 (100)
Age, years (SD)	23.6 (3.4)	28.7 (10.0)
BMI, kg/m^2 (SD)	23.6 (2.6)	25.4 (3.5)
Race (%)		
Caucasian	19 (95)	19 (95)
Black/African	1 (5)	0
Asian	0	1 (5)
Haemoglobin, g/dL (SD)	14.2 (0.42)	14.6 (0.85)
Platelet count, $x10^9$ c/L (SD)	230 (45)	221 (49)
APTT, seconds (SD)	30.9 (2.2)	30.8 (2.6)
PT, seconds (SD)	12.3 (0.9)	11.9 (0.7)

Abbreviations used: SD, standard deviation; BMI, body mass index; APTT, activated partial thromboplastin time; PT, prothrombin time

All 40 volunteers (81 volunteers were screened) completed the study in full. The demographics and baseline characteristics of study volunteers were similar in the two treatment groups (Table 6.1).

6.4.2 Pharmacokinetic profile of oral BMS-986120

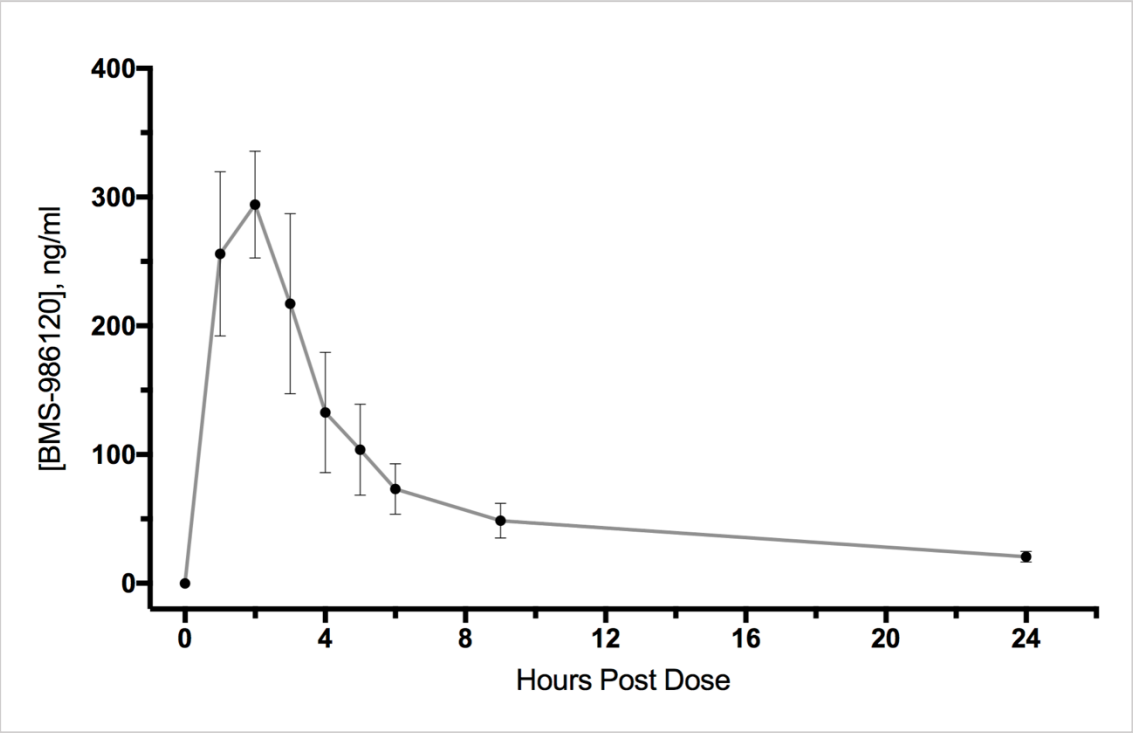


Figure 6.2. Pharmacokinetics of BMS-986120.
BMS-986120 was rapidly absorbed with a half-life of 4 hours. Data shown are mean plasma concentrations of BMS-986120 (± 95% confidence intervals) following administration of a single oral 60 mg dose (n=20).

BMS-986120 was rapidly absorbed with peak plasma concentrations occurring at 2 hours (255 ± 136 ng/mL; Figure 6.2). Plasma concentrations of BMS-986120 were halved by 4 hours (133 ± 100 ng/ml) and < 10% of the peak concentration by 24 hours (21 ± 9 ng/mL).

6.4.3 Effect of oral BMS-986120 on platelet activation and aggregation

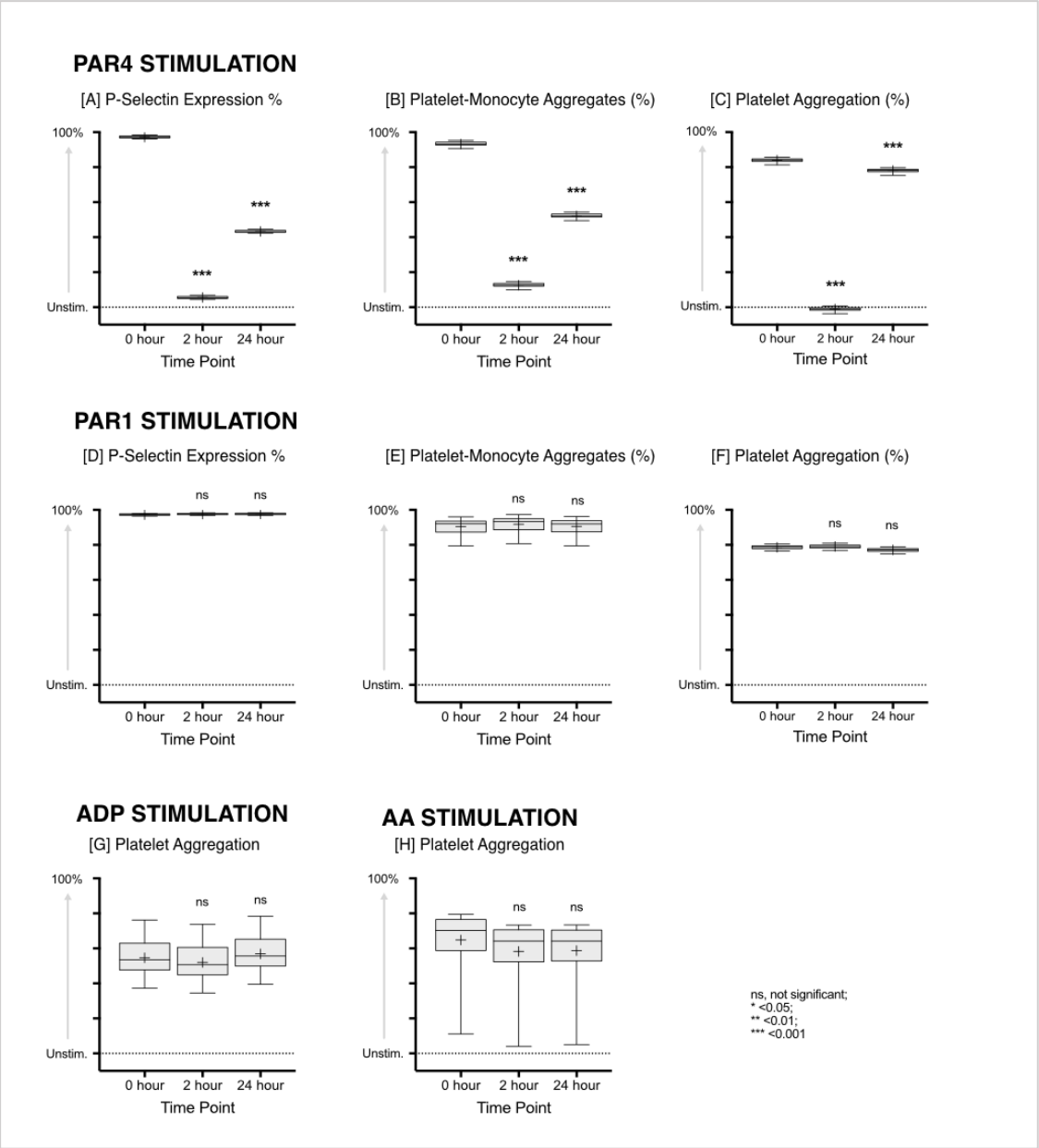


Figure 6.3. BMS-986120 demonstrated highly selective, potent and reversible inhibition of PAR4 stimulated platelet activation and aggregation.
Box plots of platelet activation and aggregation in response to (A-C) PAR4 AP [100µM], (D-E) PAR1 AP [100µM], (F) PAR1 AP [25µM], (G) ADP [10µM] and (H) AA [5mM], in volunteers randomised to BMS-986120 (n=20). Data shown are the adjusted mean (+) normalised to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (Least Significance Difference test) versus 0 hour are represented above each plot: ns=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: ADP, adenosine diphosphate; AA, arachidonic acid.

BMS-986120 demonstrated strong and reversible inhibition of PAR4-AP (100 μM) stimulated platelet activation and aggregation ($p<0.001$ for all). Compared to pre-treatment, PAR4-AP stimulated increases in platelet p-selectin expression (%), platelet-monocyte aggregates (%) and platelet aggregation (%) were reduced by 91.7% (95% CI, 81.0 to 102.4), 80.6% (95% CI, 68.6 to 92.6%) and 85.0% (95% CI, 82.0 to 88.1) at 2 hours and by 53.9% (95% CI, 43.2 to 64.7%), 41.1% (95% CI, 28.9 to 53.2%) and 6.0% (95% CI, 2.9 to 9.0%) at 24 hours ($p<0.001$ for all; Figure 6.3). There was no effect on PAR1-AP, ADP, or AA platelet responses (p=ns for all; Figure 6.3).

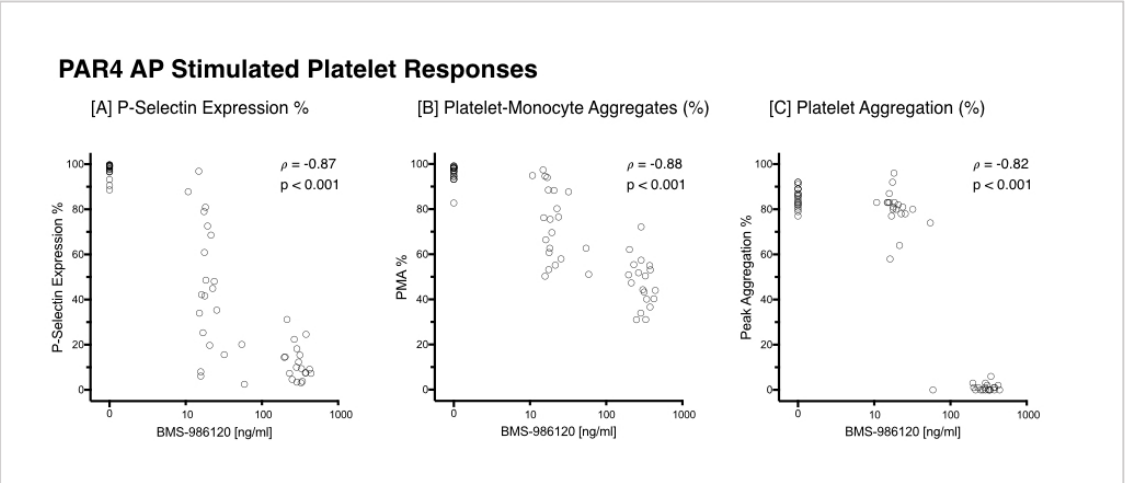


Figure 6.4. Correlations between plasma concentrations of BMS-986120 and platelet responses.
Data shown are scatter plots of [A] PAR4-AP stimulated p-selectin expression, [B] PAR4-AP stimulated platelet-monocyte aggregates, and [C] PAR4-AP stimulated platelet aggregation in volunteers randomised to BMS-986120 (n=20). Correlation coefficients (ρ) and p-values were determined by Spearman's rank-order correlation.

Plasma concentrations of BMS-986120 correlated with p-selectin expression (ρ=-0.87), platelet-monocyte aggregates (ρ=-0.88), and platelet aggregation (ρ=-0.82; p<0.001 for all).

6.4.4 Effect of aspirin ± clopidogrel on platelet aggregation

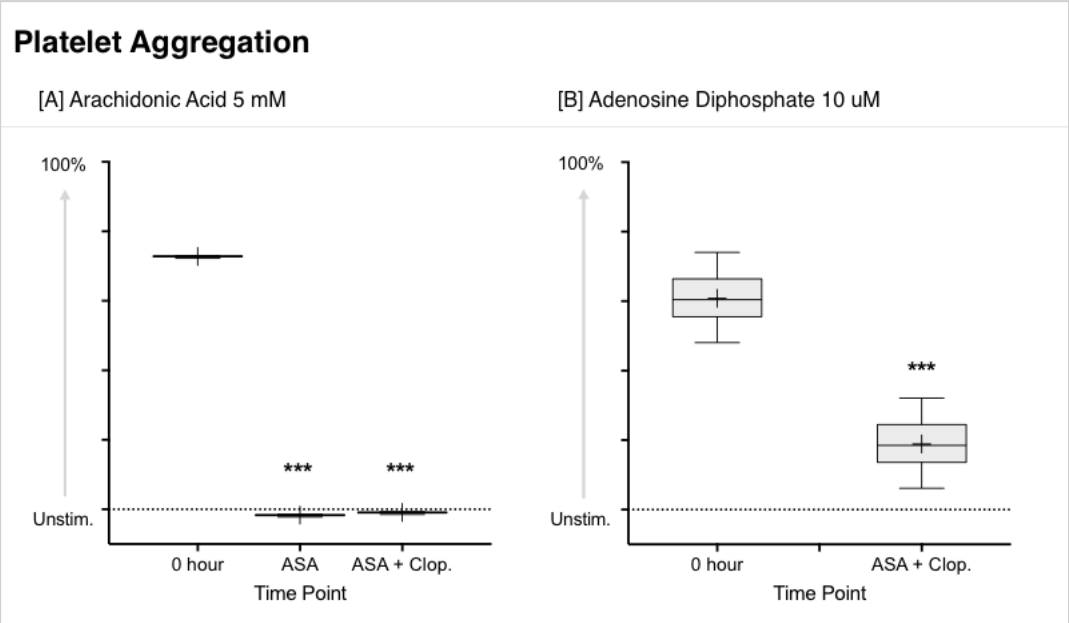


Figure 6.5. Box plots of platelet aggregation in response to (A) arachidonic acid [5 mM] and (B) adenosine diphosphate [10 µM] in volunteers randomised to aspirin ± clopidogrel (n=20).
Data shown are the adjusted means (+) normalised to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (Least Significance Difference test) versus 0 hour are shown above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Aspirin administration reduced AA stimulated platelet aggregation by 74.5% (95% CI, 71.6 to 77.3%; $p<0.001$). In combination with clopidogrel, aspirin reduced AA stimulated platelet aggregation by 73.7% (95% CI, 70.9 to 76.5%; $p<0.001$) and ADP stimulated platelet aggregation by 41.9% (95% CI, 35.2 to 48.7%; $p<0.001$) respectively (Figure 6.5).

6.4.5 Effect of BMS-986120 on ex vivo thrombus formation

6.4.5.1 Total thrombus formation

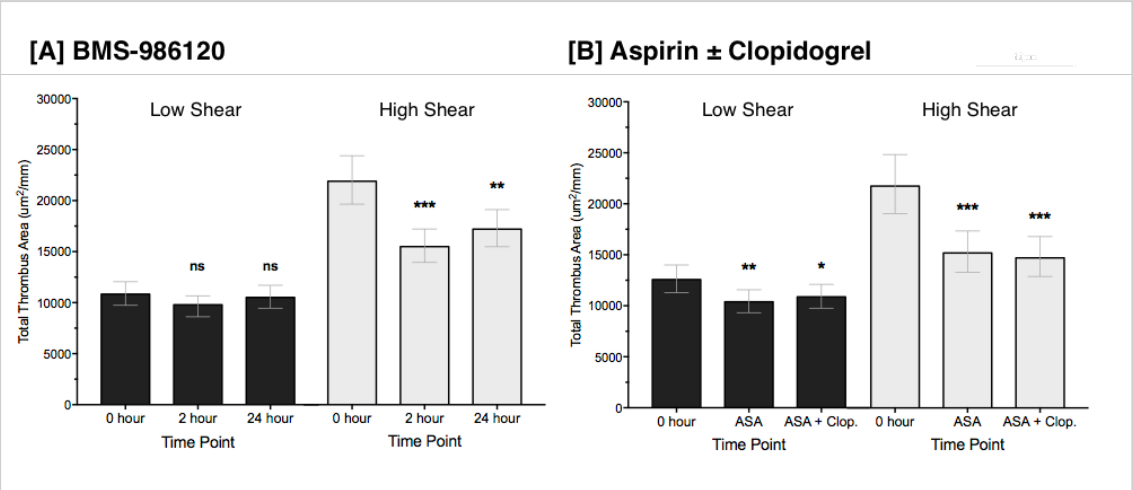


Figure 6.6. BMS-986120 reduced thrombus formation at high shear but not at low shear.
Effect of [A] BMS-986120 (n=20) and [B] aspirin ± clopidogrel (n=20) on total thrombus area at high and low shear. Statistical comparisons (Least Significance Difference test) versus 0 hour are represented above each plot: ns=not significant, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: ASA, aspirin; Clop., clopidogrel.

BMS-986120 reduced total thrombus formation at high shear (p<0.001) but not at low shear (p=ns; Figure 6.6). Compared to pre-treatment, total thrombus area (μm^2/mm) at high shear was reduced by 29.2% (95% CI, 18.3 to 38.7%; p<0.001) at 2 hours and by 21.4% (95% CI, 9.3 to 32.0%; p=0.002) at 24 hours.

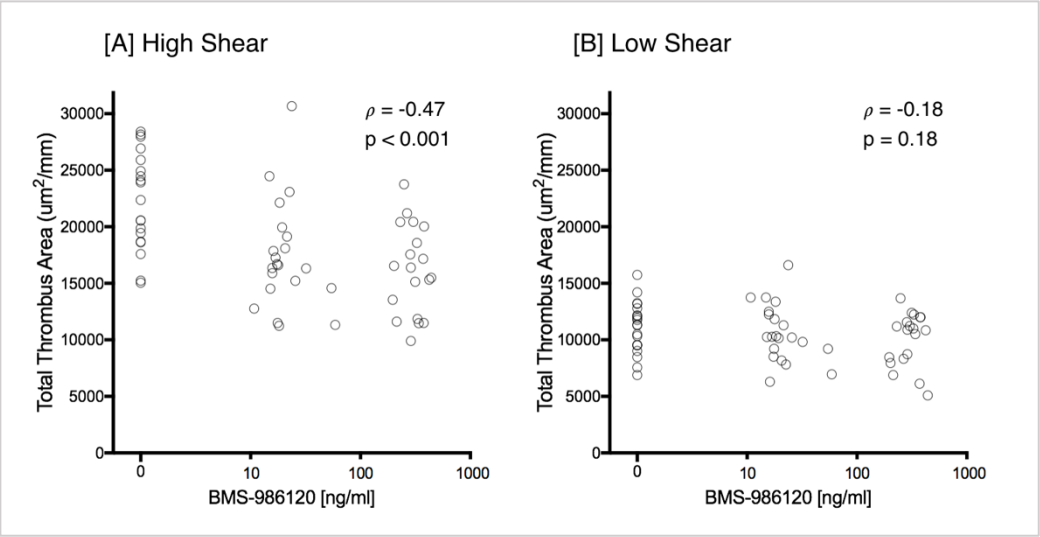


Figure 6.7. Correlations between plasma concentrations of BMS-986120 and total thrombus formation.
Data shown are scatter plots of total thrombus formation at [A] high shear and [B] low shear in volunteers randomised to BMS-986120 (n=20). Correlation coefficients (ρ) and p-values were determined by Spearman's rank-order correlation.

Plasma concentrations of BMS-986120 correlated with total thrombus formation at high shear (ρ=-0.47, p<0.001), but not at low shear (ρ=-0.18; p=ns; Figure 6.7).

6.4.5.2 Effect of BMS-986120 on platelet-rich and fibrin-rich thrombus formation

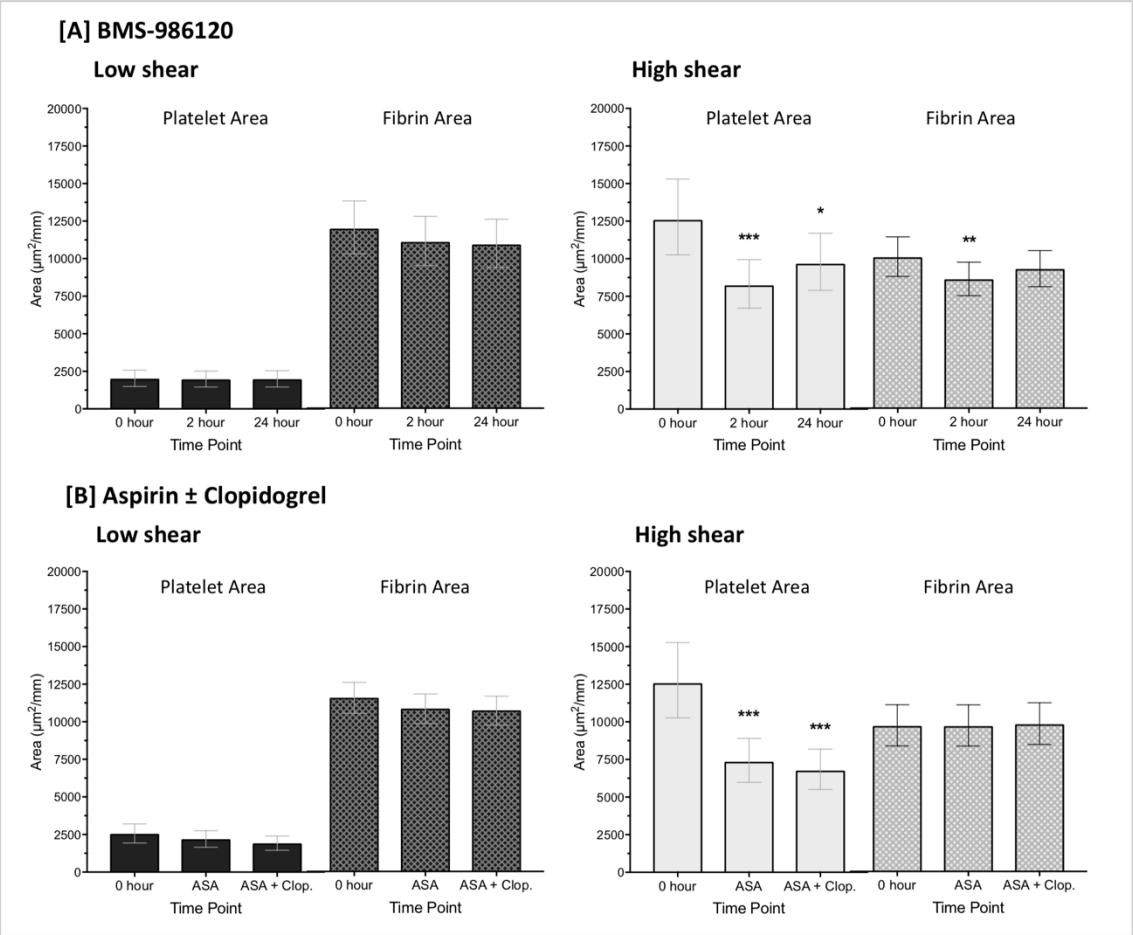


Figure 6.8. Reductions in thrombus were driven by a decrease in platelet-rich thrombus formation.
Effect of [A] BMS-986120 (n=20) and [B] aspirin ± clopidogrel (n=20) on platelet-rich and fibrin-rich thrombus formation at low and high shear. Data shown are adjusted means ± 95% confidence intervals. Statistical comparisons (Least Significance Difference test) versus 0 hour are represented above each plot: ns=not significant, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: ASA, aspirin; Clop., clopidogrel.

Reductions in total thrombus area were driven by a decrease in platelet deposition (Figure 6.8). At high shear, platelet-rich thrombus area was reduced by 34.8% (95% CI, 19.3 to 47.3%; p<0.001) at 2 hours and 23.3% (95% CI, 5.1 to 38.0%; p=0.016) at 24 hours. Reductions in fibrin-rich thrombus area at 2 h (-14.7%; 95% CI, -22.5 to -6.2%; p=0.002) and 24 h (-7.9%; 95% CI, -16.3 to 1.4%, p=0.09) were small by comparison.

BMS-986120 had no effect on platelet-rich or fibrin-rich thrombus formation at low shear (p=ns for all).

6.4.6 Effect of aspirin ± clopidogrel on ex vivo thrombus formation

Aspirin and aspirin in combination with clopidogrel both reduced thrombus development at high and low shear, also driven by decrease in platelet-rich thrombus formation. Aspirin reduced total thrombus area and platelet-rich thrombus area by 30.2% (95% CI, 15.6 to 42.2%; p<0.001) and 41.7% (95% CI, 22.9 to 56.0%; p<0.001) respectively, and by 32.4% (95% CI, 18.3 to 44.0%; p<0.001) and 46.4% (95% CI, 29.1% to 59.5%; p<0.001) respectively when used in combination with clopidogrel.

In contrast to BMS-986120, aspirin and aspirin in combination with clopidogrel both reduced total thrombus area at low shear (-17.4%; 95% CI, -27.0 to -6.5%; p=0.003 and -13.5%; 95% CI, -23.6 to -2.1%; p=0.02). There was no effect on fibrin-rich thrombus formation at low or high shear (p=ns for all).

6.4.7 Effect of study drug on coagulation and safety assessments

Table 6.2. Biochemical and ECG assessments

Test variable	BMS-986120 (n=20)			Aspirin ± Clopidogrel (n=20)		
	0 hour	2 hour	24 hour	0 hour	ASA	ASA+Clo
ALT, U/L (SD)	19.0 (6.1)	18.7 (6.2)	17.8 (6.4)**	26.3 (14.8)	25.9 (14.2)	25.4 (13.3)
AST, U/L (SD)	20.7 (4.6)	20.9 (5.7)	18.0 (3.6)***	23.6 (7.4)	23.4 (6.8)	21.0 (4.5)**
Bilirubin, mg/dL (SD)	0.70 (0.3)	0.80 (0.3)*	0.62 (0.3)	0.76 (0.2)	0.73 (0.2)	0.66 (0.2)**
Creatine Kinase, U/L (SD)	197 (190)	187 (188)	115 (83)***	186 (144)	171 (128)	108 (61)***
Urea, mmol/L (SD)	5.1 (0.96)	4.6 (0.76)**	4.1 (0.77)***	5.0 (1.0)	4.7 (0.93)	4.5 (0.98)**
Creatinine, mg/dL (SD)	0.83 (0.06)	0.81 (0.09)	0.82 (0.08)	0.89 (0.11)	0.86 (0.09)	0.86 (0.10)
Haemoglobin, g/dL (SD)	14.2 (0.4)	14.2 (0.4)	14.2 (0.3)	14.6 (0.8)	14.4 (0.8)	14.3 (1.3)
Platelet count, $x10^9$ c/L (SD)	229 (45)	227 (46)	228 (46)	221 (49)	220 (50)	216 (53)
APTT, sec (SD)	30.9 (2.1)	30.3 (2.7)	30.4 (2.1)	30.8 (2.5)	30.3 (3.3)	30.1 (2.9)
PT, sec (SD)	12.3 (0.9)	12.3 (0.9)	12.1 (0.8)	11.9 (0.7)	12.3 (0.7)**	12.1 (0.7)
QTCf interval, millisec (SD)	405 (11.8)	411 (17.9)	405 (15.9)	401 (16.3)	402 (13.4)	399 (10.8)

Data shown are the adjusted means. All statistically significant differences versus 0 hour are presented: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: ASA, aspirin; Clo, clopidogrel. ALT, alanine transaminase; ASA, Aspirin; AST, aspartate transaminase; APTT, activated partial thromboplastin time; Clop, Clopidogrel; PT, prothrombin time; QTCf, QTC interval corrected for heart rate by Fridericia's formula; and SD, standard deviation

BMS-986120 did not prolong coagulation and was not associated with clinically significant effect on any of the biochemical, haematological or ECG safety assessments conducted throughout the study (Table 6.2). Similarly, BMS-986120 was not associated with haematuria or impact on physical assessments including heart rate, blood pressure and temperature.

6.4.8 Adverse events

Table 6.3. Overview of adverse events

	BMS-986120 (n=20)	Aspirin ± Clopidogrel (n=20)
Any AE, n (%)	7 (35.0)	2 (10.0)
Headache	3 (15.0)	0
Nasopharyngitis	2 (10.0)	0
Abdominal discomfort	0	1 (5.0)
Dizziness	1 (5.0)	0
Pruritus	1 (5.0)	0
Rash	1 (5.0)	0
Rectal bleeding	0	1 (5.0)
Treatment-related AE, n (%)	2 (10.0)	2 (10.0)
SAE, n (%)	0	0
Death, n (%)	0	0
Discontinued due to AE, n (%)	0	0

Abbreviations used: AE, adverse event; SAE, serious adverse event.

BMS-986120 was well tolerated with no serious adverse events. One episode of minor bleeding was reported 12 hours following aspirin administration (Table 6.3).

6.5 DISCUSSION

In this phase 1 PROBE designed clinical trial, we have shown that PAR4 antagonism with BMS-986120 reduces ex vivo human thrombus formation under conditions representative of deep arterial injury in a stenosed coronary artery. BMS-986120 demonstrated selective and reversible antiplatelet effects with concentration-dependent inhibition of thrombus formation and PAR4-AP stimulated platelet activation and aggregation. Our results provide further insights into the role of PAR4 in human thrombogenesis and raise major promise for oral PAR4 antagonism with BMS-986120 in the treatment and prevention of arterial thrombosis.

BMS-986120 is the first orally bioavailable PAR4 antagonist [131,146]. A 60 mg oral dose of BMS-986120 resulted in near complete inhibition of PAR4-AP stimulated platelet activation and aggregation at 2 hours, with a return towards baseline at 24 hours. Consistent with earlier data from in vitro testing (Chapter 4), there was no effect on PAR1-AP, ADP or AA stimulated platelet activity. Our results therefore confirm previous reports that oral administration of BMS-986120 results in highly selective and reversible inhibition of PAR4-mediated platelet activation and aggregation in humans [146].

To examine the antithrombotic effects of PAR4 antagonism we used the Badimon perfusion chamber, a well validated model for measuring ex vivo thrombus formation [196–203]. Under similar experimental conditions, previous studies have demonstrated reductions in high shear thrombus formation of 18.7% following a 300 mg oral dose of clopidogrel, 28% with a 60 mg oral dose of edoxaban, and 56% with extracorporeal co-administration of tirofiban (50 ng/mL) [200,203,238]. In the present study, a single 60

mg dose of BMS-986120 reduced high shear thrombus formation by nearly a third. This is consistent with pre-clinical animal data [117,131] with reductions in thrombus formation also comparable to those observed with high loading doses of aspirin and clopidogrel. Thus, we have shown for the first time, that PAR4 inhibition with BMS-986120 substantially reduces ex vivo human thrombus formation. Moreover, given reductions were similar in magnitude to clinically approved antiplatelet agents, our data suggests a high probability of in vivo antithrombotic efficacy.

BMS-986120 appeared to have less of an effect on thrombus formation at low shear than either aspirin alone or in combination with clopidogrel. Low shear rates reflect flow conditions found in patent epicardial arteries and veins, whereas the majority of atherothrombotic events occur at sites of high shear stress seen in diseased arteries [239,240]. Indeed, most myocardial infarctions arise from stenotic atherosclerotic plaques with rheological conditions comparable to those in our high shear chamber [241,242]. Accordingly, antiplatelet agents that are more selective for inhibiting thrombus formation at high shear may allow at-risk vascular beds to be targeted with greater specificity and this may have significant advantages in terms of widening the safety profile given many treatment-related bleeding events occur from vessels with low shear rates [243–245]. Further studies are required to confirm if PAR4 antagonism is more high-shear selective than existing agents. However, it is noteworthy that distinct mechanisms of platelet adhesion and aggregation are known to operate under different rheological conditions [246,247].

As expected from an antiplatelet agent, reductions in thrombus were driven by a decrease in platelet aggregates. However, there was also a small but significant

reduction in fibrin-rich thrombus formation. PAR4 is reported to be the predominant platelet PAR responsible for phosphatidylserine exposure, micro-particle shedding and thrombin generation [248]. Our results add to these findings, indicating that PAR4 may have a role in platelet procoagulant activity during ex vivo human thrombus formation. Whether this is beneficial or not is uncertain, but it is notable that no bleeding-related clinical findings or serious adverse events occurred in this or a previous phase 1 single- and multiple-ascending dose study [146].

6.6 LIMITATIONS

Our study has some potential limitations. First, although the exposed porcine aortic media presents many of the common constituents of a disrupted atherosclerotic plaque, it may not contain tissue factor (TF) [249–251]. TF activates the coagulation cascade and is an important contributor to thrombogenicity [252,253]. Nevertheless, this does not overly limit our model for the assessment of thrombosis because binding of blood borne circulating TF is sufficient to allow activation of the coagulation cascade and thrombus propagation [249,250,254–256]. Indeed, previous studies have demonstrated that thrombus formed from human blood perfused over exposed porcine tunica media (devoid of TF) stains heavily for TF [249,250]. Second, we assessed a single oral dose of BMS-986120 and did not explore the effect of prolonged BMS-986120 administration on thrombus formation, such as would occur with the secondary prevention of myocardial infarction and stroke. However, as this was the phase 1 trial designed to examine the antithrombotic effects of oral PAR4 antagonism in humans for the first time, we felt our study design was appropriate. Third, BMS-986120 was dosed in isolation, and future studies to determine the antiplatelet and antithrombotic effects of PAR4 antagonism in combination with current agents would be of interest. Finally,

while no episodes of bleeding occurred in volunteers administered BMS-986120, and BMS-986120 was not associated with an increase in bleeding times in a previous phase 1 safety and tolerability study [146], the safety profile of PAR4 antagonism in humans remains to be defined.

6.7 CONCLUSION

We have demonstrated for the first time that oral PAR4 antagonism with BMS-986120 substantially reduces ex vivo human thrombus formation under conditions of high shear stress. BMS-986120 was well tolerated with no change in coagulation assays or serious adverse events. Our results suggest PAR4 antagonism has major potential as a novel antiplatelet strategy and that further investigation in clinical trials is warranted.

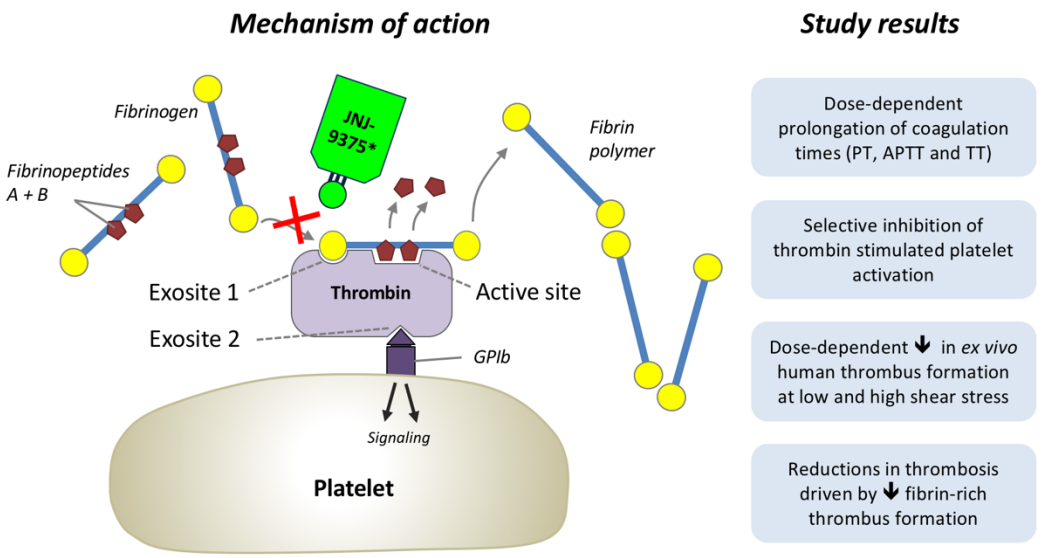
Chapter 7

Effects of Exosite 1 Thrombin Inhibition on Humans Coagulation, Platelets and Thrombosis

7 Effects of Exosite 1 Thrombin Inhibition on Human Coagulation, Platelets and Thrombosis

7.1 SUMMARY

SUMMARY FIGURE



* JNJ-9375 binds reversibly and with high affinity and specificity to the exosite 1 region of thrombin, thereby acting to inhibit the interaction of thrombin with its exosite 1 substrates (which include fibrinogen) while retaining function of both the active site and exosite 2.

Background

JNJ-64179375 (hereafter JNJ-9375) is a first-in-class, highly specific, large molecule, exosite 1 thrombin inhibitor. In preclinical studies, JNJ-9375 demonstrated robust antithrombotic protection with a wider therapeutic index when compared to apixaban.

Objective

To examine the antiplatelet, anticoagulant and antithrombotic effects of exosite 1 thrombin inhibition with JNJ-9375 in a translational model of ex vivo human thrombosis.

Methods and Results

Fifteen healthy volunteers participated in a double-blind randomized crossover study of JNJ-9375 (2.5, 25 and 250 μg/mL), bivalirudin (6 μg/mL; positive control) and matched placebo. Coagulation, platelet activation and thrombus formation were determined using coagulation assays, flow cytometry and an ex vivo perfusion chamber respectively. JNJ-9375 caused concentration-dependent prolongation of all measures of blood coagulation (prothrombin time, activated partial thromboplastin time, thrombin time; $p<0.001$ for all) and agonist selective inhibition of thrombin (0.1 U/mL) stimulated platelet p-selectin expression ($p<0.001$) and platelet-monocyte aggregates ($p=0.002$). Compared to placebo, JNJ-9375 (250 μg/mL) reduced mean total thrombus area by 41.1% (95% confidence intervals, 22.3 to 55.3%; $p<0.001$) at low shear and 32.3% (4.9 to 51.8%; $p=0.025$) at high shear. Under both shear conditions, there was a dose-dependent decrease in fibrin-rich thrombus ($p<0.001$ for both) but not platelet-rich thrombus (p=ns for both).

Conclusion

Exosite 1 inhibition with JNJ-9375 caused prolongation of blood coagulation, selective inhibition of thrombin-mediated platelet activation, and reductions in ex vivo thrombosis driven by a decrease in fibrin-rich thrombus formation. JNJ-9375 represents a novel class of anticoagulant with potential therapeutic applications.

7.2 INTRODUCTION

JNJ-64179375 (hereafter JNJ-9375) is a first-in-class, recombinant, fully human, IgG4 monoclonal antibody anticoagulant that binds reversibly and with high affinity and specificity to the exosite 1 region of thrombin [179]. JNJ-9375 therefore acts to inhibit the interaction of thrombin with its exosite 1 substrates, which include fibrinogen, but retains function of both the active site and exosite 2 [185]. It is hypothesised this unique capacity to inhibit fibrinogen binding while preserving other non-exosite 1 protease interactions will allow for a wider therapeutic index. Building on encouraging animal data [179], the present study sought to examine for the first time the anticoagulant and antithrombotic effects of exosite 1 thrombin inhibition with JNJ-9375 in human blood using a translational model of ex vivo thrombosis.

7.3 METHODS

7.3.1 Study design

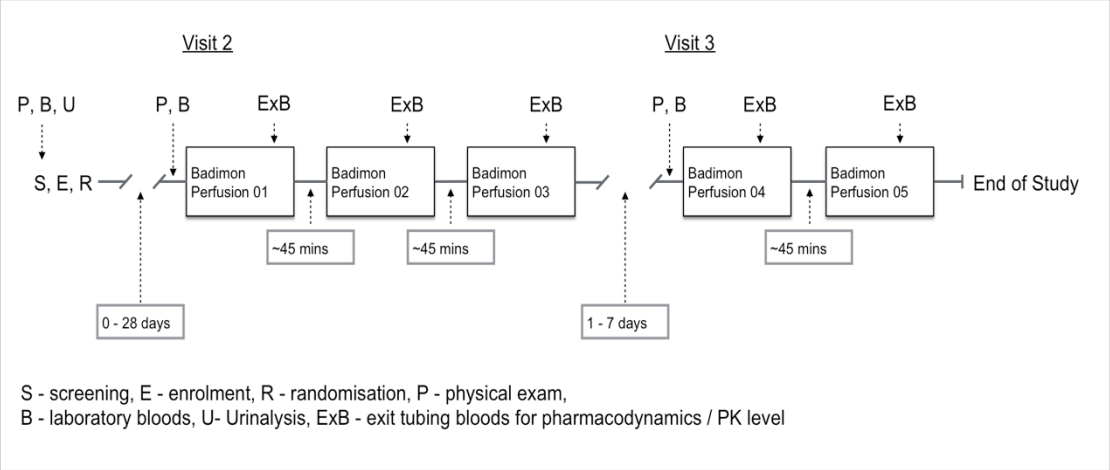


Figure 7.1. Schematic overview of study design

This was a double-blind randomised controlled five-way crossover study conducted at a single site (Clinical Research Facility, Royal Infirmary of Edinburgh, Scotland) between the 24th May 2016 and 1st July 2016. Study measures were performed during extracorporeal infusion of JNJ-9375 (estimated final concentration of 2.5, 25 and 250 μg/mL), bivalirudin (positive control; estimated final concentration of 6 μg/mL; The Medicines Company, Abingdon, UK) at a dose equivalent to recommendations at the time of percutaneous coronary intervention (PCI), and matched placebo (10 mM phosphate, 8.5% (w/v) sucrose, 0.04 % (w/v) polysorbate 20, 10 μg/mL EDTA, pH 7.1; Janssen Research and Development) upstream of the perfusion chambers. Three perfusion chamber studies were performed at the first experimental visit and two perfusion chamber studies at the second experimental visit (Figure 7.1).

7.3.2 Study objectives

The primary objective was to assess the relationship of JNJ-9375 dose concentrations to ex vivo thrombus formation under conditions of both low and high shear stress, and to compare these effects with placebo under the same rheological conditions. Bivalirudin, which blocks both exosite 1 and the active site of thrombin, was used as a positive control. Secondary objectives included a similar comparison of compound effects on platelet activation, markers of coagulation, and the fibrin and platelet components of thrombus formation. Finally, correlations between measured chamber concentrations of study drug and pharmacodynamic end-points were explored.

7.3.3 Study population

Healthy non-smoking male and female volunteers aged between 18 and 45 years (inclusive) with a body-mass index (BMI) of 18 to 35 kg/m^2 were enrolled in this study. All volunteers underwent a detailed screening assessment for eligibility. Exclusion criteria included women who were pregnant or still lactating, or any clinically significant coexisting condition including hypertension, hyperlipidaemia, diabetes mellitus, cardiovascular disease, recent infective or inflammatory condition, coagulopathy, known liver disease or screening blood tests indicative of renal, liver, clotting, thyroid or haematological abnormality. Volunteers were not permitted to take any prescription or non-prescription medication (including acetylsalicylic acid, paracetamol, vitamins and herbal supplements) within 14 days of an experimental visit. Prior to each visit, volunteers must have abstained from alcohol for 24 hours and food including caffeine-containing products for 8 hours. Informed written consent was obtained from all volunteers before enrolment. The study was approved by the local

research ethics committee (reference 16-HV-025) and conducted in accordance with the Declaration of Helsinki.

7.3.4 Sample size

A sample size of 15 volunteers was selected based on previous data the average total thrombus formation under experimental conditions is approximately 12750 μm^2 and for low shear is approximately 7500 μm^2 with a standard deviation of approximately 550 μm^2 [201]. Therefore, a sample size of 13 completers is needed to detect a 10% (750 μm^2) difference in total thrombus area with 90% power and two-sided alpha of 0.05 under low shear conditions.

7.3.5 Study outcome measures

7.3.5.1 Chamber concentrations of study drug

Blood samples for determination of serum JNJ-9375 and plasma bivalirudin concentrations were taken immediately distal to the perfusion chamber into 3.5 mL serum gel and 2.7 mL sodium citrate (3.2%) tubes (Becton-Dickinson, Cowley, UK). JNJ-9375 samples were allowed to clot for 30 minutes then centrifuged at 1500 *g* (20 °C) for 20 minutes. Bivalirudin samples were centrifuged at 1500 *g* (15 °C) for 15 minutes within 1 hour of collection. Samples were then aliquoted and stored immediately at -70 °C before analysis. Concentrations of JNJ-9375 were determined by electro-chemiluminescence using the Meso Scale Discovery platform and plate reader (Rockville, Maryland, USA). Values were then regressed from the standard curve in Watson LIMS (version 7.4.1, Thermo, PA, USA) using a five-parameter logistic regression model with $1/Y^2$ weighting.

7.3.5.2 Coagulations assays

Blood samples for coagulations assays (prothrombin time, activated partial thromboplastin time and thrombin time (undiluted and diluted)) were collected immediately distal to the final perfusion chamber into 4.5 mL sodium citrate (0.38% final v/v) tubes (Becton-Dickinson). Samples were centrifuged at 1500 *g* (15 °C) for 20 minutes within 1 hour of collection. Plasma was then aliquoted and stored immediately at -70 °C before analysis using a STA-Compact-Max analyser (Stago, Parsippany, NJ, USA). The following reagents were used, for prothrombin time, STA-Neoplastine CI Plus, for activated partial thromboplastin time, STA-PTT Automate, and for thrombin time, STA-Thrombin.

7.3.5.3 Platelet activation

Blood (2.7 mL) was collected immediately distal to the final perfusion chamber into tubes containing 0.3 mL of 3.8% sodium citrate and Pefabloc FG (final concentration 1.5 mg/mL; Quadratech Diagnostics, Surrey, UK). Pefabloc FG was added because it prevents excessive fibrin formation in response to thrombin that would preclude flow cytometry but does not interfere with platelet activation. After 5 minutes, samples were aliquoted into micro-centrifuge tubes pre-filled with or without agonist (adenosine diphosphate 20 μM, Sigma-Aldrich, Gillingham, UK; human alpha thrombin 0.1 U/mL, Enzyme Research Laboratories, Swansea, UK) and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for 15 minutes at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within

24 h using a FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

7.3.5.4 Ex vivo perfusion model of thrombosis

The effect of study compound on ex vivo thrombus formation was assessed using the Badimon perfusion chamber as described in detail in Chapter 2: Methods.

7.3.5.5 Histomorphometric analysis

Total thrombus formation, fibrin-rich thrombus formation and platelet-rich thrombus formation were assessed as described in detail in Chapter 2: Methods.

7.3.6 Statistical Analysis

After study completion, the database was locked and all statistical analyses carried out by an independent statistician. Categorical variables are expressed as percentages, continuous variables are expressed as mean ± standard deviation (SD). The effects of study compounds on study end-points were assessed by general linear mixed effect models with period and study compound as fixed effects, subjects as random effects. Chamber end-points were log-transformed and assessed separately by shear rate (low and high). From the models, point and interval estimates for means and mean differences versus placebo (absolute and %) were generated and analysed using the Least Significance Difference test. The correlation between plasma JNJ-9375 concentrations and study end-points were determined by Pearson's (r) or Spearman's rank-order correlation (ρ) as appropriate. Two-sided p values of ≤ 0.05 were considered statistically significant. All statistical calculations were performed using SAS version 9.4.

7.4 RESULTS

All 15 enrolled volunteers (10 male) completed the study in full, with no safety concerns. Mean age of the volunteers was 26 ± 5 years with a body-mass index of 24 ± 3 kg/m^2.

7.4.1 Chamber concentrations of study drug

Table 7.1. Target versus measured study drug concentrations

Concentration	JNJ-9375			Bivalirudin
Target, µg/mL	2.5	25	250	6
Measured, µg/mL (SD)	1.93 (0.68)	22.3 (5.86)	214.0 (20.8)	6.92 (11.3)

Compound concentrations in the effluent of the perfusion chamber closely matched the target (intended dose) concentrations.

7.4.2 Coagulation

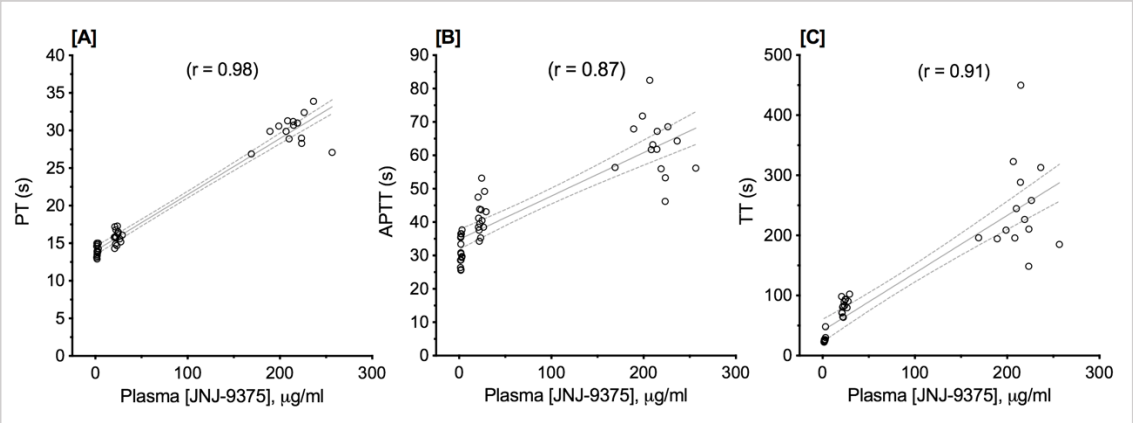


Figure 7.2. Correlation between plasma concentrations of JNJ-9375 and coagulation assays (n=15).
Plots of plasma concentrations of JNJ-9375 versus [A] prothrombin time (PT), [B] activated partial thromboplastin time (APTT), and [C] thrombin time (TT). Data shown includes the regression line ± 95% confidence intervals for Pearson's correlations. r = Pearson's correlation coefficient.

JNJ-9375 caused dose-dependent prolongation of all measured blood coagulation markers, with thrombin time the most sensitive to the anticoagulant effect (Table 7.2). Pearson's correlation coefficient between chamber plasma concentrations of JNJ-9375 and coagulation assays was 0.98 for prothrombin time, 0.87 for activated partial thromboplastin time, and 0.91 for thrombin time ($p<0.001$ for all; Figure 7.2).

Table 7.2. Effect of study drug on coagulation assays

	Placebo	**JNJ-9375 (2.5 μg/ml)**	**JNJ-9375 (25 μg/ml)**	**JNJ-9375 (250 μg/ml)**	**Bivalirudin (6 μg/ml)**
PT (secs)	13.7 *[10.5, 16.9]*	13.9 *[10.7, 17.1]*	15.8 *[12.6, 19.0]*	30.0 *[26.8, 33.2]*	36.6 *[33.4, 39.8]*
APTT (secs)	28.9 *[23.3, 34.6]*	31.4 *[25.7, 37.0]*	41.6 *[35.9, 47.3]*	63.5 *[57.8, 69.2]*	91.5 *[85.8, 97.2]*
TT (secs)	15.6 *[-12.7, 43.8]*	24.9 *[-3.3, 53.2]*	80.9 *[52.6, 109.2]*	245.6 *[217.3, 273.9]*	351.2 *[323.0, 379.5]*
Dilute TT (secs)	< LLOQ	< LLOQ	< LLOQ	151.5 *[126.5, 176.6]*	> 501*

Data shown are statistical means with 95% confidence intervals. *14 of 15 results > 501 s. Abbreviations used: PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; and LLOQ, less than lower limit of quantification.

7.4.3 Platelet activation

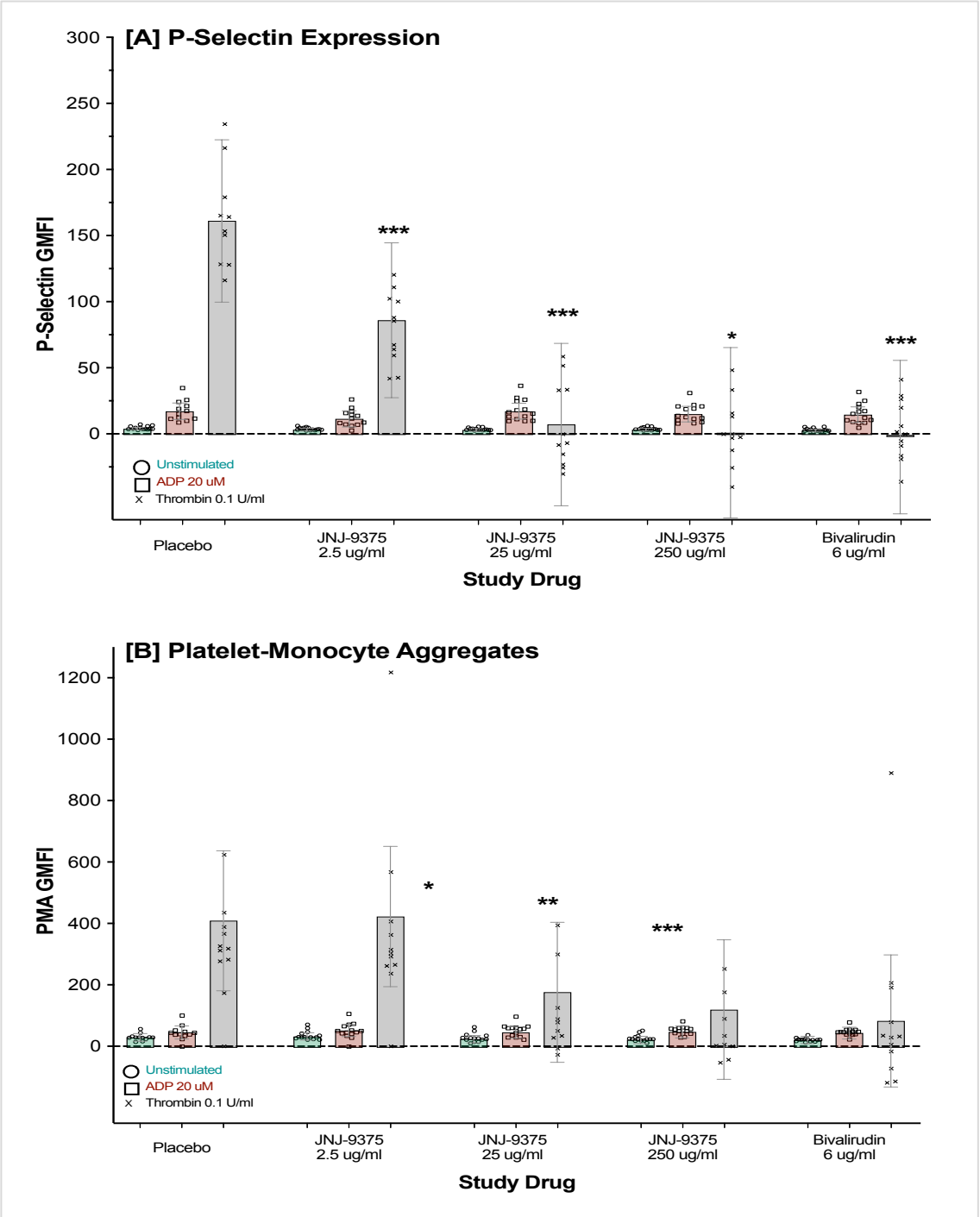


Figure 7.3. Impact of study drug on ex vivo platelet activation.
Extra-corporeal administration of JNJ-9375 inhibited thrombin-simulated [A] p-selectin expression and [B] platelet-monocyte aggregates in a dose-dependent manner but had no effect on ADP activity. Data shown are the adjusted means (± 95% confidence intervals) and individual points. Statistical comparisons (Least Significance Difference test) versus placebo are represented above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: ADP, adenosine diphosphate; PMA, platelet-monocyte aggregates; GMFI, geometric mean fluorescent intensity.

Compared to placebo, JNJ-9375 2.5, 25 and 250 μg/mL inhibited thrombin (0.1 U/mL) stimulated platelet p-selectin expression (geometric mean fluorescent intensity, GMFI) by 46.5% [95% confidence intervals (CI), 4.6 to 97.5%; p=0.07], 95.2% [95% CI, 43.2 to 147.2%; p<0.001] and 99.0% [95% CI, 46.1 to 151.9%; p<0.001] and platelet-monocyte aggregates (GMFI) by -3.4% [95% CI, -56.1 to 49.4%; p=0.90], 56.3% [95% CI, 2.2 to 110.4%; p=0.04] and 69.9% [95% CI, 16.2 to 123.6%; p=0.01] (Figure 7.3). Chamber plasma concentrations of JNJ-9375 correlated with both platelet p-selectin expression (ρ=-0.83, p<0.001) and platelet-monocyte aggregates (ρ=-0.64, p <0.001). In contrast, JNJ-9375 had no effect on ADP (20 μM) stimulated platelet activation (p=ns for all). Bivalirudin exhibited a similar selective profile (Figure 7.3).

7.4.4 Effect of JNJ-9375 on ex vivo thrombus formation

Total thrombus

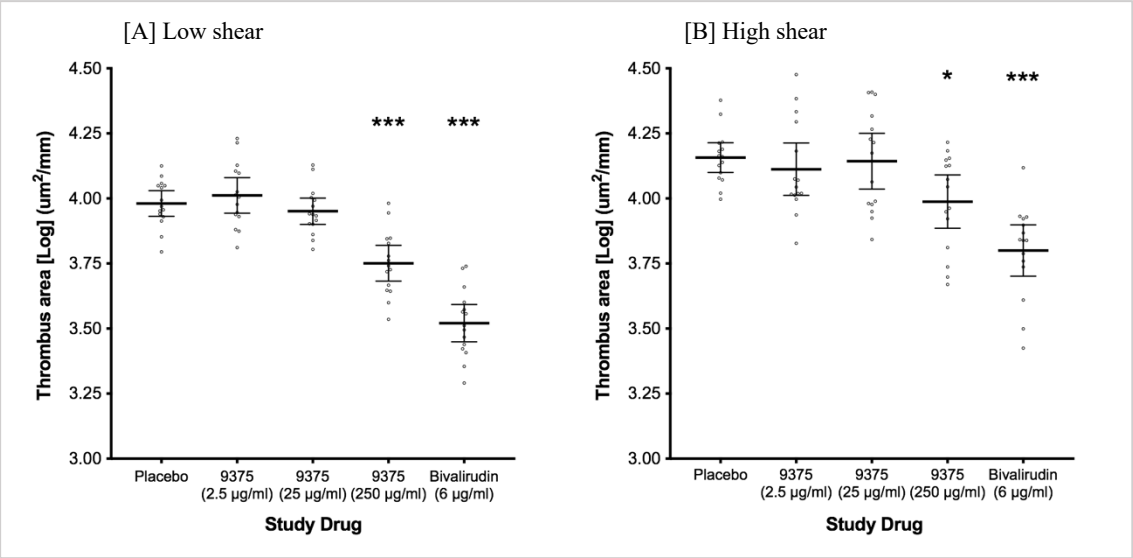


Figure 7.4. The effect of study compound on ex vivo total thrombus formation
Extra-corporeal administration of JNJ-9375 inhibited total thrombus formation in a dose-dependent manner at both [A] low shear stress (212 s^{-1}) and [B] high shear stress (1690 s^{-1}) shear stress. Data shown are the adjusted means (± 95% confidence intervals) for [Log] total thrombus area (μm^2/mm) and individual points (n=15). Statistical comparisons (Least Significance Difference test) versus placebo are represented above each plot: * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: 9375, JNJ-9375.

Ex vivo total thrombus formation was reduced at both low and high shear stress at the 250 μg/mL concentration (Figure 7.4). Compared to placebo, JNJ-9375 (2.5, 25 and 250 μg/mL) reduced mean total thrombus area by -7.4% (95% CI, -41.6 to 18.5%; p=0.60), 6.6% (95% CI, -23.1 to 29.2%; p=0.62) and 41.1% (95% CI, 22.3 to 55.3%; p<0.001) at low shear and by 9.8% (95% CI, -26.6 to 35.7%; p=0.54), 3.3% (95% CI, -35.8 to 31.1%; p=0.85) and 32.3% (95% CI, 4.9 to 51.8%; p=0.025) at high shear.

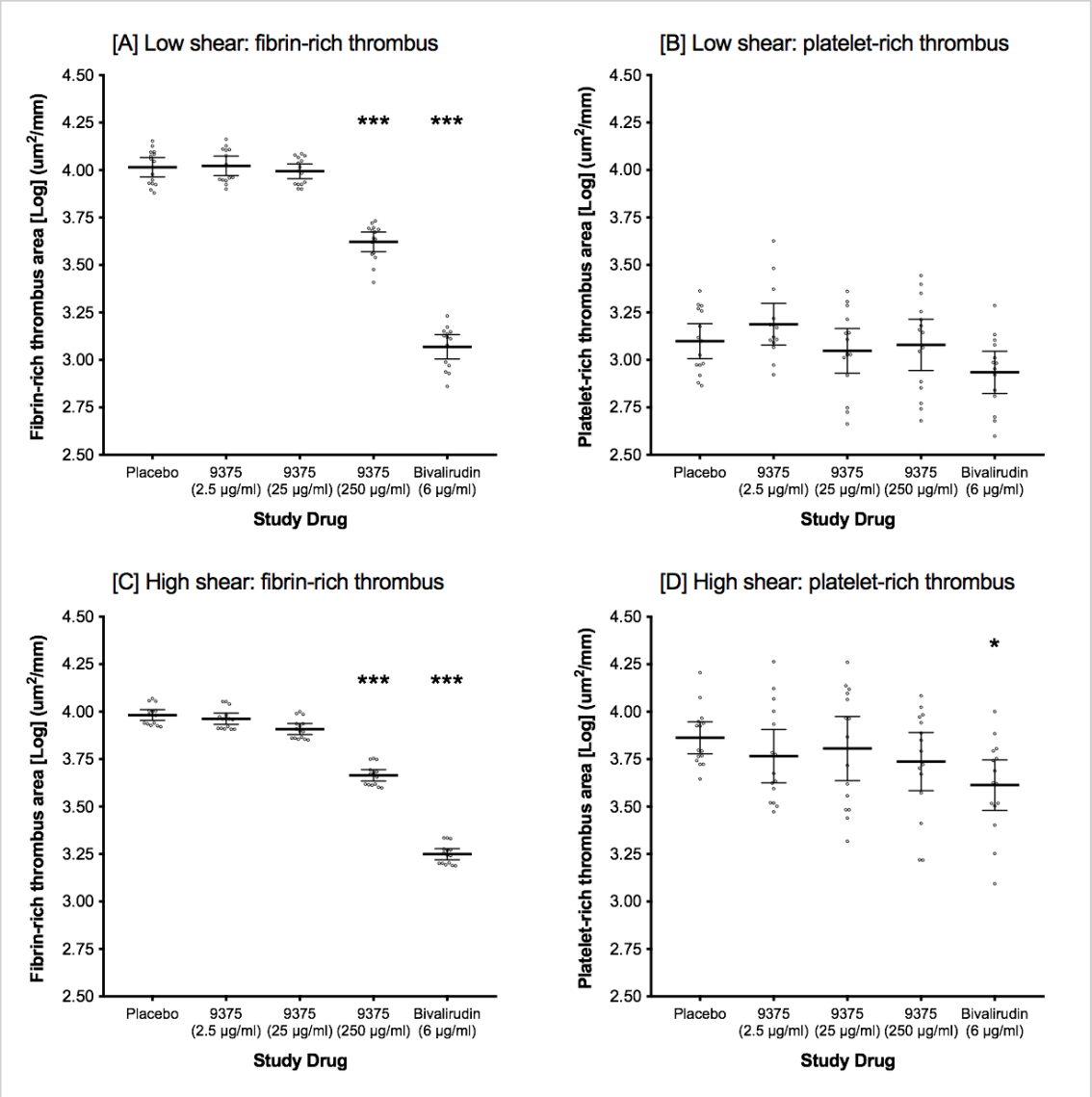


Figure 7.5. The effect of study compound on the platelet- and fibrin-rich thrombus formation

Extra-corporeal administration of JNJ-9375 inhibited fibrin-rich thrombus deposition in a dose-dependent manner at both [A] low shear stress (212 s^{-1}) and [C] high shear stress (1690 s^{-1}) shear stress, as compared to placebo. JNJ-9375 had no effect on platelet-rich thrombus deposition at either shear stress. Bivalirudin reduced fibrin-rich thrombus deposition at low and high shear stress, and platelet-rich thrombus deposition at high shear stress. Data shown are the adjusted means (± 95% confidence intervals) for [Log] fibrin- or platelet-rich thrombus area (μm^2/mm) and individual points (n=15). Statistical comparisons (Least Significance Difference test) versus placebo are represented above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations used: 9375, JNJ-9375.

Reductions in total thrombus area were driven by a dose-dependent decrease in fibrin-rich thrombus deposition under both shear conditions (Figure 7.5). At peak dose (250 μg/mL), JNJ-9375 reduced fibrin-rich thrombus area by 59.5% [95% CI, 37.8 to 73.7%; p<0.001] at low shear and 51.8% [95% CI, 37.7 to 62.7%; p<0.001] at high shear. There was no reduction in platelet-rich thrombus area (p=ns for all).

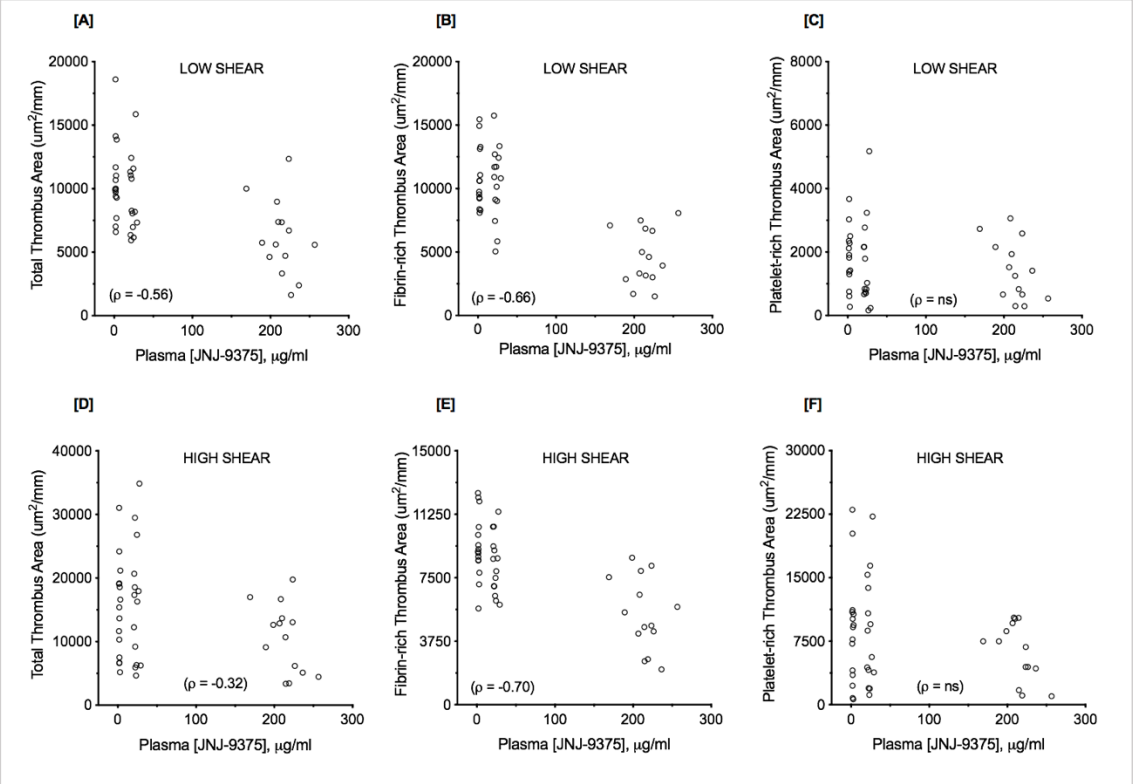


Figure 7.6. Correlation between plasma concentrations of JNJ-9375 and thrombus area.
Plots of plasma concentrations of JNJ-9375 versus [A] total thrombus formation at low shear, [B] fibrin-rich thrombus formation at low shear, [C] platelet-rich thrombus formation at low shear, [D] total thrombus formation at high shear, [E] fibrin-rich thrombus formation at high shear, and [F] platelet-rich thrombus formation at high shear (n=15). ρ = Spearman's rank order correlation.

Chamber plasma concentrations of JNJ-9375 correlated with total thrombus area at low (ρ=-0.56, p<0.001) and high (ρ=-0.32, p=0.03) shear; and fibrin-rich thrombus area at low (ρ=-0.66, p<0.001) and high (ρ=-0.70, p<0.001) shear (Figure 7.6). There was no correlation with platelet-rich thrombus area (p=ns for all).

7.4.5 Effect of bivalirudin on ex vivo thrombus formation

Bivalirudin reduced total thrombus area at both low and high shear, also driven by a decrease in fibrin-rich thrombus formation (Figures 7.4 and 7.5). In contrast to JNJ-9375, there was a modest reduction ($p=0.01$) in platelet-rich thrombus formation at high shear (Figure 7.5).

7.5 DISCUSSION

In this double-blind randomised controlled crossover study, ex vivo administration of JNJ-9375, a highly specific exosite 1 thrombin inhibitor, resulted in dose-dependent prolongation of blood coagulation and selective inhibition of thrombin-stimulated platelet activation. Thrombosis was reduced under rheological conditions of both low and high shear stress, driven principally by a reduction in fibrin-rich thrombus formation. We conclude that JNJ-9375 holds promise as an anticoagulant for the prevention and treatment of thromboembolic events and provide further insights into the role of exosite 1 in human thrombogenesis.

This is the first description of the ex vivo antithrombotic effects of exosite 1 thrombin inhibition in native human blood under flow conditions. At a dose of 250 μg/mL, JNJ-9375 reduced total thrombus area by over 40% and 30% at low and high shear respectively. Under the same conditions, high dose bivalirudin (equivalent to that used at the time of PCI) reduced thrombus formation by 65% at low shear and 56% at high shear; while in previous studies reductions of 14% with heparin (70 IU/kg bolus plus 15 IU/kg/h infusion) [198], 26-28% with oral edoxaban (60 mg) [238], and up to 40% with serial dosing of the parenteral direct factor Xa inhibitor, DX-9065a [199], were reported. Importantly, therefore, we have shown that exosite 1 thrombin antagonism alone is sufficient to substantially reduce ex vivo human thrombus formation. Moreover, given reductions were comparable (if not superior) in magnitude to the clinically approved anticoagulant edoxaban, our results suggest a high probability of in vivo antithrombotic efficacy.

JNJ-9375 resulted in dose-dependent prolongation of prothrombin time, activated partial thromboplastin time and thrombin time. As expected, thrombin time was most sensitive to the anticoagulant effect. Although direct oral anticoagulants are licensed for use without the need for routine monitoring, there are clinical situations in which readily available assays to measure anticoagulant activity may be useful. Our data suggests that if indicated, thrombin time, and to a lesser extent prothrombin time and activated partial thromboplastin time, may provide a useful assay for measuring the extent of exosite 1 inhibition and JNJ-9375 activity.

The outstanding challenge in anticoagulation is the development of drugs that can provide equivalent (or superior) antithrombotic efficacy but with a significantly lower bleeding risk. While the safety of JNJ-9375 has yet to be demonstrated in clinical trials, several lines of evidence indicate the potential for favourable outcomes. On a mechanistic level, selective inhibition of thrombin through exosite 1 specific antagonism is attractive because of the capacity to prevent fibrin formation without necessarily interfering with other (active site and exosite 2 dependent) protease interactions relating to haemostasis. For example, both the active site and exosite 2 are involved in catalytic feedback activation of clotting cofactors V, VIII, XI and XIII, with deficiencies of each of these factors associated with bleeding diatheses [257–259].

Thrombin is also a potent platelet agonist, and whereas over-aggregation may lead to pathological events, early platelet responses are central to haemostasis. Thrombin activates platelets through binding to platelet surface GPIb and protease-activated receptors 1 (PAR1) and 4 (PAR4). Exosite 1 interacts with PAR1 to facilitate efficient receptor cleavage [127], whereas PAR4 activation and GPIb binding are largely

dependent on the active site and exosite 2 respectively [116,260]. In the present study, JNJ-9375 selectively inhibited thrombin stimulated platelet activation but was not associated with a reduction in platelet deposition. This is consistent with previous reports that exosite 1 inhibition only weakly inhibits thrombin-induced platelet aggregation and does not affect platelet collagen binding [183,261]. Collectively, these results suggest potentially favourable differential effects on thrombin-platelet responses, that could be especially useful in clinical situations where combined treatment with an antiplatelet is required [262,263]. This is speculative and requires further exploration. Future studies are required to examine the effects of JNJ-9375 on platelet adhesion, thrombosis and bleeding, alone and in combination with existing antiplatelet agents.

Indirect evidence that exosite 1 thrombin inhibition may be associated with a low haemorrhagic potential is supported by data from animal studies of thrombosis and bleeding. Using a baboon arteriovenous shunt model, Cadroy and colleagues found that exosite 1 thrombin inhibition prevented thrombus formation but did not affect the ability to form haemostatic plugs [183]. More recently, JNJ-9375 demonstrated a substantially wider therapeutic index when compared to apixaban in rats and cynomolgus monkeys [179]. Further insight comes from the case report of an anti-exosite 1 thrombin IgA antibody (from which JNJ-937 was subsequently synthesized to mimic) identified in a patient presenting with a large traumatic subdural haematoma and persistently abnormal clotting studies [184]. Despite continued evidence of intense anticoagulation (prothrombin time, 40 s; activated partial thromboplastin time, 240 s; thrombin time with bovine thrombin, 173 s), the patient made a full recovery without surgical intervention and had no abnormal bleeding events during 8 years of follow up.

7.6 LIMITATIONS

Our study has some potential limitations. First, only a modest number of volunteers were studied. However, problems associated with intra-group variability were minimised by the crossover design that allowed each volunteer to serve as their own control. Second, although the exposed porcine aortic media used in the perfusion model presents many of the common constituents of an injured human blood vessel (including type I collagen), it is unlikely to contain tissue factor (TF) [249–251]. TF activates the coagulation cascade and is an important contributor to thrombogenicity [252,253]. Nevertheless, this does not overly limit our model for the assessment of thrombosis because binding of blood borne circulating TF is sufficient to allow activation of the coagulation cascade and thrombus propagation [249,250,254–256]. Indeed, previous studies have confirmed that thrombus formed from human blood perfused over porcine tunica media (devoid of TF) stains heavily for TF [249,250]. Third, we used an anti-fibrin(ogen) antibody, which recognises both fibrinogen and fibrin, to examine the fibrin component of thrombus formation. However, the chamber is perfused by saline at the end of the experiment washing away unbound cells, proteins and other molecules, such as fibrinogen, leaving only adherent thrombus. Thus, histomorphometric quantification of fibrin-rich thrombus area is unlikely to be affected by this cross-reacting antibody and our findings are consistent with previous studies using the same immunohistochemical approach [264–267]. Fourth, while we have shown that exosite 1 thrombin inhibition reduces fibrin-rich thrombus formation, determining how JNJ-9375 alters the dynamics of clot development, stabilisation and dissolution might further inform therapeutic potential and are areas for future exploration. Finally, the study included ex vivo experiments only and thus lacked hard clinical end-points necessary to draw any conclusions regarding the safety or efficacy of this novel anticoagulant in

practice. However, given this was a translational study designed to examine for the first time the effects of exosite 1 thrombin inhibition with JNJ-9375, we felt our study design appropriate.

7.7 CONCLUSION

In conclusion, JNJ-9375, a highly specific exosite 1 thrombin inhibitor, demonstrated substantial reductions in ex vivo thrombosis in native human blood under flow conditions. These reductions were driven by a decrease in fibrin-rich thrombus formation and were comparable in magnitude to clinically approved anticoagulants. Our findings suggest JNJ-9375 represents a promising novel class of anticoagulant, and that further clinical studies are warranted. A phase 2 trial comparing the safety and efficacy of JNJ-9375 to apixaban in patients undergoing elective total knee replacement surgery is currently underway (ClinicalTrials.gov, NCT03251482).

Chapter 8

Conclusions and future directions

8 Conclusions and future directions

8.1 THESIS DIRECTION AND METHODOLOGY

The primary purpose of this thesis was to examine the therapeutic potential of novel targets for the prevention of thrombosis in humans. Given the central role of platelet aggregometry in this process, it was important to first establish the repeatability and agreement between traditional LTA and the most widely used method in our institute, 96-WPA. This allowed determination of the most appropriate methodology for the remainder of the thesis and crucially provided reference values above which we could be 95% confident in future studies a true change in aggregation had occurred.

The original target of interest was PAR4, a platelet receptor for thrombin. After a series of in vitro studies exploring PAR4 agonism and antagonism in the presence and absence of other major platelet agonists and their cognate inhibitors, a phase 1 randomised open label blinded endpoint trial was designed to examine for the first time the effect of oral PAR4 antagonism in healthy volunteers on ex vivo human thrombosis. After presenting early results, we were approached to conduct a similar study with another novel antithrombotic agent, JNJ-9375. This represented an excellent opportunity on a number of levels. Like PAR4 inhibition, exosite 1 thrombin inhibition specifically targets direct functions of thrombin in mediating thrombosis. However, in contrast to PAR4 inhibition, the primary mechanism of action is one of anticoagulation rather than platelet blocking. Moreover, the antithrombotic effects of exosite 1 thrombin inhibition in native human blood under flow conditions were previously unknown.

In the early stages, the intention was to investigate the wider actions of PAR4 in the vasculature if an effect on thrombosis was demonstrated. Our group has previously shown that PAR1 is an important mediator of vascular tone and function in humans. A similar panel of studies was proposed; however, a number of significant challenges were encountered. No PAR4 agonist had ever been given to humans before. Accordingly, and in line with the phase 1 review committee, we felt it important to first conduct a safety and tolerability study in mice. Manufacturing the agonist to sufficient purity and to Current Good Manufacturing Practice regulations within Europe proved prohibitively expensive. This was overcome by sourcing from a non-European site but mandated further quality and sterility testing on arrival to the UK, followed by stability testing for several months. This process took over 12 months with a further 4 months to conduct the study. While these delays enabled the JNJ-9375 studies to be undertaken, an important part of this thesis, they invariably resulted in insufficient time to go on and examine the in vivo vascular effects of PAR4 agonism in humans. Nevertheless, the data represents the culmination of an enormous effort and provides the key groundwork for future researchers.

One of the major challenges in developing more effective antithrombotic drugs is the avoidance of increased bleeding. However, determination of bleeding risk prior to undertaking large trials is challenging. There are no safe human models, while data from animal studies has repeatedly proven to have significant limitations. Bleeding risk and the impact of an increase in bleeding on therapeutic efficacy are also person and situation specific. Key to maximising gain is to develop agents with high selectivity and specificity for the principal pathways associated with driving occlusive thrombus for that specific pathology. This had a strong influence on the methodology and materials

used, particularly the Badimon chamber. By adopting this model of thrombosis, we were not only able to determine an antithrombotic effect, but also whether this was shear dependent and driven by reductions in platelet-rich thrombus, fibrin-rich thrombus, or both. Such data provides important insights into what pathologies and patient sub-groups these drugs are most likely to be effective.

8.2 96-WELL PLATE AGGREGOMETRY

Platelet aggregometry is critical to the evaluation of patients with suspected platelet dysfunction and plays a central role in the investigation and development of antithrombotic agents. 96-WPA is a newer method of platelet aggregation that may offer advantages over the current gold standard LTA. However, data regarding the test-retest repeatability of 96-WPA and agreement between these two methods was lacking.

96-WPA demonstrated good within- and between-repeatability for the determination of peak platelet aggregation and EC_{50} with results comparable to LTA. Conversely, there was a systematic measurement bias with two of the three agonists studied and the methods should not be considered interchangeable. Bednar and colleagues reported similar results. Compared to LTA, 96-WPA was associated with a numerically lower EC_{50} in 3 out of the 4 thrombin-agonists studied with results identical for the fourth [268]. Thus, our findings are consistent with the only other published data. As to the cause of the measurement bias, this remains uncertain. 96-WPA uses shaking to induce mixing and aggregation whereas LTA uses vortexes. This can lead to differences in the physical forces applied, which are a key aspect of platelet activation [269]. However, as there was no significant bias for AA, physical forces alone are unlikely to fully explain these differences and further research is required.

It has been suggested 96-WPA may be technically easier than LTA. Given the requirement for reverse pipetting and smaller test volumes, this is not our experience. Nonetheless, 96-WPA undoubtedly facilitates a much greater throughput and in our research institute we are increasingly using this approach. 96-WPA also continues to evolve. The Optimul method is an iteration of 96-WPA in which individual wells are

precoated with one of seven concentrations of AA, ADP, collagen, epinephrine, ristocetin, TRAP-6 amide or U46619, before being lyophilised, vacuum-sealed, foil-packed and stored for up to 24 weeks [211]. By removing the need for daily preparation and pipetting of agonists, this approach further simplifies and standardizes 96-WPA. Further benefits including improved test-retest repeatability would not be surprising but remain to be evaluated.

8.3 PAR4 AGONISM AND ANTAGONISM

PAR4 is a promising novel target for antiplatelet therapy with mechanistic and animal data indicating the potential for a wide therapeutic index. A phase 1 parallel group prospective randomized open-label blinded endpoint trial was conducted to examine for the first time the antiplatelet, anticoagulant and antithrombotic effects of oral PAR4 antagonism with BMS-986120 in humans. We also sought to determine whether platelet responses to PAR4 stimulation are dependent on additional input or vice-versa.

BMS-986120 mechanism of action

To be confident in our conclusions, it was important to confirm the selectivity of BMS-986120 for inhibiting PAR4 under the study conditions. Both in vitro and oral administration of BMS-986120 markedly attenuated PAR4-AP induced platelet activation and aggregation with no effect on PAR1-AP, AA or ADP platelet stimulation. Following a single oral dose, platelet responses made an almost complete recovery within 24 hours. Our results therefore add to previous data that BMS-986120 is a highly selective, potent and reversible PAR4 antagonist [131].

PAR4 platelet activation and aggregation

There is conflicting evidence as to whether platelet responses to PAR4 signaling are dependent on additional input. Covic and colleagues reported that up to 50% of the PAR4 intracellular Ca^{2+} signal is due to released ADP acting on the $P2Y_{12}$ receptor and is conditional on prior PAR1 activation [40]. However, in a more recent study, PAR4-mediated platelet aggregation was shown not to be dependent on ADP with PAR4 acting in parallel rather than contingent on the ADP/$P2Y_{12}$ - PI3K pathway [129]. Under conditions designed to mimic thrombin levels within the local environment of a

developing clot, we found that platelet responses to PAR4 stimulation were not dependent on input from other major agonist-receptor pathways and vice-versa. This is consistent with evidence PAR4 activation occurs well after platelet degranulation [130] and induces sustained intracellular Ca^{2+} and protein kinase C signals independent of additional support [114,129,130]. Importantly, therefore, our results identify PAR4 signaling as a major mechanism through which pathological platelet activation and aggregation can continue despite current standard of care antiplatelet agents.

Coagulation

Activated platelets upregulate surface phosphatidylserine and release procoagulant microvesicles. The resulting surfaces facilitate assembly of activated clotting factor complexes leading to thrombin generation and fibrin formation. PAR4 inhibition has been shown to abolish thrombin-induced platelet phosphatidylserine exposure [248] and compared to PAR1, stimulation of PAR4 leads to faster and more robust thrombin generation [270]. These data suggest PAR4 has a role in platelet procoagulant activity. Consistent with this, BMS-986120 was associated with a small but significant reduction in fibrin-rich thrombus formation that occurred in the absence of PT or APTT prolongation.

Whether this additional effect on platelet function is therapeutically beneficial or harmful is unknown. Aspirin and $P2Y_{12}$ antagonists inhibit platelet procoagulant activity [271] and it has been suggested this may add to the increased bleeding seen with each of these agents. Scott syndrome, a congenital disorder in platelet, red cell, and lymphocyte phosphatidylserine exposure, is associated with a moderately severe bleeding phenotype [272]. However, it would seem unlikely PAR4 inhibition affects

phosphatidylserine exposure to the same broad extent as Scott syndrome and both aspirin and $P2Y_{12}$ antagonists interfere with a number of other haemostatic mechanisms.

Thrombosis

Research into the antithrombotic potential of PAR4 antagonism is of major clinical importance given the large unmet clinical need for more effective antiplatelet therapy. We examined the effect of BMS-986120 on ex vivo human thrombosis using the highly validated Badimon chamber. Under conditions representative of deep arterial injury in a stenosed coronary artery, a single 60 mg oral dose of BMS-986120 prevented thrombus formation by nearly a third. Moreover, reductions were comparable in magnitude to high loading doses of aspirin ± clopidogrel, suggesting a high likelihood of in vivo antithrombotic efficacy.

An interesting finding was that compared to current standard of care antiplatelet agents, PAR4 antagonism appeared to have greater specificity for inhibiting high versus low shear thrombus formation. Most atherothrombotic events occur at sites of high shear stress, whereas many treatment-related bleeding events occur in low shear environments [241–245,273,274]. Therefore, antiplatelet agents that are more selective for inhibiting high shear thrombus formation may allow at-risk vascular beds to be targeted with superior precision. Further research is required to confirm our findings and establish whether such an approach may translate to improved net clinical gain.

Bleeding

One of the major challenges in developing more effective antithrombotic therapies is the avoidance of increased bleeding.

Mechanistic data suggests PAR4 may be an ideal target because of its differential role in early and late platelet signaling. In animal studies, PAR4 inhibition with BMS-986120 was associated with a substantially reduced bleeding risk as compared to clopidogrel [131]. Combining our trial with a previous ascending-dose study, BMS-986120 has been administered to a total of 62 healthy volunteers with no treatment-related bleeding events recorded [146]. However, these trials were neither designed nor powered for the detection of bleeding and results from animal models do not always translate well to humans, as exemplified by PAR1 antagonists [9,80]. Thus, while data to date suggests we can be optimistic, whether or not PAR4 inhibition provides a lower risk of bleeding than existing agents remains to be determined.

Future directions

Results from this thesis have recently been confirmed in a phase 0 study in healthy volunteers examining the additional benefit of PAR4 antagonism when combined with the anticoagulant apixaban [275]. Extracorporeal administration of BMS-986141, a potent PAR4 antagonist similar to BMS-986120, substantially reduced total (-44.4%) and platelet-rich (-39.3%) ex vivo thrombus formation. BMS-986141 with apixaban caused a further modest reduction in total thrombus area (9.6% to 12.4%), especially under conditions of high shear stress.

We believe PAR4 inhibition has real potential for in vivo antithrombotic efficacy and that further investigation in larger phase II clinical trials is warranted. Often this involves combining the new agent with existing standard of care. However, this requires careful consideration, particularly if PAR4 inhibition is to be combined with a $P2Y_{12}$ antagonist. PAR4 acts independently of the $P2Y_{12}$/PI3K pathway to sustain GPIIb/IIIa activation. Thus, blocking both pathways is likely to promote potent GPIIb/IIIa inhibition [129]. As with current GPIIb/IIIa antagonists, this could provide highly efficacious antithrombotic protection but may also precipitate substantial bleeding. Consequently, we suggest combinations of PAR4 inhibition and existing antiplatelet agents are first explored in animal and human models of thrombosis and bleeding. Such studies would not only add to our understanding of thrombosis but may have important implications for future clinical trial design.

A major area for future PAR4 inhibition research is exploration of the potential for additional clinical effects beyond platelet antagonism. Data from animal models suggests PAR4 inhibition may protect against ischaemia-reperfusion injury as well as restenosis in diabetic conditions [154–156]. Such pluripotent effects would be highly advantageous. PAR4 is also thought to be involved in the control of vascular tone [276,277] and this may have important clinical applications. However, to date, there have been no studies examining the in vivo vascular effects of PAR4 activation in man.

Our research group has previously investigated the vascular actions of PAR1 through local infusion of a PAR1-AP into the dorsal hand vein and brachial artery of healthy volunteers. Results from these studies demonstrated for the first time that PAR1 activation in humans results in endothelial-dependent arterial dilatation, endothelium-

independent venoconstriction and release of tissue plasminogen activator [27]. To enable a similar future research programme into PAR4, we examined the safety and tolerability of systemic PAR4 agonism in mice with the highly selective PAR4-AP, AYPGKF-NH2. Even at suprathreshold doses, intravenous AYPGKF-NH2 was well tolerated with no evidence of acute systemic toxicity, thrombosis, inflammation, organ injury or dysfunction. Results from this thesis support the safety of AYPGKF-NH2 to investigate the in vivo effects of PAR4 in man.

8.4 EXOSITE 1 THROMBIN INHIBITION

Pre-clinical data suggests exosite 1 thrombin antagonism may enable antithrombotic efficacy without the concomitant bleeding risk seen with current agents. We conducted a double-blind randomised controlled five-way crossover study to examine the anticoagulant and antithrombotic effects of exosite 1 thrombin inhibition with JNJ-9375 in human blood using a translational model of ex vivo thrombosis.

Coagulation

Exosite 1 thrombin inhibition prolonged prothrombin time, activated partial thromboplastin time, and thrombin time in a dose-dependent manner. There was a strong correlation between plasma concentrations and coagulation times with thrombin time the most sensitive to the anticoagulant effect. These findings are consistent with previous in vitro studies [185] and suggest thrombin time could provide a useful and readily available clinical test for measuring the in vivo activity of exosite 1 thrombin inhibitors if required.

Thrombosis

This thesis provides the first evidence that exosite 1 thrombin inhibition with JNJ-9375 substantially inhibits ex vivo human thrombosis. At the highest dose studied, JNJ-9375 reduced total thrombus formation by over 40% at low shear and 30% at high shear. By applying immunofluorescent staining to histomorphometric quantification, we were able to show that reductions in thrombosis were driven by a decrease in fibrin-rich thrombus formation. This is consistent with data from animal studies [179,181,182]. Our results indicate exosite 1 has a major role in human thrombus formation and

suggest that targeting exosite 1 has significant potential for in vivo antithrombotic efficacy.

Bleeding

It has been hypothesized the distinct way in which exosite 1 thrombin inhibition modulates thrombin may reduce bleeding. This is supported by data from animal studies [179] and although clinical trial evidence is lacking, the long-term follow-up of a patient with an exosite 1 thrombin antibody has afforded unique insights into the potential haemostatic sparing effects of exosite 1 thrombin inhibition in vivo [184]. However, precisely how exosite 1 thrombin inhibition might avoid bleeding remains to be fully understood.

An obvious candidate is the greater capacity for exosite 1 thrombin inhibition to maintain adequate feedback activation and auto-amplification of thrombin production. This is because appropriate levels of propagation phase thrombin generation appear critical to stabilizing barrier function of the developing blood clot [278–281]. Alternatively, exosite 1 thrombin inhibition may avoid overly interfering with thrombin-mediated platelet responses relating to haemostasis. Previous studies have shown exosite 1 thrombin inhibition only partially inhibits thrombin-mediated platelet aggregation and does not affect platelet-collagen binding or haemostatic plug formation [183,261]. Our data supports these findings, demonstrating that in contrast to bivalirudin, JNJ-9375 was not associated with a reduction in platelet deposition.

Future directions

Following positive data from this and other preclinical studies, a phase 2 double blind RCT (NCT03251482) was undertaken comparing JNJ-9375 against apixaban for the prevention of thromboembolic events after elective knee surgery [282]. The planned enrolment was 1500 from multiple sites. The primary efficacy endpoint (asymptomatic or symptomatic venous thromboembolism) occurred in 12.2%, 33.3%, 27.3%, 30.0%, and 32.0% of patients randomised to apixaban 2.5 mg bd (n=49), JNJ-9375 0.3 mg/kg (n=30), JNJ-9375 0.6 mg/kg (n=33), JNJ-9375 1.2 mg/kg (n=30), and JNJ-9375 1.8 mg/kg (n=97), respectively. There were no major bleeds with either JNJ-9375 or apixaban. Because JNJ-9375 was statistically less effective than apixaban, the study was stopped early (n=308).

On the face of it, these results are highly disappointing. However, even at the highest dose of 1.8 mg/kg, plasma concentrations of JNJ-9375 can be estimated to be far lower than what we found to be efficacious. Thus, rather than dismissing exosite 1 thrombin antagonism and JNJ-9375 entirely, we believe results from this study simply validate our model and findings. Further studies are required with higher doses of JNJ-9375 or more potent exosite 1 thrombin inhibitors before meaningful conclusions can be drawn as to the potential of this novel class of anticoagulant.

The management of patients with an indication for anticoagulant and antiplatelet therapy is an increasingly common issue. These patients continue to present a major challenge because of the difficulty balancing efficacy for the prevention of both arterial and thromboembolic events against the increased risk of bleeding seen with all current anticoagulant and antiplatelet combinations. It is suggested this may relate to the

overlapping effect of existing anticoagulants on thrombin-mediated platelet responses leading to excessive platelet inhibition when combined with aspirin and $P2Y_{12}$ antagonists. We propose the platelet-sparing effects of exosite 1 inhibition may avoid this issue, allowing optimal anticoagulant and optimal antiplatelet therapy to be combined more readily. However, this is speculative and the combined effects of exosite 1 thrombin inhibition and antiplatelet agents remain to be explored.

Alternative strategies for more effective anticoagulation are also in development. Of primary interest are targeting factors XI and XII of the contact pathway. Importantly, factor XII appears to have a very limited role, if any, in haemostasis. However, the therapeutic potential of targeting factor XII may be severely undermined by the ability of the extrinsic pathway to activate factor XI and therefore bypass factor XII inhibition [283,284]. An exception to this may be thrombosis induced on the surface of medical devices such as vascular catheters, leads or extracorporeal circuits [285].

In contrast to factor XII, inhibition of factor XI prevents amplification of coagulation by either pathway [284]. While the risk of bleeding may be marginally higher than factor XII inhibition, the overall therapeutic index would appear to be more favourable. Consequently, strategies to target factor XI have been the primary focus of most research and development. In a phase II study of patients (n=300) undergoing elective knee replacement, factor XI inhibition with an antisense oligonucleotide reduced the risk of post-operative VTE compared to enoxaparin (4% vs. 30%) without increasing the risk of bleeding [286]. Other compounds including active site peptidomimetic inhibitors of FXIa have completed phase I clinical trials with the results awaited [287]. There remains substantial work before any of these compounds enter clinical practice

and it will be of enormous interest to see how targeting opposite ends of the coagulation cascade (intrinsic / extrinsic pathway versus exosite 1 thrombin inhibition) compare and contrast.

8.5 CONCLUSION

Cardiovascular disease remains one of the leading global causes of morbidity and mortality, driven largely by thrombotic and thromboembolic complications. Results from this thesis provide the first evidence that PAR4 inhibition with BMS-986120 and exosite 1 thrombin antagonism with JNJ-9375 substantially inhibit ex vivo human thrombosis. These emerging antiplatelet and anticoagulant strategies warrant further investigation in larger clinical trials and may provide the next paradigm shift in the treatment and prevention of thrombotic disorders.

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10 AWARDS ARISING FROM THIS THESIS

1. Winner of the 2017 European Society of Cardiology Young Investigator of the Year in Thrombosis

2. Finalist of the 2017 British Heart Foundation Reflections of Research

11 PUBLICATIONS RELATING TO THIS THESIS

Wilson, S. J., Newby, D. E., Dawson, D., Irving, J., & Berry, C. (2017). Duration of dual antiplatelet therapy in acute coronary syndrome. Heart (British Cardiac Society), 103(8), 573–580.

Wilson, S., & Newby, D. E. (2017). Predicting long-term bleeding risk after acute coronary syndrome: a step closer to optimising dual antiplatelet therapy duration? Heart 103(15), 1143–1145.

Wilson, S. J., Ismat, F. A., Wang, Z., Cerra, M., Narayan, H., Raftis, J., et al. (2018). PAR4 (Protease-Activated Receptor 4) Antagonism With BMS-986120 Inhibits Human Ex Vivo Thrombus Formation. Arteriosclerosis, Thrombosis, and Vascular Biology, 38(2), 448–456.

Wilson, S. J., Connolly, T. M., Peters, G., Ghosh, A., Johnson, M., & Newby, D. E. (2019). Exosite 1 thrombin inhibition with JNJ-64179375 inhibits thrombus formation in a human translational model of thrombosis. Cardiovascular Research, 115(3), 669–677.

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OPEN ACCESS

Duration of dual antiplatelet therapy in acute coronary syndrome

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ABSTRACT

Despite a large volume of evidence supporting the use of dual antiplatelet therapy in patients with acute coronary syndrome, there remains major uncertainty regarding the optimal duration of therapy. Clinical trials have varied markedly in the duration of therapy, both across and within trials. Recent systematic reviews and meta-analyses suggest that shorter durations of dual antiplatelet therapy are superior because the avoidance of atherothrombotic events is counterbalanced by the greater risks of excess major bleeding with apparent increases in all-cause mortality with longer durations. These findings did not show significant heterogeneity according to whether patients had stable or unstable coronary heart disease. Moreover, the potential hazards and benefits may differ when applied to the general broad population of patients encountered in everyday clinical practice who have markedly higher bleeding and atherothrombotic event rates. Clinicians lack definitive information regarding the duration of therapy in patients with acute coronary syndrome and risk scores do not appear to be sufficiently robust to address these concerns. We believe that there is a pressing need to undertake a broad inclusive safety trial of shorter durations of therapy in real world populations of patients with acute coronary syndrome. The clinical evidence would further inform future research into strategies for personalised medicine.

INTRODUCTION

A ruptured or eroded coronary atherosclerotic plaque is the principal underlying cause of an acute coronary syndrome. The greatest 'at risk' period is during this early phase of plaque instability and healing, with recurrent event rates peaking in the first month. By 3 months, the plaque has usually stabilised, healed and subsequent event rates return to the background rates seen in patients with stable coronary heart disease.[1–3] Indeed, beyond 3 months, recurrent events commonly occur on plaques at other sites within the coronary circulation.[3] From first principles, the first 3 months is the most critical time for interventions to reduce recurrent cardiovascular events after an acute coronary syndrome (ACS). This is consistent with event rates seen in all clinical trials of patients with acute coronary syndrome: an initial time-varying high event rate that reverts to a consistent linear lower event rate from 3 months onwards (table 1).[1 2 4 5]

Antiplatelet therapy

In an acute coronary syndrome, thrombus formation occurs under conditions of high shear stress and is principally driven by platelet aggregation (figure 1). This dominance of platelet aggregation during intracoronary thrombus formation reflects the dramatic effects that antiplatelet therapies have on clinical outcomes (table 2). Aspirin was the first antiplatelet therapy which induced a halving in event rates in patients with acute coronary syndrome:[6 7] such a large effect size has rarely been surpassed in other domains of cardiology.

Given aspirin's remarkable success, it is perhaps unsurprising that adjunctive antiplatelet therapies have been investigated to build on these benefits, especially as there are multiple mechanisms of platelet activation beyond the cyclo-oxygenase pathway (figure 2). However, as platelets are essential to primary haemostasis, there is a balance between reducing the incidence of future cardiovascular events and causing harm from an increased risk of bleeding. The P2Y12 receptor antagonists are a class of drugs that have gained widespread acceptance since they appear to provide additional thrombotic protection at the expense of modest increases in bleeding. Their use is principally associated with reductions in recurrent myocardial infarction[1 4 5 8] and in a few trials, reductions in cardiovascular events and mortality.[5 8] Other antiplatelet therapies (figure 2) are available but have variable net clinical benefit and for the purposes of this review, we will consider only dual antiplatelet therapy (DAPT) with aspirin and P2Y12 receptor antagonism.

Dual antiplatelet therapy

The benefit of dual antiplatelet therapy following an acute coronary syndrome was established by the CURE,[1] COMMIT/CCS-2[8] and CLARITY-TIMI 28[9] trials. Combined aspirin and clopidogrel therapy reduced the 1-year incidence of cardiovascular events by approximately 20% compared with aspirin alone. More potent and consistent P2Y12 receptor inhibition with either prasugrel or ticagrelor was superior to clopidogrel in the subsequent TRITON[4] and PLATO[5] trials.

The evidence for dual antiplatelet therapy in patients with stable coronary heart disease is less distinct. In the CHARISMA trial,[2] the addition of clopidogrel to aspirin in patients with established cardiovascular disease or at high risk of clinical atherosclerotic disease did not reduce cardiovascular events and was associated with an increase in severe bleeding. There was, however, a suggestion of improved outcomes in patients with established atherothrombotic disease, particularly those with a history of myocardial infarction. The PEGASUS-TIMI 54[10] trial compared aspirin monotherapy to a combination of aspirin and ticagrelor in patients with a previous myocardial infarction and at least one additional high-risk factor. At a mean of 33 months, ticagrelor (60 mg) reduced the

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Table 1 Temporal relationship with the clinical benefits of clopidogrel therapy

Time interval (months)	Primary endpoint* Clopidogrel (%)	Placebo (%)	ARR (%)	RRR [95% CIs] (%)	NNT (per month)
CURE trial					
0–1	4.3	5.5	1.2	22 [9, 32]	84
1–3	1.8	2.5	0.8	32 [13, 46]	240
3–6	1.8	1.8	0.0	4 [–27, 27]	5174
6–9	1.3	1.4	0.1	6 [–34, 34]	3171
9–12	1.1	1.3	0.2	14 [–32, 44]	1600
0–12	10.3	12.6	2.4	19	507
CHARISMA trial					
0–28	6.8	7.3	0.5	7*	5591
Subgroup of patients with clinically evident atherosclerotic disease					
0–28‡	6.9	7.9	1.0	12	2800

*Primary endpoint – cardiovascular death, myocardial infarction and stroke.
ARR, absolute risk reduction; CI, confidence intervals; CVD, cardiovascular death; MI, myocardial infarction; NNT, number needed to treat; RRR, relative risk reduction.

incidence of cardiovascular death, myocardial infarction or stroke (7.77% vs 9.04%) at the expense of increased thrombolysis in myocardial infarction (TIMI) major bleeding (2.30% vs 1.06%) and a neutral effect on overall mortality. On the basis of these trials, combination antiplatelet therapy would appear to confer only a small ischaemic benefit at the cost of a significant bleeding risk. European[11] and North American[12] guidelines therefore do not recommend dual antiplatelet therapy in patients with stable atherothrombotic disease but acknowledge that with careful consideration, combined antiplatelet therapy may be beneficial in some high-risk patients.

Duration of dual antiplatelet therapy: clinical trials

Current European[13] and North American[12] guidelines advise continuing dual antiplatelet therapy for 1 year following an acute coronary syndrome. These recommendations are made on the basis of early studies[4 5 14 15] demonstrating a sustained increased risk of thrombotic complications, including stent thrombosis and spontaneous cardiovascular events, beyond 6 months. However, the greatest absolute reductions in cardiovascular events with dual antiplatelet therapy are seen in the first 3 months (table 1) and since these studies, advances in drug-eluting stent technology have led to a substantially reduced incidence of late (>30 days) and very late (>1 year) stent thrombosis.[16]

In recent trials of patients treated with newer generation drug-eluting stents, shorter durations of dual antiplatelet therapy (3 months to 6 months) were non-inferior to 12[17–22] months or 24[23] months of treatment with regard to either. The primary end point was either a composite of cardiovascular events or cardiovascular events plus major bleeding. Moreover, all of these trials included patients with an acute coronary syndrome (range 23% to 74% of study population) and in those who undertook prespecified subgroup analyses, there was no heterogeneity in treatment effect between stable and unstable coronary artery disease was observed (figure 3).

Beyond 12 months, there remains a residual risk of local and systemic atherothrombotic complications[24] and a number of studies have examined whether extended dual antiplatelet therapy

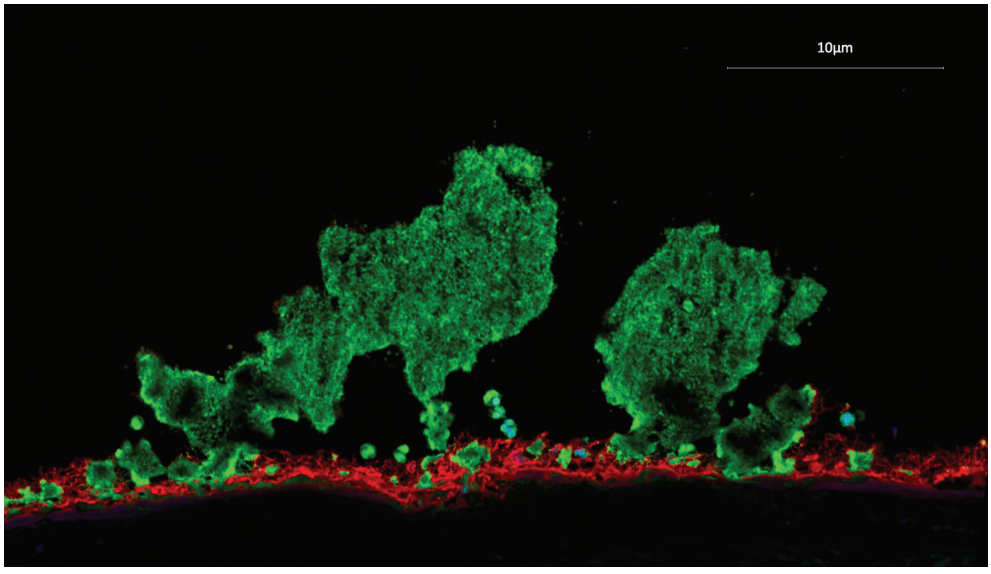

Figure 1 Immunofluorescent staining of human thrombus (platelets, green; fibrin, red) formed at high shear stress.

Table 2 Major trials of antiplatelet agents in acute coronary syndrome ± unstable angina

Antiplatelet agent	Mechanism of action	Trial	Comparison	(Primary) end point	Risk reduction (time point)
Aspirin	COX-1 inhibitor	The ISIS-2 collaborators (ISIS-2), 1979 The Risk Group, 1990 Lewis *et al*, 1983 Cairns *et al*, 1985	aspirin versus placebo aspirin versus placebo aspirin versus placebo aspirin versus placebo	Vascular mortality MI or death MI or death MI or death	23% (5 weeks) 74% (3 months) 51% (3 months) 51% (2 years)
Ticlopidine	P2Y12 antagonist	Scrutinio *et al* (STAMI), 2001	aspirin versus ticlopidine	Death, MI, stroke or angina	ns (6 months)
Clopidogrel	P2Y12 antagonist	Bertrand *et al* (CLASSICS), 2000 Yusuf *et al* (CURE), 2001 The COMMIT Group (COMMIT), 2005 Sabatine *et al* (CLARITY), 2005	aspirin + clopidogrel versus aspirin + ticlopidine aspirin + clopidogrel versus aspirin aspirin + clopidogrel versus aspirin aspirin + clopidogrel versus aspirin	Cardiac death, MI, or TLR CV death, MI or stroke Death, MI or stroke CV death, MI or urgent TVR	ns (30 days) 20% (1 year) 9% (discharge or 28 days) 20% (30 days)
Prasugrel	P2Y12 antagonist	Wiviott *et al* (TRITON), 2007	aspirin + prasugrel versus aspirin + clopidogrel	CV death, MI or stroke	19% (1 year)
Ticagrelor	P2Y12 antagonist	Steg *et al* (PLATO), 2010	aspirin + ticagrelor versus aspirin + clopidogrel	CV death, MI or stroke	13% (1 year)
Dipyridamole	PDE inhibitor	The PARIS Research Group (PARIS-1), 1980 White *et al*, 1995	aspirin + dipyridamole versus aspirin aspirin + dipyridamole versus aspirin	Cardiac death or MI Prevention of late reocclusion	ns (20 months) ns (1 year)
Cilostazol	PDE inhibitor	Lee *et al* (DECLARE-LONG II), 2011	Cilostazol + standard care versus standard care	In-stent late loss	18% (8 months)
Abciximab	GPIIb/IIIa inhibitor	The EPIC Investigators (EPIC), 1994 The CAPTURE Investigators (CAPTURE), 1997 Kastrati *et al* (ISAR-REACT 2), 2006 Simoons *et al* (GUSTO IV-ACS), 2001 Kastrati *et al* (IASR-REACT 4), 2011	12-hour infusion versus placebo 24-hour infusion versus placebo 12-hour infusion versus placebo 24-hour or 48-hour infusion versus placebo Abciximab + heparin versus bivalirudin	Death, MI or urgent revascularisation Death, MI or urgent revascularisation Death, MI or urgent TVR Death or MI Death, MI, urgent TVR or major bleeding	35% (30 days) 29% (30 days) 24% (30 days) ns (30 days) ns (30 days)
Eptifibatide	GPIIb/IIIa inhibitor	The PURSUIT Investigators (PURSUIT), 1998	Bolus and 72-hour infusion versus placebo	Death or MI	10% (30 days)
Tirofiban	GPIIb/IIIa inhibitor	The PRISM investigators (PRISM), 1998 The PRISM investigators (PRISM-PLUS), 1998	Bolus and 48-hour infusion versus placebo Bolus and 72-hour infusion versus placebo	Death, MI, refractory ischaemia or readmission for UA Death, MI or refractory ischaemia	ns (30 days) 17% (30 days)
Vorapaxar	PAR-1 antagonist	Tricoci *et al* (TRACER), 2012	vorapaxar + standard care versus standard care	CV, death, MI, readmission with ischaemia, urgent revascularisation	ns (median 502 days)

AbbreCOX-1, cyclo-oxygenase-1; CV, cardiovascular; GP, glycoprotein; MI, myocardial infarction; PAR-1, protease-activated receptor-1; PDE, phosphodiesterase; TLR, target lesion revascularisation; TVR, target vessel revascularisation; UA, unstable angina.

(>12 months) following percutaneous coronary intervention may be beneficial. In the DES-LATE[25] and ARCTIC-INTERRUPTION trials,[26] prolonged treatment with dual antiplatelet therapy (18–30 months vs 12 months) neither reduced the incidence of cardiovascular events nor increased the risk of major bleeding. Among those patients presenting with an acute coronary syndrome, primary and secondary ischaemic end points did not differ from the global treatment population. In the DAPT trial,[27] the largest and only double-blinded study, extended dual antiplatelet therapy (30 months vs 12 months) reduced the risk of major adverse cardiovascular and cerebrovascular events (4.3% vs 5.9%), myocardial infarction (2.1% vs 4.1%) and stent thrombosis (0.4% vs 1.4%) but at a cost of increased moderate or severe bleeding (2.5% vs 1.6%) and a borderline rise in all-cause mortality (2.0% vs 1.0%; p=0.05). Treatment effect did not differ between patients with or without a history of myocardial infarction for any of the co-primary end points including bleeding (figure 3).

Duration of dual antiplatelet therapy: systematic reviews and meta-analyses

Meta-analyses of trials using dual antiplatelet therapy in patients receiving intracoronary stents have compared short (3–6 months), 12-month and prolonged (>12 months) durations of therapy.[28 29] Longer treatment periods reduced the incidence of myocardial infarction and stent thrombosis but at a cost of increased major bleeding and with a tendency to increase overall mortality because of an increase in non-cardiovascular death. However, the majority of patients included in these analyses had stable coronary artery disease and few patients with acute coronary syndrome were treated with ≤6 months of dual antiplatelet therapy. In a recent meta-analysis[30] that included only patients with a history of acute coronary syndrome, prolonged dual antiplatelet therapy reduced the risk of cardiovascular death (RR 0.85; 95% CI: 0.74 to 0.98, p=0.03) without an increase in non-cardiovascular death (RR 1.03, 95% CI 0.86 to 1.23; p=0.76) or all-cause mortality (RR 0.92, 95% CI 0.83 to 1.03).

Atherothrombotic risk

The optimal duration of dual antiplatelet therapy is dependent on the balance between preventing future atherothrombotic events and the increased risk of bleeding from continued treatment. Following an acute coronary syndrome, predictors of atherothrombotic risk include ST deviation, diabetes mellitus, smoking status, left ventricular ejection fraction, stent type, number of

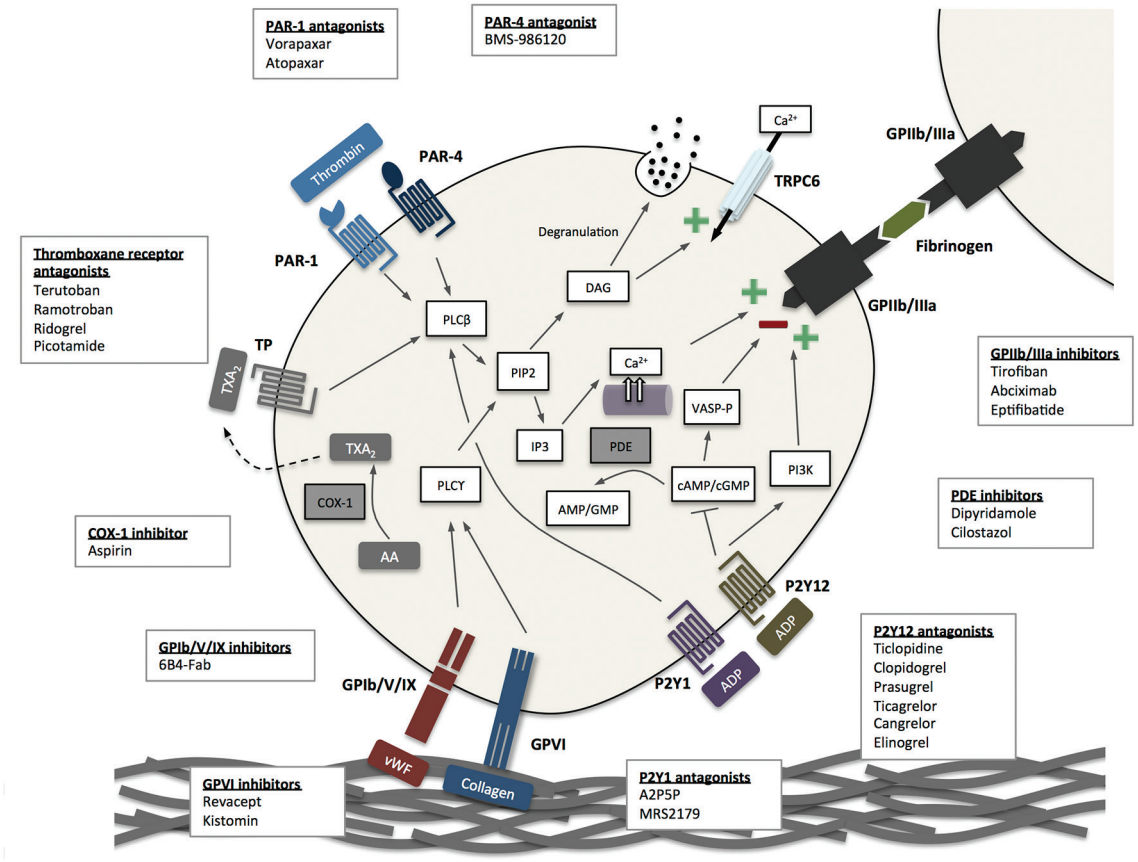

Figure 2 Platelet activation pathways and sites targeted by current and novel antiplatelet agents. Arachidonic acid; ADP, adenosine diphosphate; c, cyclic; Ca2+, calcium; AMP, adenosinemonophosphate; COX-1, cyclo-oxygenase-1; DAG, diacylglycerol; GMP, guanosine monophosphate; GP, glycoprotein; IP3: inositol trisphosphate; PAR, protease activated receptor; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PIP2: phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TP, thromboxane receptor; TRPC, transient receptor potential channel; TXA2, thromboxane A2; vWF, von Willebrand factor.

stents and complexity of coronary artery disease. Subgroup analyses have attempted to identify if any of these factors influence outcomes with regard to duration of dual antiplatelet treatment.

In the EXCELLENT trial,[22] there was a threefold increase in the incidence of cardiovascular events ($p<0.001$ for interaction) among pat ients with diabetes mellitus treated with 6 months as compared with 12 months of dual antiplatelet therapy. In the ISAR-SAFE trial,[20] rates of death, myocardial infarction, stent thrombosis, stroke or TIMI major bleeding tended to be higher in patients aged <67 years and lower in patients aged ≥67 years with 6 months compared with 12 months of dual antiplatelet treatment ($p=0.03$). These differences were driven by ischaemic complications rather than bleeding events. In the DAPT trial, men were nearly five times less likely to suffer from stent thrombosis if dual antiplatelet therapy was extended beyond 12 months ($p=0.04$) and the reduction in major adverse cardiovascular and cerebrovascular events with prolonged therapy (30 months) was greater in those treated with prasugrel (vs clopidogrel; $p=0.03$) or a first-generation drug-eluting stent ($p=0.048$). Similar trends were observed in DES-LATE[25] and ARCTIC-INTERRUPTION.[26]

Audit of the British Cardiovascular Society Intervention database indicates that in 2013/2014, a half of all percutaneous coronary intervention procedures were associated with residual disease (≥1 stenosis of >50% severity), fulfilling the criteria for incomplete revascularisation. Patients with acute coronary syndrome and incomplete revascularisation have a residual burden of coronary disease that is a substrate for recurrent plaque rupture, coronary thrombosis and future cardiac events.[31 32] Prolonged dual antiplatelet treatment may mitigate this risk but whether this translates to a more favourable risk-to-benefit balance for patients with incomplete revascularisation remains an area for future research.

Bleeding and total mortality

Large registries and trials have shown that major bleeding is associated with an increase in mortality that could potentially negate the benefits of dual antiplatelet therapy in acute coronary syndrome.[33–36] Importantly, these bleeding risks are not confined to the initial hospitalisation phase.[33 35] The association between bleeding and mortality has been a consistent feature of acute coronary syndrome trials irrespective of whether the intervention being assessed and improvements in outcome are seen with interventions that are associated with a lower bleeding risk. For example, in the OASIS-5 trial,[33] fondaparinux had similar antithrombotic benefits to enoxaparin but was associated with lower rates of major bleeding and marked reductions in all-cause mortality. Similar benefits have also been reported for randomised controlled trials of arterial access sites in patients treated with an invasive strategy for either ST-segment[37] or non-ST-segment[38] myocardial infarction. Again, because radial artery access was associated with less bleeding, overall all-cause mortality was lower.[37 38] There have been various mechanisms proposed for the link between bleeding and mortality that

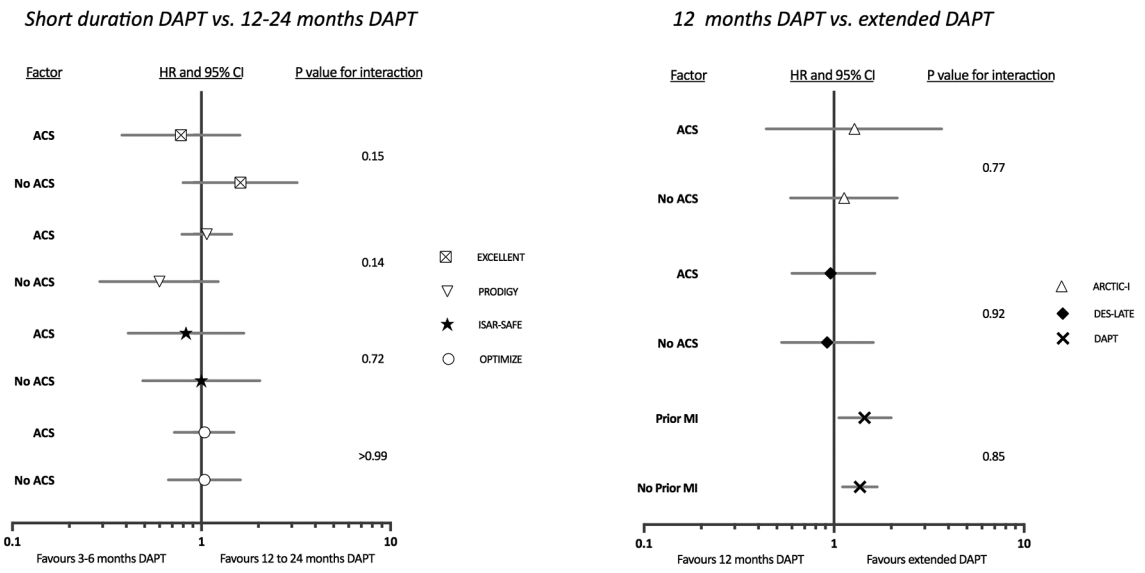

Figure 3 Hazard ratios for the composite primary end-point from sub-group analyses of patents presenting with and without an acute coronary syndrome. EXCELLENT trial (n=1443), 6 vs 12 months, patients presenting with ACS = 52% of study population; PRODIGY trial (n=2013), 6 vs 24 months, patients presenting with ACS subgroup = 74% of study population; ISAR-SAFE trial (n=4000), 6 vs 12 months, patients presenting with ACS = 40% of study population; OPTIMIZE trial (n=3119), 3 vs 12 months, patients presenting with ACS = 37% of study population; ARTIC INTERRUPTION (n=1259), 12 vs 30 months, patients presenting with ACS = 26% of study population; DES-LATE (n=5045), 12 vs 24 months, patients presenting with ACS = 61% of study population and DAPT (n=9961), 12 vs 30 months, patients presenting with ACS = 43% of study population.

include rebound hypercoagulability, discontinuation of antithrombotic treatments, inflammation and ischaemia.[39] The European Society of Cardiology Working Group on Thrombosis has called for clinical trials to address bleeding in acute coronary syndrome including the exploration of the duration of dual antiplatelet therapy.[39]

Duration uncertainty

Currently there are variations in local and regional dual antiplatelet therapy practices that are confusing for patients, primary care physicians and cardiologists. Indeed, while European[13] and North American[12] guidelines recommend dual antiplatelet therapy for 12 months after an acute coronary syndrome, both acknowledge that shorter or longer durations may be appropriate. Duration of therapy is seen as a major priority for future research by numerous national and international guideline committees as well as having considerable financial implications, especially for the latest generation of P2Y12 receptor antagonists. However, major pharmaceutical companies have to date not funded trials comparing shorter (<12 months) durations of dual antiplatelet therapy, since, arguably, it may not be in their commercial interest to do so.

For clinicians and healthcare providers, there remains much uncertainty regarding the default duration of dual antiplatelet therapy for most patients with acute coronary syndrome. Current guidelines are largely based on evidence that predates potentially important technological advances, including second-generation drug-eluting stents, while in recent trials, only a minority of patients presented with an acute coronary syndrome and many of these studies were underpowered to detect differences due to low event rates. Selected populations included in randomised controlled trials have lower rates of bleeding and non-cardiovascular death than the general population (table 3), since patients with any history of bleeding or major comorbidity were specifically excluded from such trials. On the other hand, shorter durations of dual antiplatelet therapy may expose medically managed patients (such as due to complex disease) to an increased risk of atherothrombotic events. This is because stenting in acute coronary syndrome confers protection against atherothrombotic events[40 41] and in the vast majority of clinical trials, only patients who underwent stent implantation were included. There is therefore a major concern that the evidence to date has been extrapolated to a broader population with a higher risk of both atherothrombotic events and adverse outcomes from bleeding. Accordingly, this has left uncertainty as to the relative benefits and risks of one period of treatment versus another in real-world patients with acute coronary syndrome.

Increased thrombin generation[42] and platelet reactivity[43] have been demonstrated for up to 2 years following a plaque rupture myocardial infarction. This may explain potentially more favourable outcomes with longer durations of dual antiplatelet theraphy in patients with acute coronary syndrome as compared to those with stable ischaemic heart disease undergoing percutaneous coronary intervention. However, switching from dual antiplatelet treatment to monotherapy (usually aspirin alone) is associated with a rebound prothrombotic effect, especially with regard to an excess of stent thrombosis.[10 27] In the DAPT trial,[27] this phenomenon occurred irrespective of the timing of switching to monotherapy. Thus, unless dual antiplatelet therapy is continued indefinitely, there will remain a small persistent short-term 3-month risk of rebound stent thrombosis and myocardial infarction following the transition from dual to single antiplatelet therapy.

Newer generation P2Y12 inhibitors provide more effective antithrombotic protection than clopidogrel but at the cost of increased bleeding. Given the greater expense of these agents

Table 3 Temporal relationship with the clinical benefits of clopidogrel therapy

Outcome	CURE (clopidogrel)	TRITON (prasugrel)	TRILOGY (prasugrel)	PLATO (ticagrelor)	Randomised controlled trial average	Scotland 2006–2010* (clopidogrel)
All-cause mortality	5.8%	3.0%	8.3%	4.5%	5.40%	25.4%
Cardiovascular death	5.1%	2.1%	6.6%	4.0%	4.45%	17.8%
Non-cardiovascular death	0.7%	0.9%	1.7%	0.5%	0.95%	7.6%
Fatal bleeding	0.2%	0.4%	0.2%	0.3%	0.28%	0.9%

*From Information and Statistics Division of NHS Scotland.

and the marked temporal decline in thrombotic risk that is evident over the first few months, an early 'switch' from ticagrelor or prasugrel to clopidogrel after 1 month to 6 months has been advocated. While evidence from small cohort[44] and pharmacodynamic studies[45] suggest such an approach may be safe and reduce bleeding events, an overall lack of data limits any meaningful recommendations. TROPICAL-ACS (NCT 01959451) is an ongoing clinical trial investigating whether a switch to clopidogrel treatment after 1 week of prasugrel is non-inferior to 12 months of standard treatment with prasugrel. Results from this and other similar randomised studies (SWAP-4, NCT 02287909) should provide insights into defining the best strategy for switching between P2Y12 antagonists.

Personalised medicine

Generic recommendations for length of dual antiplatelet therapy derived from a protocolised intervention in clinical trials will inevitably expose some patients to an excessive duration of treatment and disadvantage other patients by withdrawing therapy that protects them from myocardial infarction. The critical clinical question is therefore: can individuals who are more or less likely to benefit from shorter or longer durations of treatment be identified? While several risk tools have been developed to help determine the future incidence of coronary thrombotic events and major bleeding episodes for an individual, the DAPT Score[46] and PARIS registry risk Score[47] were specifically designed to predict medium-term to long-term risks that are directly modified by continuing or interrupting dual antiplatelet therapy.

Using data from the DAPT trial, the DAPT Score was developed to determine the net clinical benefit of extending dual antiplatelet therapy from 12 months to 30 months (figure 4). Thirty-seven candidate variables were considered with 8 included in the final model. In the validation cohort the c-statistic was 0.64 for ischaemia and 0.64 for bleeding. The PARIS registry risk score was

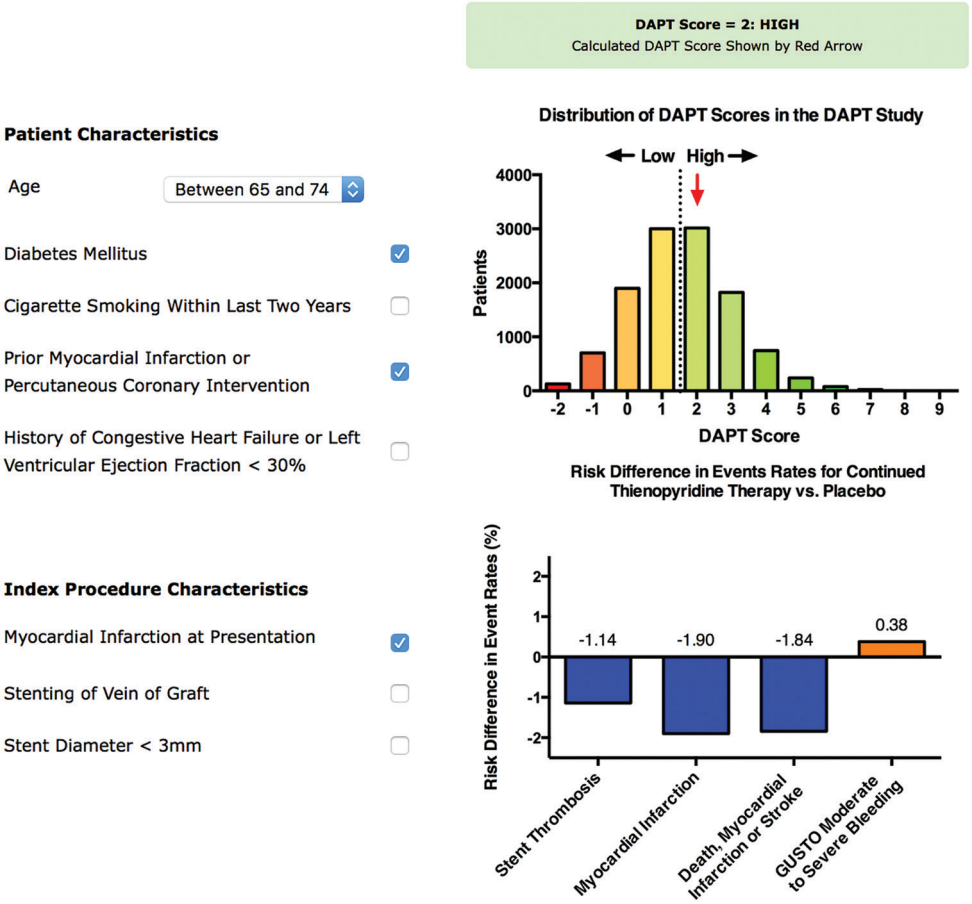

Figure 4 DAPT Score calculator for predicting risk/benefit of extending dual antiplatelet therapy from 12 months to 30 months.Available at www.daptstudy.org.

similarly developed but was based on prospective observational data. Model discrimination for ischaemia (c-statistic 0.65) and bleeding (c-statistic 0.64) events were comparable to the DAPT score. Thus while these and other similar risk / benefit tools may provide a step forward, they do not as yet offer sufficiently robust predictive value for everyday clinical use.

Variables included in risk tools tend to predict both bleeding and myocardial infarction. High platelet reactivity was originally reported to identify patients at an increased risk of future atherothrombotic events.[48] However, subsequent studies have failed to show an association between platelet reactivity and atherothrombotic outcomes[49] or demonstrate that dose adjustment on the basis of on-treatment platelet reactivity is beneficial.[50] Routine platelet testing is therefore not recommended in patients prescribed P2Y12 and is unlikely to deliver a clinically useful prediction score. Further research is needed to define prediction tools with high discriminatory value in real-world patients, and the potential for net-clinical benefit according to the length of dual anti-platelet therapy.

CONCLUSIONS

It has been 15 years since the CURE trial demonstrated the benefit of dual antiplatelet therapy following an acute coronary syndrome and yet the optimal duration remains uncertain. With regard to thrombotic complications, recent clinical trials and meta-analyses suggest that with newer generation drug-eluting stents, 3 months to 6 months of dual antiplatelet therapy is non-inferior to 12 months of treatment. Prolonged treatment (>12 months) reduces the risk of stent thrombosis, myocardial infarction and possibly cardiovascular death but at the cost of increased major bleeding and with no net mortality benefit. However, these potential hazards and benefits of intervention may differ when applied to the general broad population of patients encountered in everyday clinical practice who have higher bleeding and atherothrombotic event rates.

While ongoing randomised clinical trials may address some of the residual uncertainties in select subgroups, we believe there is a pressing need to undertake a broad inclusive trial of shorter durations of therapy in broad populations of patients with acute coronary syndrome. Such a trial will need to be able to explore specific subgroups, such as those who are medically managed, undergo percutaneous coronary intervention or have coronary artery bypass graft surgery, as well as enable better identification of atherothrombotic and bleeding risks from real world data to inform a more personalised approach to decisions regarding treatment duration.

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Predicting long-term bleeding risk after acute coronary syndrome: a step closer to optimising dual antiplatelet therapy duration?

Simon Wilson, David E Newby

Dual antiplatelet therapy (DAPT) with aspirin and a P2Y12 antagonist improves cardiovascular outcomes in patients with acute coronary syndrome but at a cost of an increased risk of bleeding complications.[1] There is marked variability in the thrombotic and bleeding risk between individuals with multiple patient and procedural factors identified but not yet fully understood. Generic recommendations for DAPT duration inevitably expose some patients to an excessive duration of treatment and bleeding risk while simultaneously disadvantaging other patients by withdrawing therapy that could protect them from atherothrombotic events. This is increasingly recognised as a major clinical problem, and current European and North American Acute Coronary Syndromes (ACS) guidelines now acknowledge that shorter durations of DAPT may be considered in patients at high risk of bleeding while extended treatment (>12 months) is an option in selected patients. To implement these guideline recommendations, robust prediction tools are required to facilitate the accurate identification of individuals who are more or less likely to benefit from shorter or longer durations of treatment.

The association between bleeding and mortality has been a consistent feature of acute coronary syndrome trials, irrespective of the intervention being assessed. The present paper by Alfredsson and colleagues[2] fills an important gap since it provides the first longitudinal long-term bleeding risk score (0–30 months) for medically managed ACS patients treated with DAPT. Previous post-ACS bleeding risk tools have informed on short-term bleeding risk in patients predominantly managed with percutaneous coronary intervention[3–5] or at a single time point (2 years) in a population with a majority of stable coronary artery disease (~70%).[6] From 12 preselected candidate variables and using the Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage (TRILOGY) ACS study database, the authors identified 10 baseline predictors of Global Use of Strategies to Open Occluded Arteries (GUSTO) moderate/severe/life-threatening and five baseline predictors of thrombolysis in myocardial infarction (TIMI) major or minor bleeding (table 1). While many of the predictors for bleeding were common to those previously reported, other variables such as a history of peptic ulcer disease and angiography before randomisation (unique to the TRILOGY ACS database) were also identified. In sharp contrast to results from short-term bleeding risk tools and studies of long-term bleeding in mixed populations,[6 7] female gender predicted a reduced long-term bleeding risk. These and other differences highlight the potentially time-dependent and population-specific contribution of risk factors for bleeding, emphasising the possible hazard of applying prediction tools to patient groups distinct from the reference cohort.

The bleeding risk prediction models derived by Alfredsson and colleagues showed moderate to good predictive accuracy with C-indices of 0.78 (95% CI 0.75 to 0.80) for the GUSTO model and 0.67 (95% CI 0.65 to 0.69) for the TIMI model. Internal validation with bootstrapping gave bias-adjusted C-index values of 0.77 and 0.65 for the GUSTO and TIMI models, respectively, comparing favourably to existing prediction tools. However, if risk scores are to be of clinical utility they must also be easy to estimate, generalisable to populations treated in clinical practice and perhaps most importantly, actionable.

All the current risk models for bleeding are based on metrics measured at a set time point, typically within the index presentation. Measures of admission blood pressure or heart rate and cardiovascular or periprocedural medication can be difficult and time consuming to retrieve, particularly as patients are often transferred to and from a tertiary centre for angiography. Furthermore, given the high turnover of most cardiology departments, formally estimating an individual's relative risk and benefit of continuing DAPT will often default to the outpatient clinic. Thus, even at the expense of a degree of model discrimination, prediction tools with a modest number of easily identifiable risk factors (eg, TIMI minor or major bleeding model in the current paper—five variables; Patterns of Non-Adherence to Anti-Platelet Regimens in Stented Patients (PARIS) registry risk score—six variables) may be of greater clinical utility, as they are more likely to be adopted in the 'real world'.

Selected populations included in randomised controlled trials have lower rates of bleeding and non-cardiovascular death than the general population, since patients with any history of bleeding or major comorbidity are often specifically excluded from such trials. Applying risk tools derived from clinical trial data to broader populations may therefore underestimate true bleeding hazards. A relative strength of the prediction models derived by Alfredsson and colleagues is the high number of female patients (~40%), patients aged over 75 years (>22%) and important comorbidities in the study population, comparable to those found in large observational ACS registries.[1] External validation in a predominantly invasively managed ACS trial population (Thrombin-Receptor Antagonist Vorapaxar in Acute Coronary Syndromes trial) demonstrated good generalisability of both risk tools to other acute coronary syndrome (trial) populations with a C-index that was essentially unchanged for the TIMI minor or major bleeding model (0.68) and modestly attenuated for the GUSTO moderate/severe/life-threatening bleeding model (0.69).

Patients with cerebrovascular disease are particularly sensitive to the bleeding risks of DAPT and a past history of transient ischaemic attack (TIA) or stroke is both a commonly encountered comorbidity and important risk factor for intracranial haemorrhage (ICH) in ACS populations. Unfortunately, many clinical trials of DAPT, including TRILOGY ACS, exclude such patients from the study population. Management of ACS patients with an indication for anticoagulation remains unclear with considerable heterogeneity in clinical practice reflecting the limited available data. This is even more problematic given

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Table 1 Examples of baseline predictors of bleeding from current risk tools

Study	Alfredsson *et al*[2]		CRUSADE bleeding score[3]	Mehran *et al*[4]	Mathews *et al*[5]	PARIS registry bleeding score[6]
Population	NSTEMI/UA		NSTEMI	ACS	ACS	~38% ACS;~72% SCAD
Bleeding definition	GUSTO mod/sev/LT bleeding: 0–30 months	TIMI minor/major bleeding: 0–30 months	In-hospital non-CABG major bleeding*	30 day non-CABG major bleeding†	In-hospital major bleeding‡	Major bleeding within 2 years§
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Age	1.27 (1.16 to 1.37) per 5 year ↑	1.18 (1.1 to 1.27) per 5 year ↑		1.17 (1.13 to 1.21) per 5 year ↑	1.04 (1.03 to 1.05) per 5 year ↑	1.02 (1.00 to 1.04) per 1 year ↑
Female gender	0.67 (0.47 to 0.96)	0.59 (0.41 to 0.84)	1.31 (1.23 to 1.39)	2.32 (1.98 to 2.72)	1.37 (1.29 to 1.45)	
Presentation	1.58 (1.04 to 2.42) NSTEMI vs UA			1.26 (1.04 to 1.54) NSTEMI vs UA	1.26 (1.04 to 1.54) STE-ACS vs non STE-ACS	
				1.92 (1.52 to 2.44) STEMI vs UA		
Haemoglobin	1.34 (1.48 to 1.2) per 1 g/dL ↓	1.13 (1.25 to 1.03) per 1 g/dL ↓	2.28 (2.11 to 2.46) haematocrit <36%	1.98 (1.65 to 2.37) <13 g/dL, men; <12 g/dL, women	2.29 (2.08 to 2.52) <12 g/dL	2.72 (1.83 to 4.04) <12 g/dL, men; <11 g/dL, women
Creatinine / GFR	1.88 (1.12 to 3.17) per 1 mg/dL ↑	2.11 (1.28 to 3.5) per 1 mg/dL ↑	1.12 (1.10 to 1.13) per 10mL/min ↓	1.09 (1.07 to 1.12) per 0.1 mg/dL ↑	1.17 (1.14 to 1.20) per 1 mg/dL ↑	1.81 (1.16 to 2.82) <60 mL/min
Weight / BMI	1.04 (1.1 to 0.99) per 5 kg ↓				1.02 (1.01 to 1.03) per 5 kg ↓	1.68 (1.09 to 2.60) BMI <25 vs 25 to 29.9 kg/mm^2
Diabetes mellitus			1.25 (1.12 to1.40)		1.21 (1.15 to 1.28)	
Current smoking						1.94 (1.18 to3.20)
Prior vascular disease			1.10 (0.98 to 1.24)		1.27 (1.17 to 1.37)	
History of peptic ulcer disease	1.70 (0.98 to 2.94)					
Signs of CHF at presentation			1.23 (1.15 to 1.31)		3.87 (3.36 to 4.45) shocked vs non-shocked	
BP at presentation	1.25 (1.47 to 1.06) per 10 mm Hg ↓ when <130 mm Hg		1.26 (1.16 to 1.36) ≤110 mm Hg vs 110 to 180 mm Hg		1.15 (1.09 to 1.21) ≤130 mm Hg vs 130 to 160 mm Hg	
	0.66 (0.85 to 0.52) per 10 mm Hg ↓ when >130 mm Hg		1.24 (1.14 to 1.35) ≥180 mm Hg vs 110 to 180 mm Hg		1.09 (1.03 to 1.16) ≥160 mmHg vs 130 to 160 mm Hg	
Heart rate at presentation			1.08 (1.07 to 1.10) per 10 bpm increase		1.11 (1.10 to 1.12) per 10 bpm increase	
Beta-blocker at randomisation	1.68 (1.07 to 2.62)					
Angiography performed	1.70 (1.23 to 2.34)	1.51 (1.12 to 2.04)				
Triple therapy on discharge						1.93 (1.08 to 3.43)

*Intracranial haemorrhage, documented retroperitoneal bleed, haematocrit drop ≥12% (baseline to nadir), any red blood cell transfusion when baseline haematocrit was≥28% or any red blood cell transfusion when baseline haematocrit was <28% with witnessed bleed.
†Intracranial or intraocular bleeding, access site haemorrhage requiring intervention, reduction in haemoglobin of ≥4 g/dL without or ≥3 g/dL with an overt bleeding source, reoperation for bleeding or blood product transfusion.
‡Absolute Hgb decrease of ≥4 g/dL (baseline to nadir), intracranial haemorrhage, documented or suspected retroperitoneal bleed, any red cell blood transfusion with baseline Hgb≥9 g/dL or any red cell transfusion with Hgb<9 g/dL and a suspected bleeding event.
§Occurrence of Bleeding Academic Research Consortium type 3 or 5 bleed.
ACS, acute coronary syndrome; BP, blood pressure; CABG, coronary artery bypass graft; CHF, congestive heart failure; CRUSADE, Can Rapid Risk Stratification of Unstable Angina Patients Suppress ADverse Outcomes with Early Implementation of the ACC/AHA Guidelines; GFR, glomerular filtration rate; GUSTO, Global Use of Strategies to Open Occluded Arteries; LT, life threatening; mod, moderate; sev, severe; NSTEMI, Non ST-elevation myocardial infarction; PARIS, Patterns of Non-Adherence to Anti-Platelet Regimens in Stented Patients; SCAD, stable coronary artery disease; STEMI; ST-elevation myocardial infarction; STE-ACS, ST-elevation-acute coronary syndrome; TIMI, thrombolysis in myocardial infarction. UA, unstable angina.

the recent widespread uptake of contemporary direct oral anticoagulants. In the PARIS registry risk score,[6] triple therapy (DAPT + oral anticoagulant) at discharge was associated with a near twofold increased risk of major bleeding within 2 years; although given the recruitment window was between 2009 and 2010, it is unlikely that individuals were receiving an oral anticoagulant other than a vitamin K antagonist. Prediction tools that inform on the short-, medium- and long-term risks and benefits of triple therapy are to be strongly welcomed as well as ongoing trials to assess the long-term discontinuation or resumption of antithrombotic therapies in patients with a history of ICH [ISRCTN71907627].

The CHA_2DS_2-VASc score has a pooled C-statistic of approximately 0.68 for predicting the risk of ischaemic stroke in non-anticoagulated patients, lower than most bleeding prediction scores. Nevertheless, this clinical tool is widely used because not only is it is easy to calculate, the results are easy to translate into patient management. Presently, this is a shortcoming of all bleeding risk scores. Optimal DAPT duration depends on the balance between preventing future atherothrombotic events and the increased risk of bleeding from continued treatment. Without knowing the consequences of discontinuing therapy, how then can a 2-year risk of major bleeding, for example of 5%, inform treatment? This is even more challenging if we consider that many of the risk factors for bleeding (eg, age, gender, type of presentation, renal dysfunction) predict higher atherothrombotic risk and vice-versa. On the basis of a high bleeding risk alone, prescribing shorter durations of DAPT will inevitably disadvantage some patients who stand to gain the most anti-ischaemic benefit. Clinical tools that can predict the trade-off between ischaemic event reduction and occurrence of major bleeding events from continuing DAPT may be of greater practical use. Using data from the DAPT trial, the DAPT score was developed to help determine the net clinical benefit of extending dual antiplatelet therapy from 12 to 30 months in both ACS and non-ACS patients who underwent PCI.[7] However this and other similar risk/benefit tools remain to be validated in broader populations and are perhaps too immature at present for mainstream use.

Currently, bleeding and thrombotic risk prediction models for DAPT in post-ACS patients are based on variables collected at a single time point and therefore provide a static determination of the risk of future events. In reality though the bleeding risk for an individual is dynamic, modified by the accumulation of positive or negative predictors of risk and the relative contributions of these factors over time. The consequences of an event can also be time dependent, and it is important to keep in context the merits to the individual of increasing the risk of one type of event to prevent another. For example, the risk of death associated with early stent thrombosis is markedly higher than the risk of death associated with very late stent thrombosis.[8] In contrast, there is much less temporal variability in the mortality from bleeding.[9]

Ideally, risk prediction tools are developed that allow modifiable longitudinal risk estimates to be generated at any given point, taking into account known dynamic variables and the time dependent severity of an event. Whether it is feasible to assimilate all these factors into one unifying and implementable model remains to be determined. Until then, the TRILOGY ACS bleeding risk models presented by Alfredsson and colleagues represent a significant step forward in predicting long-term bleeding risks with DAPT after ACS to facilitate clinical decision making on an individual level.

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OPEN

PAR4 (Protease-Activated Receptor 4) Antagonism With BMS-986120 Inhibits Human Ex Vivo Thrombus Formation

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Objective—BMS-986120 is a novel first-in-class oral PAR4 (protease-activated receptor 4) antagonist with potent and selective antiplatelet effects. We sought to determine for the first time, the effect of BMS-986120 on human ex vivo thrombus formation.

Approach and Results—Forty healthy volunteers completed a phase 1 parallel-group PROBE trial (Prospective Randomized Open-Label Blinded End Point). Ex vivo platelet activation, platelet aggregation, and thrombus formation were measured at 0, 2, and 24 hours after (1) oral BMS-986120 (60 mg) or (2) oral aspirin (600 mg) followed at 18 hours with oral aspirin (600 mg) and oral clopidogrel (600 mg). BMS-986120 demonstrated highly selective and reversible inhibition of PAR4 agonist peptide (100 μM)-stimulated P-selectin expression, platelet-monocyte aggregates, and platelet aggregation (P<0.001 for all). Compared with pretreatment, total thrombus area (μm²/mm) at high shear was reduced by 29.2% (95% confidence interval, 18.3%–38.7%; P<0.001) at 2 hours and by 21.4% (9.3%–32.0%; P=0.002) at 24 hours. Reductions in thrombus formation were driven by a decrease in platelet-rich thrombus deposition: 34.8% (19.3%–47.3%; P<0.001) at 2 hours and 23.3% (5.1%–38.0%; P=0.016) at 24 hours. In contrast to aspirin alone, or in combination with clopidogrel, BMS-986120 had no effect on thrombus formation at low shear (P=nonsignificant). BMS-986120 administration was not associated with an increase in coagulation times or serious adverse events.

Conclusions—BMS-986120 is a highly selective and reversible oral PAR4 antagonist that substantially reduces platelet-rich thrombus formation under conditions of high shear stress. Our results suggest PAR4 antagonism has major potential as a therapeutic antiplatelet strategy.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT02439190.

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Key Words: antiplatelet ■ human ■ novel ■ protease-activated receptor 4 ■ thrombosis

Platelets are central to thrombus formation, the leading cause of global mortality.[1] Antiplatelet drugs are of proven benefit for the treatment and prevention of atherothrombotic events in many clinical settings, but despite the introduction of newer agents in the last decade, important limitations persist. Aspirin and P2Y12 antagonists, the current standard of care oral antiplatelet agents in patients with acute coronary syndrome, stroke, and peripheral arterial disease, prevent thromboxane A2 and ADP platelet activation, respectively.[2–7] However, neither is effective against thrombin, the most potent of all platelet agonists,[8] and both are associated with an increased incidence of bleeding that restricts their use in sensitive populations (eg, elderly, cerebrovascular disease) and reduces their net clinical benefit.[5,9–12] Thus, despite contemporary antiplatelet pharmacotherapy, many patients remain at high risk of future atherothrombotic events,[5–7,10,13,14] and there is a clear need for newer agents that can provide equivalent (or superior) antithrombotic efficacy with an improved safety profile.

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In recent years, PAR4 (protease-activated receptor 4) antagonism has emerged as promising new antiplatelet strategy. PAR4 is a G-protein coupled receptor expressed on the platelet surface that together with PAR1 (protease-activated receptor 1) is responsible for thrombin-mediated platelet activation and aggregation.[15] Thrombin has a key role in the coagulation cascade, but by targeting the platelet receptor rather than the protease, this avoids directly interfering with thrombin-induced fibrin production. PAR1 has greater affinity for thrombin than PAR4, but

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Nonstandard Abbreviations and Acronyms	
AP	agonist peptide
CI	confidence interval
ns	nonsignificant
PAR	protease-activated receptor
PI3K	phosphatidylinositol 3-kinase
PROBE	prospective randomized open-label blinded end point
TF	tissue factor

despite early clinical promise, the addition of vorapaxar (the only licensed PAR1 antagonist) to standard care failed to meet its primary efficacy outcome in patients with acute coronary syndrome and was associated with an excess of major bleeding, especially intracranial hemorrhage, in phase3 clinical trials.[13,16] PAR4 was originally thought to simply provide some redundancy to PAR1 platelet signaling at high thrombin concentrations.[17] However, due to differences in activation kinetics and downstream pathways, it is now evident that PAR1 and PAR4 have distinct and complementary roles in the early and late phases of platelet activation and aggregation, respectively.[18–20] PAR1 activation is brisk but transient and requires input from the P2Y12-PI3K (phosphatidylinositol 3-kinase) pathway to maintain platelet aggregation.[19,20] In contrast, PAR4 is activated at higher thrombin concentrations and induces a slow but prolonged intracellular signal that acts independently to sustain irreversible aggregation.[17,18,20] Furthermore, PAR4 activation occurs after ADP secretion, and thrombin depends on PAR4 but not PAR1 to induce full platelet spreading.[21] Thus, several lines of evidence indicate that while PAR1 and other agonist-signaling pathways may have important roles in initiating platelet activation, the primary function of PAR4 appears to be in sustaining irreversible platelet aggregation and thrombus propagation. This suggests that selectively targeting PAR4-mediated thrombin activity may protect against occlusive thrombus formation while avoiding interfering with hemostatic platelet responses to the same extent as PAR1 antagonists and other antiplatelet agents.[22]

BMS-986120 is a first-in-class, oral, highly selective, and reversible PAR4 antagonist antiplatelet agent. In preclinical animal models, BMS-986120 demonstrated potent antithrombotic activity with a substantially wider therapeutic window when compared with clopidogrel.[22] The purpose of the present phase 1 parallel-group PROBE trial (Prospective Randomized Open-Label Blinded End Point) was to build on these observations and examine for the first time, the antiplatelet and antithrombotic effects of BMS-986120 in humans using a translational model of ex vivo thrombosis. We determined whether reductions in thrombus formation were driven by a decrease in platelet-rich or fibrin-rich thrombus formation and whether these effects were greater under rheological conditions of low or high shear stress.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

All 40 volunteers (81 volunteers were screened) completed the study in full. The demographics and baseline characteristics of study volunteers were similar in the 2 treatment groups (Table). BMS-986120 was well tolerated with no clinically significant effect on any of the biochemical, hematologic, coagulation, physical, or ECG safety assessments conducted throughout the study (Table I in the online-only Data Supplement). There were no serious adverse events. One episode of minor bleeding was reported. This occurred 12 hours after aspirin administration, self-resolved, and did not recur.

Pharmacokinetic Profile of Oral BMS-986120

BMS-986120 was rapidly absorbed with peak plasma concentrations occurring at 2 hours (255±136 ng/mL; Figure 1). Plasma concentrations of BMS-986120 were halved by 4 hours (133±100 ng/mL) and <10% of the peak concentration by 24 hours (21±9 ng/mL).

Effect of BMS-986120 on Platelet Activation and Aggregation

BMS-986120 demonstrated strong and reversible inhibition of PAR4 agonist peptide (AP; 100 μM)-stimulated platelet activation and aggregation (P<0.001 for all). Compared with pretreatment, PAR4 AP-stimulated increases in platelet P-selectin expression (%), platelet-monocyte aggregates (%), and platelet aggregation (%) were reduced by 91.7% (95% confidence interval [CI], 81.0–102.4), 80.6% (95% CI, 68.6%–92.6%), and 85.0% (95% CI, 82.0–88.1) at 2 hours and by 53.9% (95% CI, 43.2%–64.7%), 41.1% (95% CI, 28.9%–53.2%), and 6.0% (95% CI, 2.9%–9.0%) at 24 hours (P<0.001 for all; Figure 2). Plasma concentrations of BMS-986120 correlated with P-selectin expression (ρ=−0.87), platelet-monocyte aggregates (ρ=−0.88), and platelet aggregation (ρ=−0.82; P<0.001 for all; Figure III in the online-only Data Supplement). There was no effect on PAR1 AP, ADP, or arachidonic acid platelet responses (P=nonsignificant [ns] for all; Figure 2).

Effect of Aspirin±Clopidogrel on Platelet Aggregation

Aspirin administration reduced arachidonic acid-stimulated platelet aggregation by 74.5% (95% CI, 71.6%–77.3%; P<0.001). In combination with clopidogrel, aspirin reduced arachidonic acid-stimulated platelet aggregation by 73.7% (95% CI, 70.9%–76.5%; P<0.001) and ADP-stimulated platelet aggregation by 41.9% (95% CI, 35.2%–48.7%; P<0.001), respectively (Figure IV in the online-only Data Supplement).

Effect of BMS-986120 on Ex Vivo Thrombus Formation

BMS-986120 reduced total thrombus formation at high shear (P<0.001) but not at low shear (P=ns; Figure 3). Compared with pretreatment, total thrombus area (μm²/mm) at high shear was reduced by 29.2% (95% CI, 18.3%–38.7%; P<0.001) at 2 hours and by 21.4% (95% CI, 9.3%–32.0%; P=0.002) at 24 hours. Plasma concentrations of BMS-986120 correlated with total thrombus formation at high shear (ρ=−0.47; P<0.001) but not at low shear (ρ=−0.18; P=ns; Figure III in the online-only Data Supplement).

Table. Baseline Characteristics of Study Volunteers

Test Variable	BMS-986120 (n=20)	Aspirin±Clopidogrel (n=20)
Men (%)	20 (100)	20 (100)
Age, y (SD)	23.6 (3.4)	28.7 (10.0)
BMI, kg/m^2 (SD)	23.6 (2.6)	25.4 (3.5)
Race (%)		
White	19 (95)	19 (95)
Black/African	1 (5)	0
Asian	0	1 (5)
Hemoglobin, g/dL (SD)	14.2 (0.42)	14.6 (0.85)
Platelet count, ×10^9 c/L (SD)	230 (45)	221 (49)
APTT, s (SD)	30.9 (2.2)	30.8 (2.6)
PT, s (SD)	12.3 (0.9)	11.9 (0.7)

APTT indicates activated partial thromboplastin time; BMI, body mass index; and PT, prothrombin time.

Reductions in total thrombus area were driven by a decrease in platelet deposition (Figure 4). At high shear, platelet-rich thrombus area was reduced by 34.8% (95% CI, 19.3%–47.3%; *P*<0.001) at 2 hours and 23.3% (95% CI, 5.1%–38.0%; *P*=0.016) at 24 hours. Reductions in fibrin-rich thrombus area at 2 (−14.7%; 95% CI, −22.5% to −6.2%; *P*=0.002) and 24 hours (−7.9%; 95% CI, −16.3% to 1.4%; *P*=0.09) were small by comparison. BMS-986120 had no effect on either platelet-rich or fibrin-rich thrombus formation at low shear (*P*=ns for all).

Effect of Aspirin±Clopidogrel on Ex Vivo Thrombus Formation

Aspirin and aspirin in combination with clopidogrel both reduced thrombus formation at high and low shear, also driven by decrease in platelet-rich thrombus. Aspirin reduced total thrombus area and platelet-rich thrombus area by 30.2% (95% CI, 15.6%–42.2%; *P*<0.001) and 41.7% (95% CI, 22.9%–56.0%; *P*<0.001), respectively, and by 32.4% (95% CI, 18.3%–44.0%; *P*<0.001) and 46.4% (95% CI, 29.1%–59.5%; *P*<0.001), respectively, when used in combination with clopidogrel.

In contrast to BMS-986120, aspirin and aspirin in combination with clopidogrel both reduced total thrombus area at low shear (−17.4%; 95% CI, −27.0% to −6.5%; *P*=0.003 and −13.5%; 95% CI, −23.6% to −2.1%; *P*=0.02). There was no effect on fibrin-rich thrombus deposition at low or high shear (*P*=ns for all).

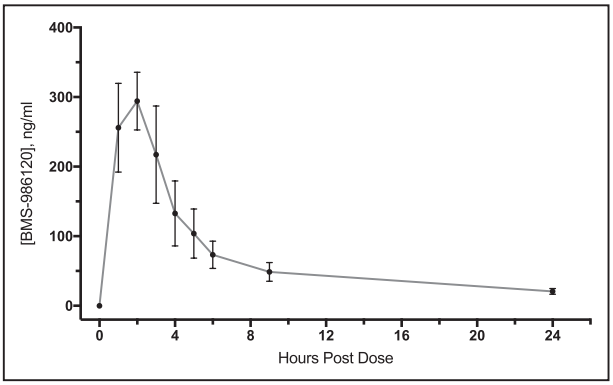

Figure 1. Pharmacokinetics of BMS-986120. BMS-986120 was rapidly absorbed with a half-life of 4 h. Data shown are mean plasma concentrations of BMS-986120 (±95% confidence intervals) after administration of a single oral 60-mg dose.

Discussion

In this phase 1 PROBE designed clinical trial, we have shown for the first time that PAR4 antagonism with BMS-986120 reduces ex vivo human thrombus formation under conditions representative of deep arterial injury in a stenosed coronary artery. BMS-986120 demonstrated selective and reversible antiplatelet effects with concentration-dependent inhibition of thrombus formation and PAR4 AP-stimulated platelet activation and aggregation. Our results provide further insights into the role of PAR4 in human thrombogenesis and raise major promise for BMS-986120 as an antiplatelet agent in the treatment and prevention of arterial thrombosis.

Assessment of the antiplatelet and antithrombotic effects of PAR4 inhibition has previously been limited by a lack of compound specificity and availability.[23–25] In comparison with earlier compounds, including P4pal-10, YD-3, and its derivative ML354,[25–27] BMS-986120 has antiplatelet activity against α thrombin, demonstrated greater potency and selectivity of effect in preclinical and phase 1 studies of platelet inhibition, and is the first orally bioavailable PAR4 antagonist.[22,28] In the present study, a single dose of BMS-986120 resulted in near complete inhibition of PAR4 AP-stimulated platelet activation and aggregation at 2 hours, with a return toward baseline at 24 hours. Importantly, there was no effect on PAR1 AP, ADP, or arachidonic acid-stimulated platelet activity. Our data, therefore, add to previous studies indicating that BMS-986120 is a highly selective and reversible antiplatelet agent with potent activity against PAR4-stimulated platelet activation and aggregation in humans.

The antithrombotic effects of BMS-986120 in humans were examined using the Badimon perfusion chamber—a well validated model for measuring ex vivo thrombus formation in humans.[29–36] Using the same model and under the same flow conditions, previous studies in healthy volunteers have demonstrated reductions in high shear thrombus formation of 18.7% after a single 300-mg oral dose of clopidogrel, 28% with a 60-mg oral dose of edoxaban and 56% with extracorporeal coadministration of tirofiban (50 ng/mL).[29,36,37] In the present study, a single dose of BMS-986120 (60 mg) reduced high shear thrombus formation by nearly a third. This is consistent with preclinical animal data[22,23] and comparable with reductions in thrombus formation we observed with high loading doses of aspirin and clopidogrel. Importantly, therefore, we have shown that oral PAR4 antagonism with BMS-986120 substantially reduces ex vivo human thrombus formation. Moreover, reductions were similar in magnitude to clinically approved antiplatelet agents suggesting a high probability of in vivo antithrombotic efficacy.

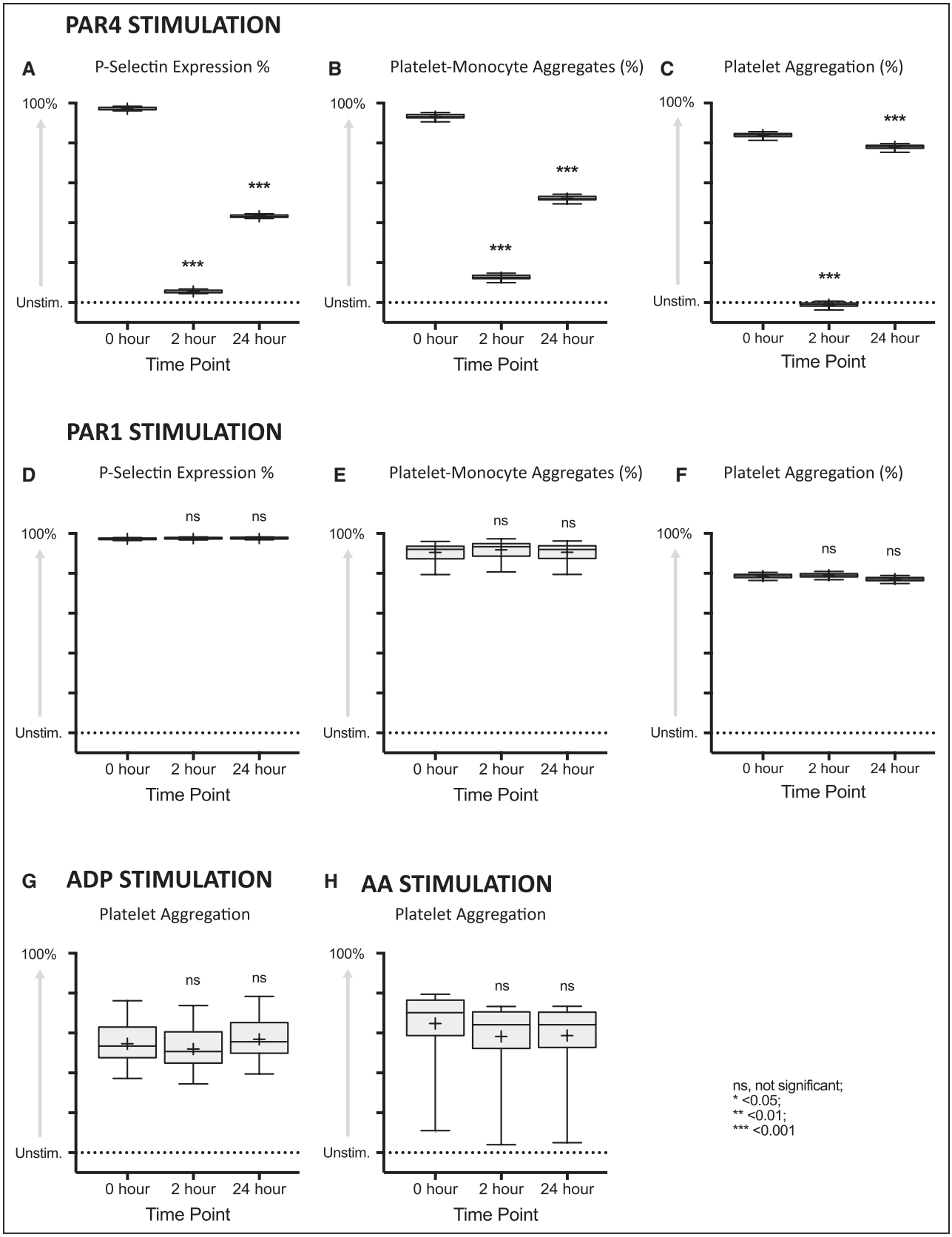

Figure 2. BMS-986120 demonstrated highly selective, potent, and reversible inhibition of PAR (protease-activated receptor) 4-stimulated platelet activation and aggregation. Box plots of platelet activation and aggregation in response to (**A**–**C**) PAR4 Agonist peptide (AP; 100 μM), (**D** and **E**) PAR1 AP (100 μM), (**F**) PAR1 AP (25 μM), (**G**) ADP (10 μM), and (**H**) arachidonic acid (AA; 5 mmol/L), in volunteers (*Continued*)

Figure 2 Continued. randomized to BMS-986120. Data shown are the adjusted mean (+) normalized to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. **P*<0.05, ***P*<0.01, ****P*<0.001.

BMS-986120 seemed to have less of an effect on thrombus formation at low shear than either aspirin alone or aspirin in combination with clopidogrel. Although further studies are required to confirm whether PAR4 antagonism is more selective for inhibiting thrombus formation at high shear than existing agents, distinct mechanisms of platelet aggregation are known to operate under different rheological conditions.[38,39] Low shear rates reflect flow conditions found in patent epicardial arteries and some veins, whereas the majority of atherothrombotic events invariably occur at areas of high shear stress seen in diseased arteries.[40,41] Indeed, most myocardial infarctions arise from stenotic atherosclerotic plaques with rheological conditions comparable with those in our high shear chamber.[42–44] Antiplatelet agents that are more selective for inhibiting thrombus formation at high shear may allow at-risk vascular beds to be targeted with greater specificity. Given many treatment-related bleeding events are likely to occur from vessels with low shear rates,[45–49] this could facilitate a wider safety profile.

As expected from an antiplatelet agent, reductions in thrombus were driven by a decrease in platelet deposition; however, there was also a small but significant reduction in fibrin-rich thrombus formation. PAR4 is reported to be the predominant platelet PAR responsible for phosphatidylserine exposure, microparticle shedding, and thrombin generation.[50] Our results add to these studies, indicating that PAR4 may have a role in platelet procoagulant activity during ex vivo human thrombus formation. Whether this is beneficial or not is uncertain, but it is worth noting BMS-986120 was not associated with an increase in coagulation assay times, and no bleeding-related clinical findings or adverse events were reported in a previous phase 1 single- and multiple-ascending dose study.[28]

PAR4 is expressed within the vasculature, and PAR4 antagonism may, in addition to protecting against thrombosis, serve to limit vascular complications in at-risk patients. Human vascular smooth muscle cells upregulate PAR4 in response to glucose,[51] and elevated expression of

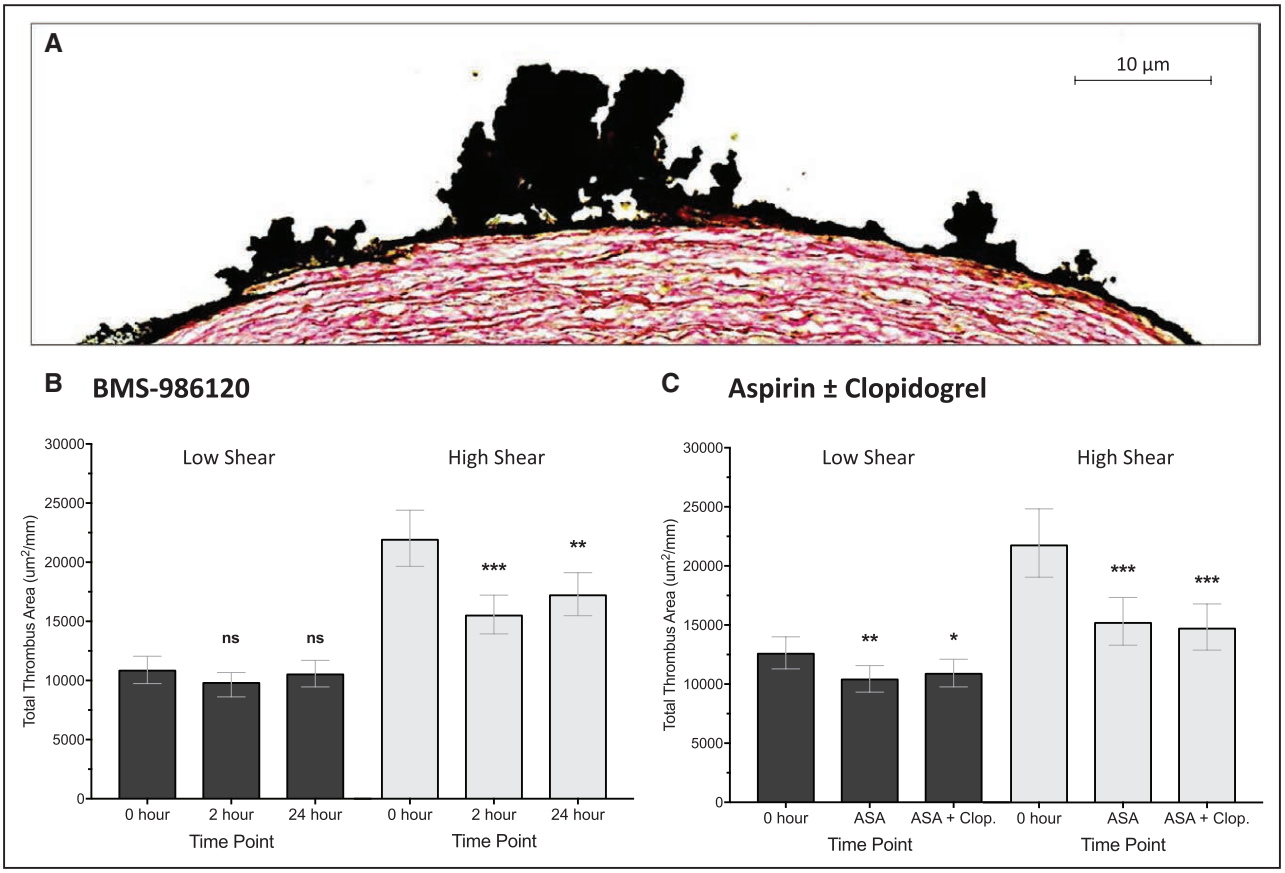

Figure 3. BMS-986120 reduced thrombus formation at high shear but not at low shear. **A**, Representative image of porcine aortic media exposed to human blood at high shear stained to quantify total thrombus area. Sections were stained with polyclonal goat antihuman fibrin(ogen) antibody and CD61 monoclonal mouse antihuman antibody before treatment with 3,3′-diaminobenzidine substrate chromogen. Sections were then counterstained with a modified Masson trichrome (hematoxylin and sirius red, 0.1%). Effect of (**B**) BMS-986120 and (**C**) aspirin (ASA)±clopidogrel (Clop.) on total thrombus area at high and low shear. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. **P*<0.05, ***P*<0.01, ****P*<0.001.

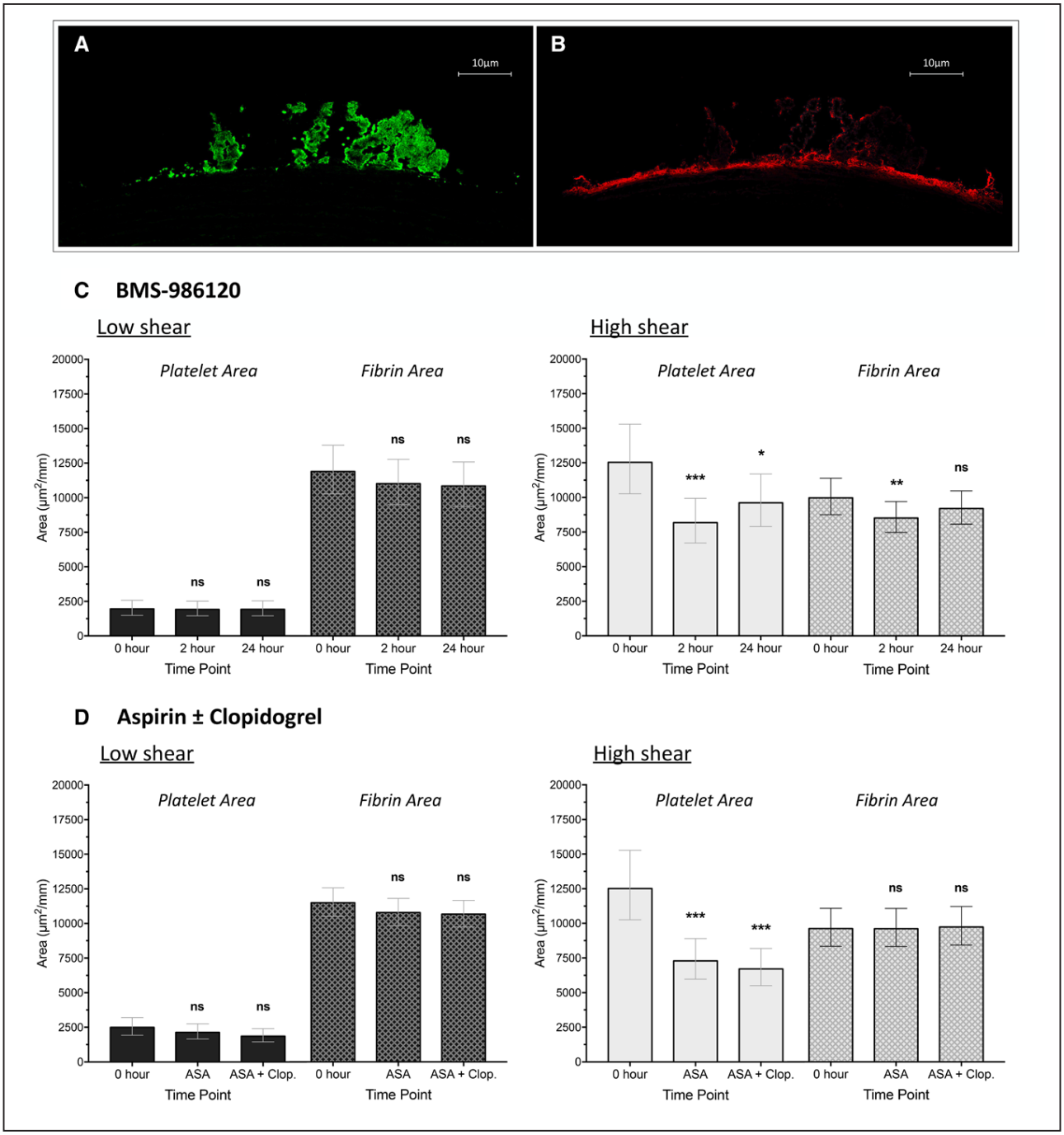

Figure 4. Reductions in thrombus formation were driven by a decrease in platelet-rich thrombus formation. Representative image of thrombus formed at high shear stained to allow quantification of (**A**) platelet-rich and (**B**) fibrin-rich thrombus area. Sections were stained with polyclonal goat antihuman fibrin(ogen) antibody and CD61 monoclonal mouse antihuman antibody before counterstaining with tyramide Cy3 (cyanine 3) and FITC (fluorescein isothiocyanate). Effect of (**C**) BMS-986120 and (**D**) aspirin (ASA)±clopidogrel (Clop.) on platelet and fibrin deposition at low and high shear. Data shown are adjusted means±95% confidence intervals. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. **P*<0.05, ***P*<0.01, ****P*<0.001.

PAR4 has been reported in the tunica media of atherectomy and saphenous vein tissue from patients with diabetes mellitus.[51] Moreover, PAR4 deficiency protected against excessive remodeling induced by carotid artery ligation in streptozotocin-diabetic mice.[52] PAR4, therefore, seems to be a key regulator of exaggerated intimal thickening in diabetes mellitus, and future studies examining the antiproliferative potential of PAR4 antagonism would be of significant therapeutic interest.

Our study has some limitations. First, although the exposed porcine aortic media presents many of the common constituents of a disrupted atherosclerotic plaque, including type I collagen, it may not contain tissue factor (TF).[53–55] TF activates the coagulation cascade and is an important

contributor to thrombogenicity.[56,57] Nevertheless, this does not overly limit our model for the assessment of thrombosis because binding of blood-borne circulating TF is sufficient to allow activation of the coagulation cascade and thrombus propagation.[53,54,58–60] Indeed, previous studies have demonstrated that thrombus formed from human blood perfused over exposed porcine tunica media (devoid of TF) stains heavily for TF.[53,54] Second, we assessed a single oral dose of BMS-986120 and did not explore the effect of prolonged BMS-986120 administration on thrombus formation, such as would occur with the secondary prevention of myocardial infarction and stroke. However, because this was the phase 1 trial designed to examine the antithrombotic effects of oral PAR4 antagonism in humans for the first time, we felt our study design was appropriate. Third, BMS-986120 was dosed in isolation, and future studies to determine the antiplatelet and antithrombotic effects of PAR4 antagonism in combination with current agents would be of interest. Finally, although no episodes of bleeding occurred in volunteers administered BMS-986120 and BMS-986120 was not associated with an increase in bleeding times in a previous phase 1 safety and tolerability study,[28] the safety profile of PAR4 antagonism in humans remains to be defined.

In conclusion, we have demonstrated that PAR4 antagonism with BMS-986120—a highly selective and reversible oral PAR4 antagonist—substantially reduces ex vivo thrombus formation in healthy volunteers under conditions of high shear stress. BMS-986120 was well tolerated with no change in coagulation assays or serious adverse events. Given the potential hemostatic sparing effects of PAR4 antagonism, our results suggest that BMS-986120 has major potential as a novel antiplatelet agent and that further investigation in clinical trials is warranted.

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Highlights

- Inhibition of thrombin-mediated platelet activation through PAR4 (protease-activated receptor 4) antagonism represents a promising new antiplatelet strategy because of the potential for reduced bleeding.
- BMS-986120 is a first-in-class, oral, highly selective, and reversible PAR4 antagonist antiplatelet agent.
- A single dose of BMS-986120 substantially reduced ex vivo thrombus formation in healthy volunteers under conditions of high shear stress, driven by a reduction in platelet-rich thrombus deposition.
- Our results suggest PAR4 antagonism with BMS-986120 holds major promise as a novel antiplatelet strategy because of the potential for a wider therapeutic window in terms of antithrombotic efficacy and bleeding risk.

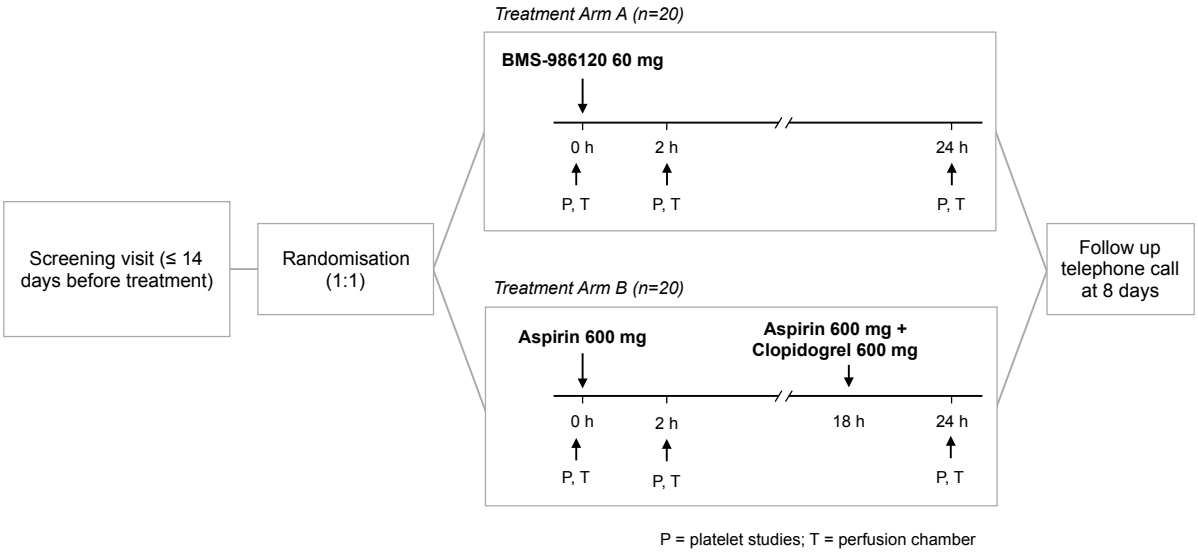

Figure I. Schematic overview of study design.

Table I. Safety Assessments

Test variable	BMS-986120 (n=20)			Aspirin ± Clopidogrel (n=20)		
	0 hour	*2 hour*	*24 hour*	*0 hour*	*ASA*	*ASA + Clop*
ALT, U/L (SD)	19.0 (6.1)	18.7 (6.2)	17.8 (6.4)**	26.3 (14.8)	25.9 (14.2)	25.4 (13.3)
AST, U/L (SD)	20.7 (4.6)	20.9 (5.7)	18.0 (3.6)***	23.6 (7.4)	23.4 (6.8)	21.0 (4.5)**
Bilirubin, mg/dL (SD)	0.70 (0.3)	0.80 (0.3)*	0.62 (0.3)	0.76 (0.2)	0.73 (0.2)	0.66 (0.2)**
Creatine Kinase, U/L (SD)	197 (190)	187 (188)	115 (83)***	186 (144)	171 (128)	108 (61)***
Urea, mmol/L (SD)	5.1 (0.96)	4.6 (0.76)**	4.1 (0.77)***	5.0 (1.0)	4.7 (0.93)	4.5 (0.98)**
Creatinine, mg/dL (SD)	0.83 (0.06)	0.81 (0.09)	0.82 (0.08)	0.89 (0.11)	0.86 (0.09)	0.86 (0.10)
Haemoglobin, g/dL (SD)	14.2 (0.4)	14.2 (0.4)	14.2 (0.3)	14.6 (0.8)	14.4 (0.8)	14.3 (1.3)
Platelet count, $x10^9$ c/L (SD)	229 (45)	227 (46)	228 (46)	221 (49)	220 (50)	216 (53)
APTT, seconds (SD)	30.9 (2.1)	30.3 (2.7)	30.4 (2.1)	30.8 (2.5)	30.3 (3.3)	30.1 (2.9)
PT, seconds (SD)	12.3 (0.9)	12.3 (0.9)	12.1 (0.8)	11.9 (0.7)	12.3 (0.7)**	12.1 (0.7)
QTCf interval, milliseconds (SD)	405 (11.8)	411 (17.9)	405 (15.9)	401 (16.3)	402 (13.4)	399 (10.8)

Data shown are the adjusted means with standard deviation. All significant differences (Least Significance Difference test) versus 0 hour are presented: * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: ASA, aspirin; Clo, clopidogrel. ALT, alanine transaminase; ASA, Aspirin; AST, aspartate transaminase; APTT, activated partial thromboplastin time; Clop, Clopidogrel; PT, prothrombin time; QTCf, QTC interval corrected for heart rate by Fridericia's formula; and SD, standard deviation

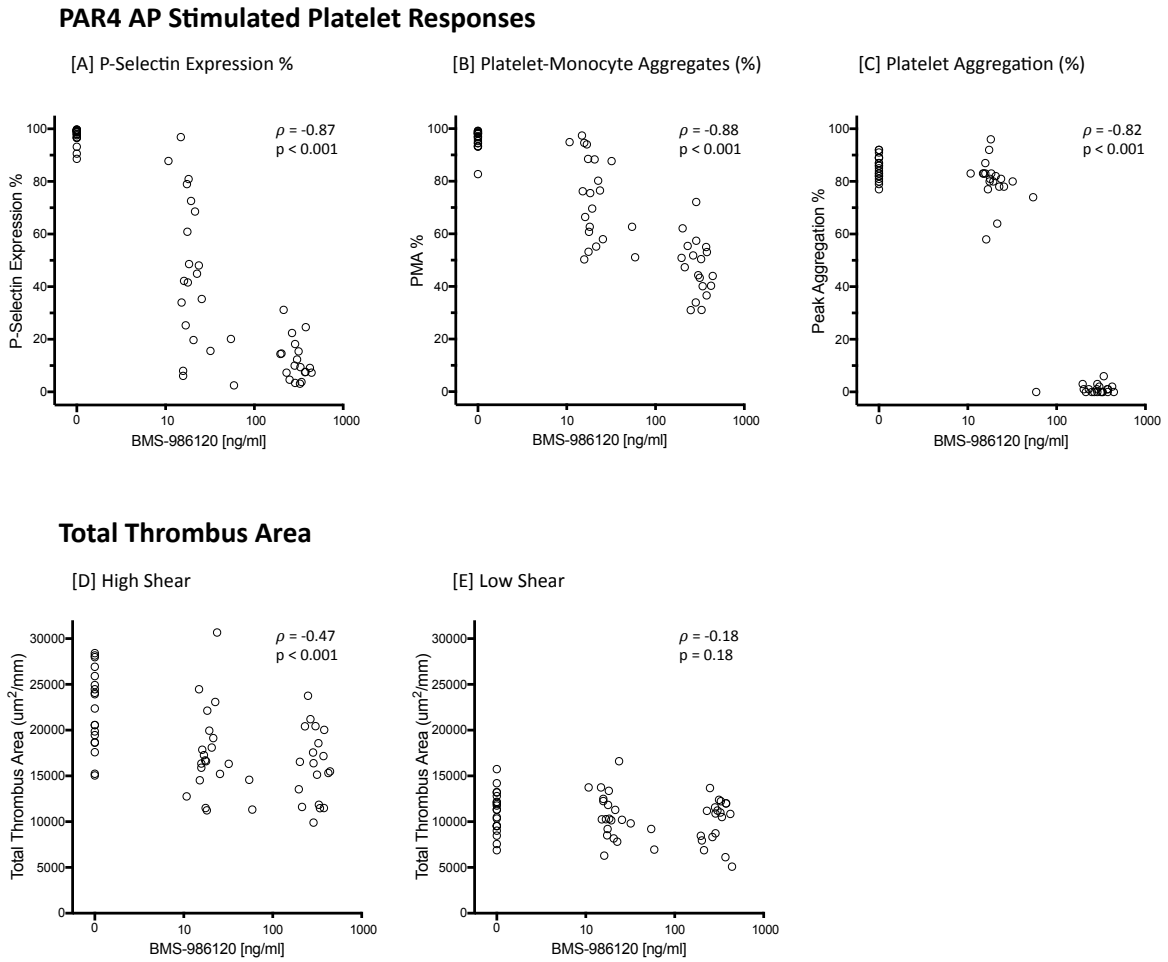

Figure II. Correlations between plasma concentrations of BMS-986120 and selected study endpoints.

Data shown are scatter plots of [A] PAR4 AP stimulated p-selectin expression, [B] PAR4 AP stimulated platelet-monocyte aggregates, [C] PAR4 AP stimulated platelet aggregation, [D] total thrombus area at high shear, and [E] total thrombus at low shear in volunteers randomised to BMS-986120. Correlation coefficients (ρ) and p-values were determined by Spearman's rank-order correlation.

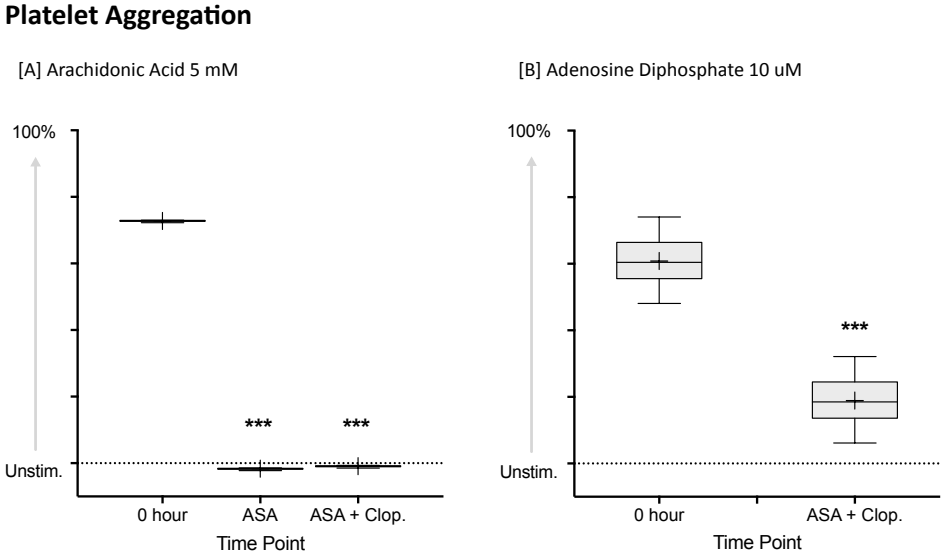

Figure III. Box plots of platelet aggregation in response to (A) arachidonic acid [5 mM] and (B) adenosine diphosphate [10 μM] in volunteers randomised to aspirin ± clopidogrel.

Data shown are the adjusted means (+) normalised to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (Least Significance Difference test) versus 0 hour are shown above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

SUPPLEMENTAL MATERIAL

Methods

Study Design

This was a phase I parallel group (n=20 per treatment arm) prospective randomized open-label blinded endpoint (PROBE) trial conducted at a single site (Clinical Research Facility, Royal Infirmary of Edinburgh, Scotland) between the 23rd September 2015 and 1st March 2016. *Ex vivo* platelet aggregation, platelet activation and thrombus formation were measured at 0 (pre-treatment), 2 and 24 h after oral administration of (a) 60 mg of BMS-986120 or (b) 600 mg aspirin with a second 600 mg aspirin and 600 mg clopidogrel at 18 h (Figure I in the online-only Data Supplement). Aspirin ± clopidogrel were included as a positive control and assay validation tool.

The trial was sponsored by Bristol-Myers Squibb (BMS) and was designed collaboratively with the host academic center. The study was approved by the local research ethics committee, conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Clinical Trial Authorization was provided by the Medicines and Healthcare products Regulatory Authority (MHRA) of the United Kingdom.

Study End-Points

The primary outcome was the effect of BMS-986120 on total thrombus area as compared to pre-treatment. Secondary and exploratory end-points included the effect of study drug (BMS-986120 or aspirin ± clopidogrel) on platelet aggregation, p-selectin expression, platelet-monocyte aggregates, and thrombus composition (platelet- and fibrin-rich thrombus area).

Study Population

Healthy non-smoking male and female volunteers between the ages of 18 and 65 years (inclusive) and with a body mass index (BMI) of 18 to 32 kg/m^2 underwent screening including detailed medical history, physical examination, laboratory blood tests, urinalysis and 12-lead electrocardiogram (ECG). Exclusion criteria were women of child-bearing potential and any clinically significant coexisting condition including hypertension, hyperlipidemia, diabetes mellitus, gastrointestinal disease that could affect drug absorption, coagulopathy, recent infective or inflammatory condition, known liver disease or screening blood tests indicative of renal, liver, clotting, thyroid or hematological abnormality. Volunteers must not have been taking any prescription medications for 4 weeks, over-the-counter medications, herbal supplements and vitamins for 1 week, and alcohol or caffeine containing products for 72 hours prior to and for the duration of the study.

Dose Selection

BMS-986120 is a competitive, reversible inhibitor of PAR4 AP induced platelet aggregation (K_{on}=0.12 ± 0.043 $nM^{-1}min^{-1}$, K_{off}=0.0082 ± 0.0016 min^{-1}, K_d=0.098 ± 0.016 nM). In cynomolgus monkeys, BMS-986120 demonstrated dose-dependent (0.2-1.0 mg/kg) preservation of carotid arterial flow following

electrolytic injury at the expense of a slight increase in mesenteric and kidney bleeding times[1]. In a single ascending (0.5-180 mg) and multiple ascending dose study (2.5-100 mg daily for up to 14 days) in healthy volunteers, BMS-986120 was found to be safe and well tolerated with complete and reversible inhibition of PAR4 agonist peptide (AP) stimulated platelet aggregation at ≥10 mg daily[2]. On the basis of these studies, a 60 mg dose was selected for the present phase 1 trial as this was calculated to be sufficient to inhibit platelet aggregation 2 h post dose and would be at the edge of a potential pharmacodynamic effect at 24 h. This would allow for "dose ranging" with a single dose of BMS-986120 whilst remaining well within the safety experience.

Doses of aspirin (600 mg) and clopidogrel (600 mg) were selected to reflect the maximal antithrombotic efficacy that might reasonably be expected in clinical practice following initiation of these antiplatelet agents in an acute setting.

Study Outcome Measures

Blood Sampling and Agonists

All blood samples for pharmacodynamic and pharmacokinetic assessments were drawn uncuffed through a 17G cannula in the ante-cubital fossa. For each time point, the first 2.5 mL of blood was discarded. PAR1 and PAR4 APs (SFLLRN and A-Phe(4-F)-PGWLVKNG respectively) were provided by Bristol-Myers Squibb (Princeton, USA), adenosine diphosphate (ADP) by Sigma-Aldrich (Gillingham, UK) and arachidonic acid (AA) by Alpha Laboratories (Eastleigh, UK).

Pharmacokinetic Assessment

Plasma concentrations of BMS-986120 were determined at 0, 1, 2, 3, 4, 5, 6, 9 and 24 h post treatment using a validated liquid chromatography tandem-mass spectrometry (LC-MS/MS) method with a lower limit of quantification (LLQ) of 0.250 ng/mL, with an accuracy coefficient of variation of <5 % and precision (intra- and inter-assay) coefficients of variation of <10 %. Blood samples were collected into 3 mL K_2EDTA vacutainers (Becton-Dickinson, Cowley, UK) and placed on wet ice. Within 1 h of collection, samples were centrifuged at 1200 *g* (2-8 °C) for 10 min. Plasma was decanted and stored at -20 °C before analysis.

Platelet Aggregation

Platelet aggregation was assessed by optical aggregometry (PAP-8E; Bio/Data Corp, Horsham, PA, USA) of platelet-rich plasma (PRP). To obtain PRP, 18 mL of blood was collected, mixed immediately with 2 mL of 3.8 % sodium citrate, and then centrifuged at 300 *g* (room temperature) for 15 min. For reference, 2 mL of PRP was centrifuged at 5500 *g* for 6 min to obtain platelet-poor plasma (PPP). All samples were allowed to equilibrate for 10 min (37 °C) after the addition of agonist and the peak aggregation recorded.

Platelet Activation

Platelet p-selectin expression and platelet-monocyte aggregates were determined by flow cytometry. Blood (5 mL) was collected into 50 μL of 75

mM D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Enzo Life Sciences, Exeter, UK) then immediately aliquoted into eppendorfs pre-filled with or without agonist and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for 20 min at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within 24 h using a FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

Ex Vivo Perfusion Model of Thrombosis

The effect of study compound on ex vivo thrombus formation was assessed using the Badimon perfusion chamber as previously described[3]. In brief, a pump was used to draw native (unanticoagulated) blood directly from an antecubital vein through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. Each chamber contained a strip of porcine aorta from which the intima and a thin layer of media had been removed. The ultrastructure of porcine aorta closely resembles that of human arteries and by removing the intima and a thin layer of media, blood is exposed to collagen fibres (type I and type III), proteoglycans, basement membrane, elastin, smooth muscle cells and other constituents common to an atherosclerotic plaque[4-8]. Rheological conditions in the first chamber were set to simulate those of patent medium-sized coronary arteries (inner lumen diameter, 2.0 mm; vessel wall shear rate, 212 s^{-1}; mean blood velocity, 5.3 cm/s; Reynolds number: 30), whereas those in the second and third chambers simulate those of mild to moderately stenosed coronary arteries (inner lumen diameter, 1.0 mm; vessel wall shear rate: 1690 s^{-1}; mean blood velocity, 21.2 cm/s; Reynolds number: 60). Shear conditions at the vessel wall were calculated from the expression for shear rate given for a Newtonian fluid in tube flow[9,10]. Each study lasted for exactly 5 min during which flow was maintained at a constant rate of 10 mL/min. All studies were performed using the same perfusion chamber and by the same operator.

Histomorphometric Analysis

As thrombus forms along the entire length of the exposed porcine aortic strip, the mean transverse cross-sectional area gives a reliable reflection of total thrombus[6]. Following fixation, the proximal and distal 1 mm of the exposed substrate were discarded and the remainder cut into eight segments. Individual segments were then embedded in paraffin wax from which 4-μm sections were prepared for histomorphometric analysis.

To detect total thrombus area, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (Leica Microsystems GmbH, Wetzlar, Germany) for 5 minutes. Sections were then incubated at room temperature for 1 hour with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL, Dako, Glostrup, Denmark; Cat. No. A0080) and monoclonal mouse anti-human CD61 antibody (1.28 μg/ml, Dako; Cat. No. M0753). Antigen visualisation was performed using a Bond Polymer refine detection kit

(Leica Microsystems GmbH) and treatment with 3,3'-diaminobenzidine substrate chromogen (66 mM, Dako). Finally, sections were counterstained with a modified Masson's trichrome (hematoxylin and sirius red 0.1 %; Figure II in the online-only Data Supplement).

To examine the effect of study drug(s) on fibrin-rich and platelet-rich thrombus formation, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (VWR, Radnor, PA, USA) for 10 min and non-specific binding blocked using 20 % normal goat serum (Biosera, Nuaille, France) in Tris-Buffered Saline with 0.01% Tween (TBST)). Sections were then incubated with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/ml) to detect fibrin and CD61 monoclonal mouse anti-human antibody (0.32 μg/ml) to detect platelets. Following TBST washes, goat anti-rabbit peroxidase (1:500; Abcam, Cambridge, UK) was applied and the presence of antigen visualized with Tyramide Cy3 (1:50; Perkin Elmer, Boston, MA, USA; Cat. no. NEL744B001KT) and FITC (1:50; Perkin Elmer, Waltham, MA, USA; Cat. no. NEL741B001KT) before nuclear counterstaining with DAPI (5 μg/ml; Sigma-Aldrich; Cat. No. D9542).

Prior to the first experimental sample, non-specific binding of the primary antibodies was excluded using tissue negative controls (perfusion chamber porcine sections exposed to saline rather than blood). To ensure staining for platelets and fibrin(ogen) antigen was the result of detection of the antigen, secondary antibody controls (with the primary antibody absent) were run in parallel for each volunteer. No labelling was observed.

A semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany) and image analysis software (Definiens, Munich, Germany) were used by a blinded operator to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established to detect total thrombus, platelet and fibrin area.

Safety and tolerability

The primary safety end-point was the incidence of serious adverse events (SAEs) or death during and for up to 30 days post dosing. Adverse events (AEs) not meeting the SAE threshold were also recorded. All volunteers received telephone follow up on day 8. Reports of SAEs and AEs could originate from the volunteer, investigator or study personnel. Additional safety endpoints included changes in hematological and biochemical indices, hematuria (including microhematuria), alteration in the 12-lead electrocardiogram (ECG), or abnormal findings on physical examination performed at baseline, 2 and 24 h post dosing.

Statistical Analysis

Following study completion, the database was locked and all statistical analyses carried out independent of the sponsor. The demographic and baseline characteristics of volunteers are expressed as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. The effect of study drug(s) on endpoints was assessed by general

linear mixed effects models, with perfusion procedure (baseline, 2 and 24 h) as fixed effects and subjects as random effects. Mean within-subject differences for the change from baseline were generated and analysed using the Least Significance Difference (LSD) test. Prior to model fitting, total thrombus area, platelet area and fibrin area were log-transformed. Associations between plasma concentrations of BMS-986120 and study end-points were determined by Spearman's rank-order correlation (ρ). Two sided p-values of ≤0.05 were considered statistically significant. Analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, New York) and R version 3.3.1 (R Project for Statistical Computing, Vienna, Austria).

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Exosite 1 thrombin inhibition with JNJ-64179375 inhibits thrombus formation in a human translational model of thrombosis

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Aims JNJ-64179375 (hereafter JNJ-9375) is a first-in-class, highly specific, large molecule, exosite 1 thrombin inhibitor. In preclinical studies, JNJ-9375 demonstrated robust antithrombotic protection with a wider therapeutic index when compared to apixaban. The purpose of the present study was to examine for the first time the antiplatelet, anticoagulant and antithrombotic effects of JNJ-9375 in a translational model of *ex vivo* human thrombosis.

Methods and results Fifteen healthy volunteers participated in a double-blind randomized crossover study of JNJ-9375 (2.5, 25, and 250 μg/mL), bivalirudin (6 μg/mL; positive control), and matched placebo. Coagulation, platelet activation, and thrombus formation were determined using coagulation assays, flow cytometry, and an *ex vivo* perfusion chamber, respectively.
JNJ-9375 caused concentration-dependent prolongation of all measures of blood coagulation (prothrombin time, activated partial thromboplastin time, and thrombin time; $P < 0.001$ for all) and agonist selective inhibition of thrombin (0.1 U/mL) stimulated platelet p-selectin expression ($P < 0.001$) and platelet-monocyte aggregates ($P = 0.002$). Compared to placebo, JNJ-9375 (250 μg/mL) reduced mean total thrombus area by 41.1% (95% confidence intervals 22.3 to 55.3%; $P < 0.001$) at low shear and 32.3% (4.9 to 51.8%; $P = 0.025$) at high shear. Under both shear conditions, there was a dose-dependent decrease in fibrin-rich thrombus ($P < 0.001$ for both) but not platelet-rich thrombus ($P =$ ns for both).

Conclusion Exosite 1 inhibition with JNJ-9375 caused prolongation of blood coagulation, selective inhibition of thrombin-mediated platelet activation, and reductions in *ex vivo* thrombosis driven by a decrease in fibrin-rich thrombus formation. JNJ-9375 represents a novel class of anticoagulant with potential therapeutic applications.

Keywords JNJ-9375 • Exosite 1 thrombin • Thrombosis • Novel • Anticoagulant

Introduction

The coagulation cascade plays a central role in thrombosis and the pathophysiology of thrombo-embolic events, the leading cause of global mortality.[1] Anticoagulants are of proven benefit in a wide range of thrombo-embolic disorders, but despite recent improvements, important limitations persist. All the currently licensed agents, including direct oral anticoagulants (DOACs), act to either inhibit thrombin generation or block the active site of the protease directly.[2] Consequently, they provide broad inhibition of all thrombin activity, which although efficacious, invariably fails to discriminate between protease interactions relating to thrombosis and those essential to haemostasis. Treatment related bleeding remains a major concern and for many patients this leads to dosing restrictions or exclusion from anticoagulation altogether.[3–9]

JNJ-64179375 (hereafter JNJ-9375) is a first-in-class, recombinant, fully human, IgG4 monoclonal antibody anticoagulant that binds reversibly and with high affinity and specificity to the exosite 1 region of thrombin.[10] Exosite 1 is a positively charged domain on the surface of thrombin

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that together with exosite 2 serves to regulate enzymatic activity of the protease by providing an initial binding site for substrates, co-factors, and inhibitors.[11–13] JNJ-9375 therefore acts to inhibit the interaction of thrombin with its exosite 1 substrates, which include fibrinogen, but retains function of both the active site and exosite 2.[10] This capacity to inhibit fibrinogen binding while preserving other (non-exosite 1) protease interactions offers the potential for a wider therapeutic index, and in preclinical animal models JNJ-9375 was associated with substantially less bleeding when compared to apixaban at doses of equivalent antithrombotic efficacy.[10] In the present study, we sought to examine for the first time the anticoagulant and antithrombotic effects of exosite 1 thrombin inhibition with JNJ-9375 in human blood using a translational model of *ex vivo* thrombosis.

Methods

Study population

Healthy non-smoking male and female volunteers aged between 18 and 45 years (inclusive) with a body mass index (BMI) of 18–35 kg/m^2 were enrolled in this study. All volunteers underwent a detailed screening assessment for eligibility. Exclusion criteria included women who were pregnant or still lactating, or any clinically significant coexisting condition including hypertension, hyperlipidaemia, diabetes mellitus, cardiovascular disease, recent infective or inflammatory condition, coagulopathy, known liver disease or screening blood tests indicative of renal, liver, clotting, thyroid, or haematological abnormality. Volunteers were not permitted to take any prescription or non-prescription medication (including acetylsalicylic acid, paracetamol, vitamins, and herbal supplements) within 14 days of an experimental visit. Prior to each visit, volunteers must have abstained from alcohol for 24 h and food including caffeine-containing products for 8 h. Informed written consent was obtained from all volunteers before enrolment. The study was approved by the local research ethics committee (reference 16-HV-025) and conducted in accordance with the Declaration of Helsinki.

Study design

This was a double-blind randomized controlled five-way crossover study conducted at a single site (Clinical Research Facility, Royal Infirmary of Edinburgh, Scotland) between the 24 May 2016 and 1 July 2016. Study measures were performed during extracorporeal infusion of JNJ-9375 (estimated final concentration of 2.5, 25, and 250 μg/mL), bivalirudin (positive control; estimated final concentration of 6 μg/mL; The Medicines Company, Abingdon, UK) at a dose equivalent to recommendations at the time of percutaneous coronary intervention (PCI), and matched placebo [10 mM phosphate, 8.5% (w/v) sucrose, 0.04% (w/v) polysorbate 20, 10 μg/mL ethylenediaminetetraacetic acid, pH 7.1; Janssen Research and Development] upstream of the perfusion chambers. Three perfusion chamber studies were performed at the first experimental visit and two perfusion chamber studies at the second experimental visit.

Study objectives

The primary objective was to assess the relationship of JNJ-9375 dose concentrations to *ex vivo* thrombus formation under conditions of both low and high shear stress, and to compare these effects with placebo under the same rheological conditions. Bivalirudin, which blocks both exosite 1 and the active site of thrombin, was used as a positive control. Secondary objectives included a similar comparison of compound effects on platelet activation, markers of coagulation, and the fibrin and platelet components of thrombus formation. Finally, correlations between measured chamber concentrations of study drug and pharmacodynamic endpoints were explored.

Perfusion chamber experiment

Thrombus formation was assessed using the Badimon chamber, a well-validated perfusion model for measuring the effect of study drugs on *ex vivo* human thrombus formation.[14–21] In brief, a pump was used to draw native (unanticoagulated) blood from an antecubital vein directly through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. Each chamber contained a strip of porcine aorta from which the intima and a thin layer of media had been removed. Rheological conditions in the first chamber were set to simulate those of patent medium-sized arteries (inner lumen diameter 2.0 mm; vessel wall shear rate 212 s^{-1}; mean blood velocity 5.3 cm/s; Reynolds number 30), whereas those in the second and third chambers were set to simulate those of mild to moderately stenosed coronary arteries (inner lumen diameter 1.0 mm; vessel wall shear rate 1690 s^{-1}; mean blood velocity 21.2 cm/s; Reynolds number 60). Shear conditions at the vessel wall were calculated from the theoretical expression for shear rate given for a Newtonian fluid in tube flow.[22,23] Each study lasted for exactly 5 min during which flow was maintained at a constant rate of 10 mL/min. All studies were performed using the same perfusion chamber and by the same operator.

Study outcome measures

Chamber concentrations of study drug

Blood samples for determination of serum JNJ-9375 and plasma bivalirudin concentrations were taken immediately distal to the perfusion chamber into 3.5 mL serum gel and 2.7 mL sodium citrate (3.2%) tubes (Becton-Dickinson, Cowley, UK). JNJ-9375 samples were allowed to clot for 30 min then centrifuged at 1500 *g* (20°C) for 20 min. Bivalirudin samples were centrifuged at 1500 *g* (15°C) for 15 min within 1 h of collection. Samples were then aliquoted and stored immediately at -70°C before analysis. Concentrations of JNJ-9375 were determined by electrochemiluminescence using the Meso Scale Discovery platform and plate reader (Rockville, MD, USA). JNJ-9375 concentrations were regressed from the standard curve in Watson LIMS (version 7.4.1, Thermo, PA, USA) using a five-parameter logistic regression model with 1/Y^2 weighting.

Coagulations assays

Blood samples for coagulations assays [prothrombin time, activated partial thromboplastin time, and thrombin time (undiluted and diluted)] were collected immediately distal to the final perfusion chamber into 4.5 mL sodium citrate (0.38% final v/v) tubes (Becton-Dickinson). Samples were centrifuged at 1500 *g* (15°C) for 20 min within 1 h of collection. Plasma was then aliquoted and stored immediately at -70°C before analysis using a STA-Compact-Max analyser (Stago, Parsippany, NJ, USA). The following reagents were used, for prothrombin time, STA-Neoplastine CI Plus, for activated partial thromboplastin time, STA-PTT Automate, and for thrombin time, STA-Thrombin.

Platelet activation

Platelet p-selectin expression and platelet-monocyte aggregates are sensitive markers of *in vivo* platelet activation.[24–26] Blood (2.7 mL) was collected immediately distal to the final perfusion chamber into tubes containing 0.3 mL of 3.8% sodium citrate and Pefabloc FG (final concentration 1.5 mg/mL; Quadratech Diagnostics, Surrey, UK). After 5 min, samples were aliquoted into Eppendorfs pre-filled with or without agonist [adenosine diphosphate (ADP) 20 μM, Sigma-Aldrich, Gillingham, UK; human alpha thrombin 0.1 U/mL, Enzyme Research Laboratories, Swansea, UK] and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P, and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for 15 min at room temperature before fixing with 1% paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within 24 h using a

Table 1 Effects of JNJ-64179375 on indices of thrombosis and coagulation

	Placebo	JNJ-9375 (2.5 μg/mL)	JNJ-9375 (25 μg/mL)	JNJ-9375 (250 μg/mL)	Bivalirudin (6 μg/mL)
PT (s)	13.7 (10.5, 16.9)	13.9 (10.7, 17.1)	15.8 (12.6, 19.0)	30.0 (26.8, 33.2)	36.6 (33.4, 39.8)
APTT (s)	28.9 (23.3, 34.6)	31.4 (25.7, 37.0)	41.6 (35.9, 47.3)	63.5 (57.8, 69.2)	91.5 (85.8, 97.2)
TT (s)	15.6 (-12.7, 43.8)	24.9 (-3.3, 53.2)	80.9 (52.6, 109.2)	245.6 (217.3, 273.9)	351.2 (323.0, 379.5)
Dilute TT (s)	<LLOQ	<LLOQ	<LLOQ	151.5 (126.5, 176.6)	>501[a]
P-Selectin GMFI					
Unstimulated	4.6 (3.6, 5.6)	3.9 (2.9, 4.9)	3.7 (2.7, 4.7)	3.9 (2.9, 4.9)	3.2 (2.2, 4.2)
ADP 20 μM	17.8 (11.6, 24.0)	12.1 (6.0, 18.1)	17.8 (11.9, 23.8)	15.6 (9.7, 21.6)	15.3 (9.4, 21.2)
Thrombin 0.1 U/mL	161.6 (100.3, 222.8)	86.5 (28.0, 145.0)	7.8 (-53.5, 69.1)	1.6 (-62.8, 65.9)	-1.6 (-59.6, 56.3)
PMA GMFI					
Unstimulated	33.0 (20.6, 45.4)	35.6 (24.0, 47.3)	27.6 (15.9, 39.3)	25.0 (13.3, 36.7)	25.0 (13.3, 36.7)
ADP 20 μM	48.9 (28.2, 69.5)	54.9 (35.8, 73.9)	48.5 (30.1, 67.0)	50.1 (31.7, 68.6)	46.4 (28.0, 64.8)
Thrombin 0.1 U/mL	401.0 (180.3, 621.6)	414.6 (193.3, 635.8)	175.3 (-45.7, 396.3)	120.7 (-99.5, 340.9)	85.0 (-123.2, 293.3)
Total thrombus area (μm^2/mm)					
Low shear	9571 (7669, 11 945)	10 283 (8239, 12 834)	8936 (7161, 11 153)	5640 (4519, 7039)	3318 (2659, 4141)
High shear	14 367 (10 734, 19 229)	12961 (9684, 17 347)	13 898 (10 384, 18 602)	9729 (7269, 13 022)	6312 (4716, 8448)
Platelet-rich thrombus area (μm^2/mm)					
Low shear	1255 (834, 1889)	1610 (1055, 2456)	1117 (742, 1681)	1200 (798, 1806)	832 (545, 1269)
High shear	7302 (4790, 11 131)	5844 (3834, 8909)	6405 (4202, 9763)	5463 (3584, 8327)	4111 (2697, 6267)
Fibrin-rich thrombus area (μm^2/mm)					
Low shear	10 349 (7535, 14 212)	10 634 (7651, 14 782)	9865 (7183, 13 547)	4190 (3051, 5755)	1162 (836, 1616)
High shear	9598 (7997, 11 521)	9176 (7645, 11 014)	8100 (6749, 9722)	4625 (3854, 5552)	1776 (1480, 2132)

Data shown are means with 95% confidence intervals.
ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; GMFI, geometric mean fluorescent intensity; LLOQ, less than lower limit of quantification; PT, prothrombin time; TT, thrombin time.
[a]14 of 15 results >501 s.

FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

Thrombus formation

After each perfusion experiment, the porcine strips with attached thrombus were removed and fixed in 4% paraformaldehyde for 24 h at 4°C prior to being prepared for histological analysis. As thrombus forms longitudinally along the entire length of the exposed porcine aortic strip, the mean cross-sectional area gives a reliable representation of total thrombus formation.[27] Following fixation, the proximal and distal 1 mm of the exposed substrate were discarded and the remainder cut into eight segments. Segments were embedded in paraffin wax and 4-μm sections prepared.

To detect total thrombus area, endogenous hydrogen peroxide activity was blocked using 3% hydrogen peroxide solution (Leica Microsystems GmbH, Wetzlar, Germany) for 5 min. Sections were then incubated at room temperature for 1 h with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL, Dako, Glostrup, Denmark; Cat. No. A0080) and monoclonal mouse anti-human CD61 antibody (1.28 μg/mL, Dako; Cat. No. M0753). Antigen visualization was performed using a Bond Polymer refine detection kit (Leica Microsystems GmbH) and treatment with 3,3′-diaminobenzidine substrate chromogen (66 mM, Dako). Finally, sections were counterstained with a modified Masson's trichrome (haematoxylin and sirius red 0.1%).

To examine the effect of study drug(s) on fibrin-rich and platelet-rich thrombus formation, endogenous hydrogen peroxide activity was blocked using 3% hydrogen peroxide solution (VWR, Radnor, PA, USA) for 10 min and non-specific binding blocked using 20% normal goat serum (Biosera, Nuaille, France) in Tris-Buffered Saline with 0.01% Tween (TBST). Sections were then incubated with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL) to detect fibrin and CD61 monoclonal mouse anti-human antibody (0.32 μg/mL) to detect platelets. Following TBST washes, goat anti-rabbit peroxidase (1:500; Abcam, Cambridge, UK) was applied and the presence of antigen visualized with Tyramide Cy3 (1:50; Perkin Elmer, Boston, MA, USA; Cat. no. NEL744B001KT) and FITC (1:50; Perkin Elmer, Waltham, MA, USA; Cat. no. NEL741B001KT) before nuclear counterstaining with 4′,6-diamidino-2-phenylindole (5 μg/mL; Sigma-Aldrich; Cat. No. D9542).

A semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany) and image analysis software (Definiens, Munich, Germany) were used by a blinded operator to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established to detect total thrombus area, fibrin-rich thrombus area, and platelet-rich thrombus area.

Statistical analysis

After study completion, the database was locked and all statistical analyses carried out by an independent statistician. Categorical variables are expressed as percentages, and continuous variables are expressed as mean ± standard deviation (SD). The effects of study compounds on study endpoints were assessed by general linear mixed effect models with period and study compound as fixed effects, subjects as random effects. Chamber endpoints were log-transformed and assessed separately by shear rate (low and high). From the models, point and interval estimates for means and mean differences vs. placebo (absolute and %) were generated and analysed using the Least Significance Difference (LSD) test. The correlation between plasma JNJ-9375 concentrations and study endpoints were determined by Pearson's (r) or Spearman's rank-order correlation (ρ) as appropriate. Two-sided P-values of ≤ 0.05 were

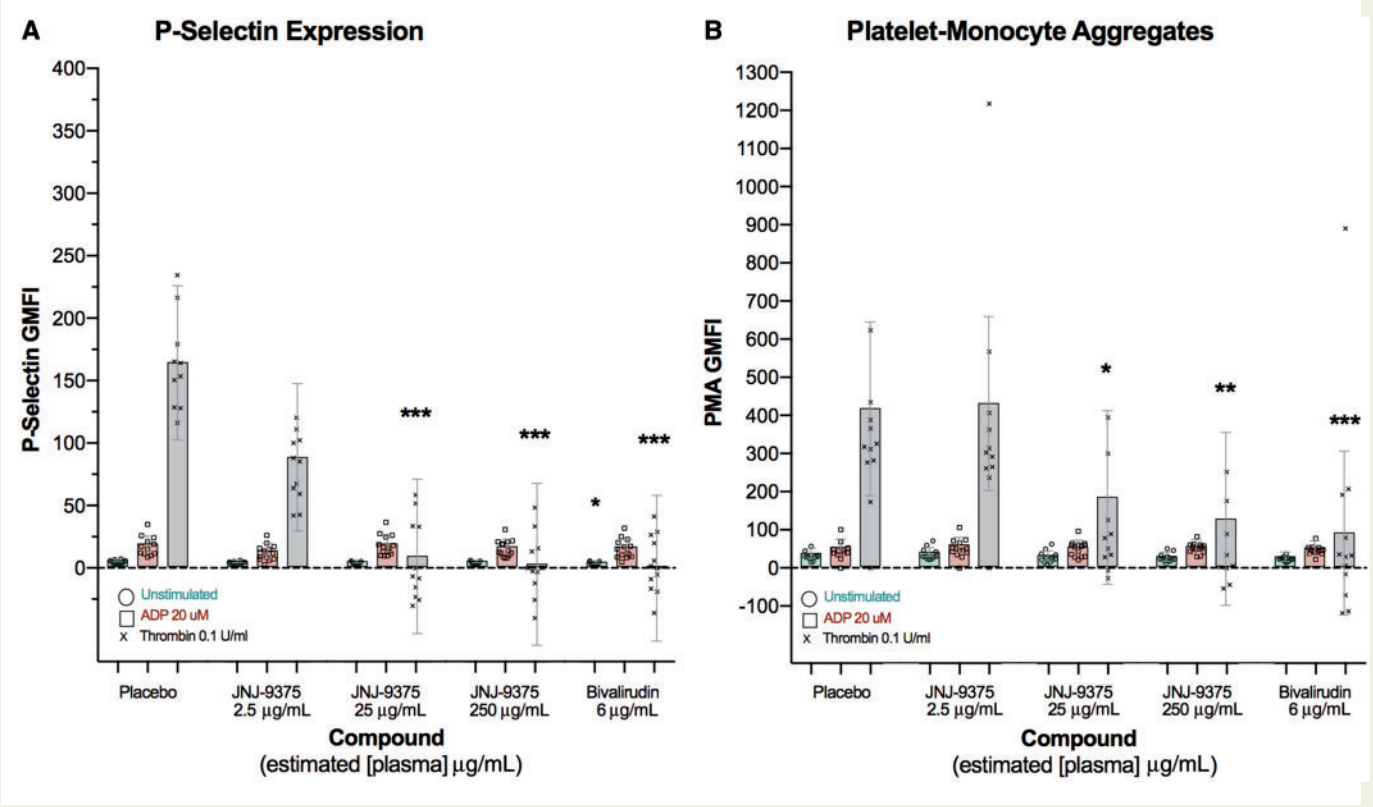

Figure 1 The effect of study compound on *ex vivo* platelet activation. Extra-corporeal administration of JNJ-9375 inhibited thrombin-simulated (*A*) p-selectin expression and (*B*) platelet-monocyte aggregates in a dose-dependent manner but had no effect on ADP activity. Data shown are the adjusted means (±95% confidence intervals) and individual points. Statistical comparisons (Least Significance Difference test) vs. placebo are represented above each plot: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ADP, adenosine diphosphate; GMFI, geometric mean fluorescent intensity; PMA, platelet-monocyte aggregates.

considered statistically significant. All statistical calculations were performed using SAS version 9.4.

Results

All 15 enrolled volunteers (10 male) completed the study in full, with no safety concerns. Mean age of the volunteers was 26 ± 5 years with a BMI of 24 ± 3 kg/m^2.

Chamber concentrations of study drug

Compound concentrations in the effluent of the perfusion chamber (JNJ-9375 1.93 ± 0.68, 22.3 ± 5.86, and 214.0 ± 20.8 µg/mL; bivalirudin 6.92 ± 11.3 µg/mL) closely matched the targeted concentrations (JNJ-9375 2.5, 25, and 250 µg/mL; bivalirudin 6 µg/mL).

Effect of JNJ-9375 on coagulation assays

JNJ-9375 caused dose-dependent prolongation of all measured blood coagulation markers, with thrombin time the most sensitive to the anticoagulant effect (*Table 1*). Pearson's correlation coefficient between chamber plasma concentrations of JNJ-9375 and coagulation assays was 0.98 for prothrombin time, 0.87 for activated partial thromboplastin time, and 0.91 for thrombin time ($P < 0.001$ for all; Supplementary material online, *Figure S1*).

Effect of JNJ-9375 on *ex vivo* platelet activation

Compared to placebo, JNJ-9375 2.5, 25, and 250 µg/mL inhibited thrombin (0.1 U/mL) stimulated platelet p-selectin expression [geometric mean fluorescent intensity (GMFI)] by 46.5% [95% confidence intervals (CI) 4.6 to 97.5%; $P = 0.07$], 95.2% (95% CI 43.2 to 147.2%; $P < 0.001$), and 99.0% (95% CI 46.1 to 151.9%; $P < 0.001$) and platelet-monocyte aggregates (GMFI) by -3.4% (95% CI -56.1 to 49.4%; $P = 0.90$), 56.3% (95% CI 2.2 to 110.4%; $P = 0.04$), and 69.9% (95% CI 16.2 to 123.6%; $P = 0.01$). Chamber plasma concentrations of JNJ-9375 correlated with both platelet p-selectin expression ($\rho = -0.83$, $P < 0.001$) and platelet-monocyte aggregates ($\rho = -0.64$, $P < 0.001$). In contrast, JNJ-9375 had no effect on ADP (20 µM) stimulated platelet activation (P = ns for all). Bivalirudin exhibited a similar selective profile (*Table 1*; *Figure 1*).

Effect of JNJ-9375 on *ex vivo* thrombus formation

Ex vivo total thrombus formation was reduced at both low and high shear stress at the 250 µg/mL concentration (*Figure 2*). Compared to placebo, JNJ-9375 (2.5, 25, and 250 µg/mL) reduced mean total thrombus area by -7.4% (95% CI -41.6 to 18.5%; $P = 0.60$), 6.6% (95% CI -23.1 to 29.2%; $P = 0.62$), and 41.1% (95% CI 22.3 to 55.3%; $P < 0.001$) at low shear and by 9.8% (95% CI -26.6 to 35.7%; $P = 0.54$), 3.3% (95% CI -35.8 to 31.1%; $P = 0.85$), and 32.3% (95% CI 4.9 to 51.8%; $P = 0.025$) at high shear.

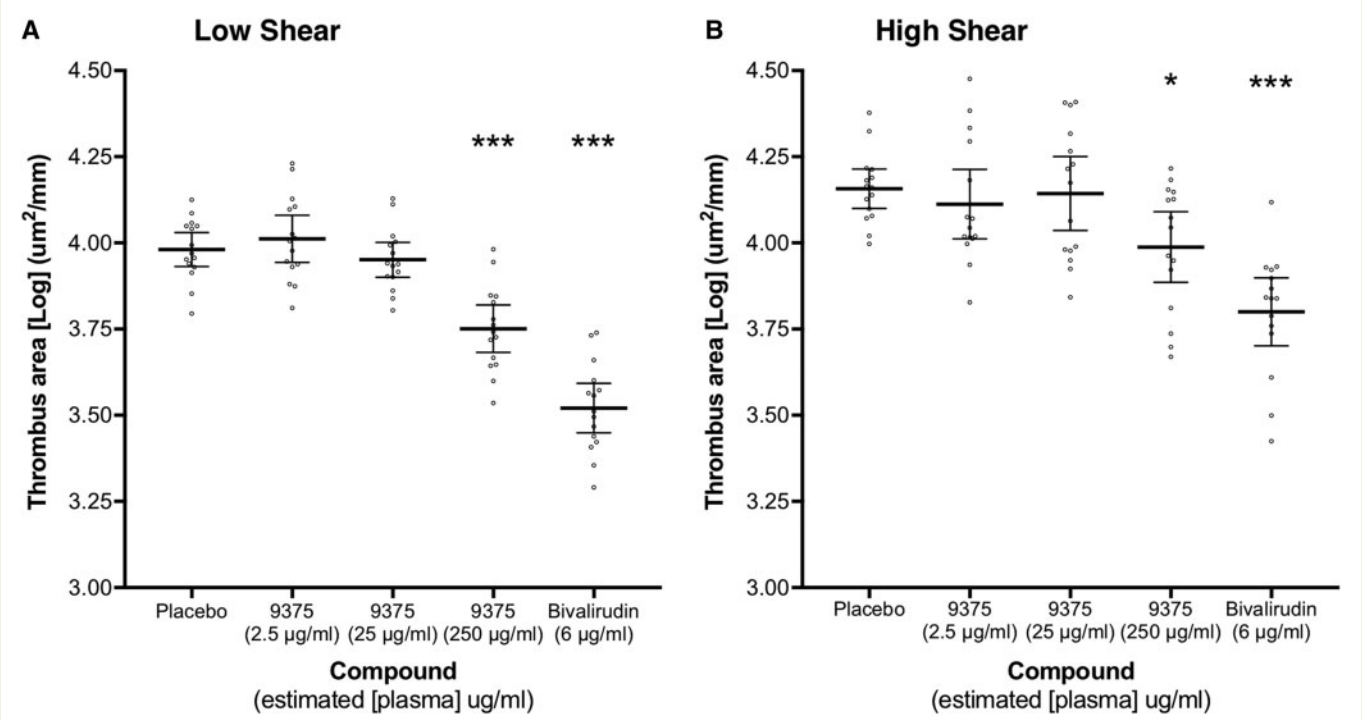

Figure 2 The effect of study compound on *ex vivo* total thrombus formation. Extra-corporeal administration of JNJ-9375 inhibited total thrombus formation in a dose-dependent manner at both (*A*) low shear stress (212 s^{-1}) and (*B*) high shear stress (1690 s^{-1}) shear stress. Data shown are the adjusted means (±95% confidence intervals) for [Log] total thrombus area (µm^2/mm) and individual points. Statistical comparisons (Least Significance Difference test) vs. placebo are represented above each plot: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 9375, JNJ-9375.

Chamber plasma concentrations of JNJ-9375 correlated with total thrombus area at low ($\rho = -0.56$, $P < 0.001$) and high ($\rho = -0.32$, $P = 0.03$) shear (Supplementary material online, *Figure S1*).

Reductions in total thrombus area were driven by a dose-dependent decrease in fibrin-rich thrombus deposition under both shear conditions (*Figure 3*). At peak dose (250 µg/mL), JNJ-9375 reduced fibrin-rich thrombus area by 59.5% (95% CI 37.8 to 73.7%; $P < 0.001$) at low shear and 51.8% (95% CI 37.7 to 62.7%; $P < 0.001$) at high shear. There was no reduction in platelet-rich thrombus area (P = ns for all). Chamber plasma concentrations of JNJ-9375 correlated with fibrin-rich thrombus area at low ($\rho = -0.66$, $P < 0.001$) and high ($\rho = -0.70$, $P < 0.001$) shear (Supplementary material online, *Figure S1*).

Effect of bivalirudin on *ex vivo* thrombus formation

Bivalirudin reduced total thrombus area at both low and high shear, also driven by a decrease in fibrin-rich thrombus formation (*Figures 2* and *3*). In contrast to JNJ-9375, there was a modest reduction ($P = 0.01$) in platelet-rich thrombus formation at high shear (*Figure 3*).

Discussion

In this double-blind randomized controlled crossover study, *ex vivo* administration of JNJ-9375, a highly specific exosite 1 thrombin inhibitor, resulted in dose-dependent prolongation of blood coagulation and selective inhibition of thrombin-stimulated platelet activation. Thrombosis was reduced under rheological conditions of both low and high shear stress, driven principally by a reduction in fibrin-rich thrombus formation. We conclude that JNJ-9375 holds promise as an anticoagulant for the prevention and treatment of thrombo-embolic events, and our results provide further insights into the role of exosite 1 in human thrombogenesis.

The outstanding challenge in anticoagulation is the development of drugs that can provide equivalent (or superior) antithrombotic efficacy but with a significantly lower bleeding risk. While the safety of JNJ-9375 has yet to be demonstrated in clinical trials, several lines of evidence indicate the potential for favourable outcomes. On a mechanistic level, selective inhibition of thrombin through exosite 1 specific antagonism is attractive because of the potential to inhibit fibrinogen binding without overly interfering with other (active site and exosite 2 dependent) protease interactions relating to haemostasis. For example, both the active site and exosite 2 are involved in catalytic feedback activation of clotting cofactors V, VIII, XI, and XIII, with deficiencies of each of these factors associated with bleeding diatheses.[28–31]

Thrombin is also a potent platelet agonist, and whilst over-aggregation may lead to pathological events, early platelet responses are central to haemostasis. Thrombin activates platelets through binding to platelet surface GPIb and protease-activated receptors 1 (PAR1) and 4 (PAR4).[32] Exosite 1 interacts with PAR1 to facilitate efficient receptor cleavage,[33] whereas PAR4 activation and GPIb binding are largely dependent on the active site and exosite 2, respectively.[34,35] In the present study, JNJ-9375 selectively inhibited thrombin-stimulated platelet activation but was not associated with a reduction in platelet deposition. This is consistent with previous reports that exosite 1 inhibition only weakly inhibits thrombin-induced platelet aggregation and does not affect

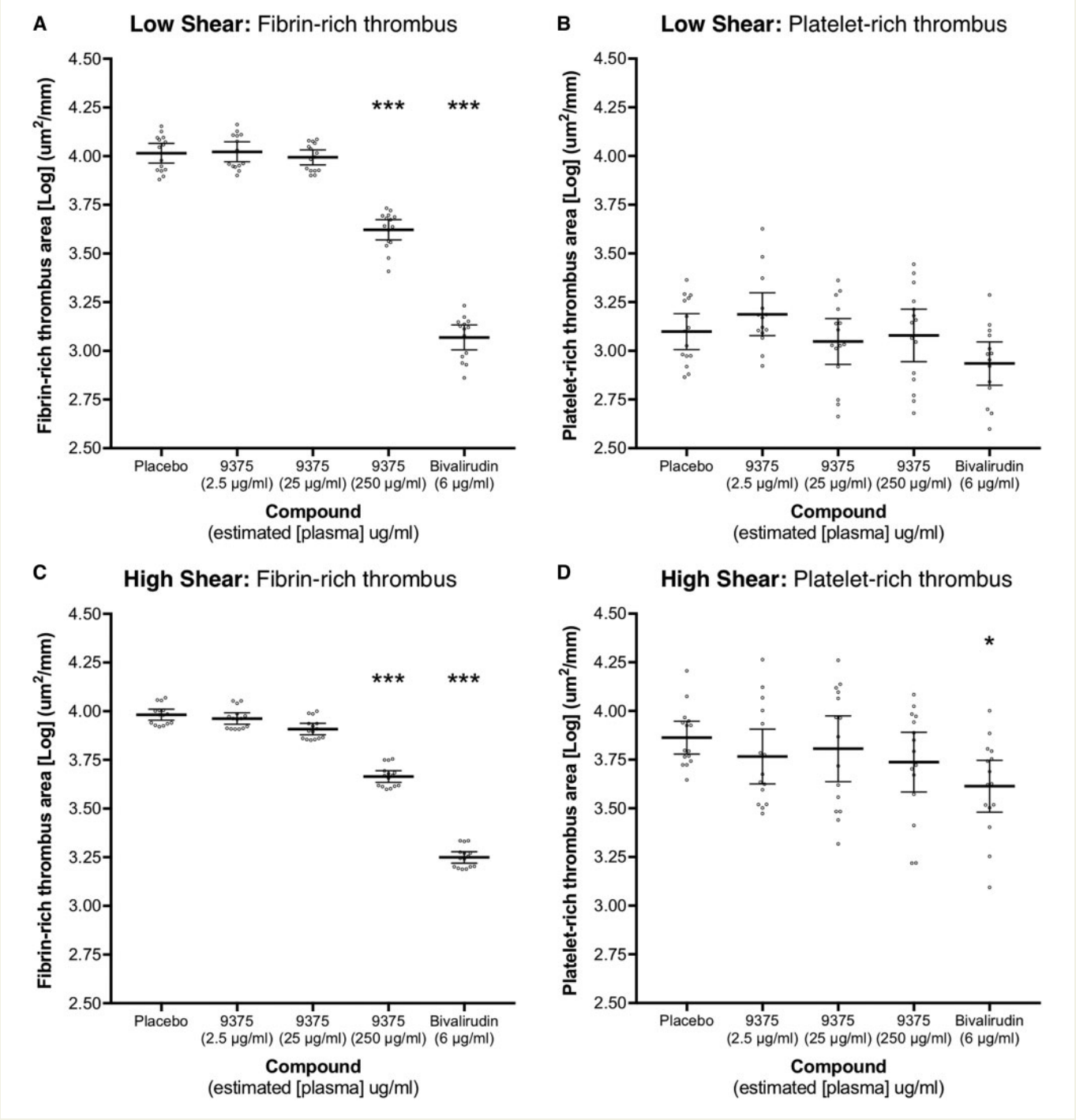

Figure 3 The effect of study compound on the components of thrombus formation. Extra-corporeal administration of JNJ-9375 inhibited fibrin-rich thrombus deposition in a dose-dependent manner at both (*A*) low shear stress (212 s^{-1}) and (*C*) high shear stress (1690 s^{-1}) shear stress, as compared to placebo. JNJ-9375 had no effect on platelet-rich thrombus deposition at either shear stress. Bivalirudin reduced fibrin-rich thrombus deposition at low and high shear stress, and platelet-rich thrombus deposition at high shear stress. Data shown are the adjusted means (±95% confidence intervals) for [Log] fibrin- or platelet-rich thrombus area (µm^2/mm) and individual points. Statistical comparisons (Least Significance Difference test) vs. placebo are represented above each plot: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 9375, JNJ-9375.

platelet collagen binding.[36,37] Collectively, these results suggest potentially favourable differential effects on thrombin-platelet responses, that could be especially useful in clinical situations where combined treatment with an antiplatelet is required.[38,39] This is speculative and requires further exploration. Future studies examining the effects of JNJ-9375 on platelet adhesion, thrombosis, and bleeding, alone and in combination with existing antiplatelet agents would be of interest.

Mechanistic evidence that exosite 1 thrombin inhibition may be associated with a low haemorrhagic potential is supported by data from animal studies of thrombosis and bleeding. Using a baboon arteriovenous

shunt model, Cadroy *et al.*[37] found that exosite 1 thrombin inhibition prevented thrombus formation but did not affect the ability to form haemostatic plugs. More recently, JNJ-9375 demonstrated a substantially wider therapeutic index when compared to apixaban in rats and cynomolgus monkeys.[10] Further insight comes from the case report of an anti-exosite 1 thrombin IgA antibody (from which JNJ-937 was subsequently synthesized to mimic) identified in a patient presenting with a large traumatic subdural haematoma and persistently abnormal clotting studies.[40] Despite evidence of intense anticoagulation (prothrombin time 40 s; activated partial thromboplastin time >240 s; thrombin time with bovine thrombin 173 s), the patient made a full recovery without surgical intervention and had no abnormal bleeding events during 8 years of follow-up.

Anticoagulants must in addition to avoiding unwanted bleeding provide clinically efficacious antithrombotic protection. Examination of the effect of exosite 1 thrombin inhibition on human thrombosis has previously been limited to studies using heparinised blood in a rabbit aortic angioplasty model[41] and cone and plate chamber.[42] This is the first description of the *ex vivo* antithrombotic effects of exosite 1 thrombin inhibition in native human blood under flow conditions. At a dose of 250 μg/mL, JNJ-9375 reduced total thrombus area by over 40% and 30% at low and high shear, respectively. Under the same conditions, high dose bivalirudin (equivalent to that used at the time of PCI) reduced thrombus formation by 65% at low shear and 56% at high shear; while in previous studies reductions of 14% with heparin (70 IU/kg bolus plus 15 IU/kg/h infusion),[19] 26–28% with oral edoxaban (60 mg)[43] and up to 40% with serial dosing of the parenteral direct factor Xa inhibitor, DX-9065a,[20] were reported. Importantly therefore, we have shown that exosite 1 thrombin antagonism alone with JNJ-9375 substantially reduces *ex vivo* human thrombus formation. Moreover, reductions were comparable (if not superior) in magnitude to the clinically approved anticoagulant edoxaban suggesting a high probability of *in vivo* antithrombotic efficacy.

JNJ-9375 resulted in dose-dependent prolongation of prothrombin time, activated partial thromboplastin time, and thrombin time. As expected, thrombin time was most sensitive to the anticoagulant effect. Although DOACs are licensed for use without the need for routine monitoring, there are clinical situations in which readily available assays to measure anticoagulant activity may be useful. Our data suggest that if indicated, thrombin time, and to a lesser extent prothrombin time and activated partial thromboplastin time, may provide a useful assay for measuring the effect of exosite 1 inhibition and JNJ-9375 activity.

Our study has some potential limitations. First, only a modest number of volunteers were studied. However, problems associated with intragroup variability were minimized by the crossover design that allowed each volunteer to serve as their own control. Second, although the exposed porcine aortic media used in the perfusion model presents many of the common constituents of an injured human blood vessel (including type I collagen), it is unlikely to contain tissue factor (TF).[44–46] Tissue factor (TF) activates the coagulation cascade and is an important contributor to thrombogenicity.[47,48] Nevertheless, this does not overly limit our model for the assessment of thrombosis because binding of blood borne circulating TF is sufficient to allow activation of the coagulation cascade and thrombus propagation.[44,45,49–51] Indeed, previous studies have confirmed that thrombus formed from human blood perfused over porcine tunica media (devoid of TF) stains heavily for TF.[44,45] Third, we used an anti-fibrin(ogen) antibody, which recognizes both fibrinogen and fibrin, to examine the fibrin component of thrombus formation. However, the chamber is perfused by saline at the end of the experiment washing away unbound cells, proteins, and other molecules, such as fibrinogen, leaving only adherent thrombus. Thus, histomorphometric quantification of fibrin-rich thrombus area is unlikely to be affected by this cross-reacting antibody, and our findings are consistent with previous studies using the same immunohistochemical approach.[52–55] Fourth, while we have shown that exosite 1 thrombin inhibition reduces fibrin-rich thrombus formation, determining how JNJ-9375 alters the dynamics of clot development, stabilization, and dissolution might further inform therapeutic potential and are areas for future exploration. Finally, the study included *ex vivo* experiments only, and thus lacked hard clinical endpoints necessary to draw any conclusions regarding the safety or efficacy of this novel anticoagulant in practice. However, given this was a translational study designed to examine for the first time the effects of exosite 1 thrombin inhibition with JNJ-9375, we felt our study design appropriate.

In conclusion, JNJ-9375, a highly specific exosite 1 thrombin inhibitor, demonstrated substantial reductions in *ex vivo* thrombosis in native human blood under flow conditions. These reductions were driven by a decrease in fibrin-rich thrombus formation and were comparable in magnitude to clinically approved anticoagulants. Our findings suggest JNJ-9375 represents a promising novel class of anticoagulant, and that further clinical studies are warranted. A Phase 2 trial comparing the safety and efficacy of JNJ-9375 to apixaban in patients undergoing elective total knee replacement surgery is currently underway (ClinicalTrials.gov: NCT03251482).

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Conflict of interest: S.J.W. and D.E.N. were supported by, and have undertaken consultancy for, Janssen. T.M.C., G.P., A.G., and M.J. are employees of Janssen.

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FORUM REVIEW ARTICLE

Effects of Diesel Exhaust on Cardiovascular Function and Oxidative Stress

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Abstract

Significance: Air pollution is a major global health concern with the particulate matter (PM) being especially associated with marked increases in cardiovascular morbidity and mortality. Diesel exhaust emissions are a particularly rich source of the smallest sizes of PM (''fine'' and ''ultrafine'') in urban environments, and it is these particles that are believed to be the most detrimental to cardiovascular health.
Recent Advances: Controlled exposure studies to diesel exhaust in animals and man demonstrate alterations in blood pressure, heart rate, vascular tone, endothelial function, myocardial perfusion, thrombosis, atherogenesis, and plaque stability. Oxidative stress has emerged as a highly plausible pathobiological mechanism by which inhalation of diesel exhaust PM leads to multiple facets of cardiovascular dysfunction.
Critical Issues: Diesel exhaust inhalation promotes oxidative stress in several biological compartments that can be directly associated with adverse cardiovascular effects.
Future Directions: Further studies with more sensitive and specific *in vivo* human markers of oxidative stress are required to determine if targeting oxidative stress pathways involved in the actions of diesel exhaust PM could be of therapeutic value. *Antioxid. Redox Signal.* 00, 000–000.

Keywords: diesel exhaust, cardiovascular function, oxidative stress

Background

Overview

In 2012, the World Health Organization estimated that air pollution was responsible for up to 7.3 million premature deaths globally, predominantly from cardiovascular disease (178). Air pollution is a major public health concern with both indoor and outdoor air pollution ranking in the top 10 risk factors for all-cause death worldwide (89). Increasing industrialization and the rapid expansion of urban environments in developing countries means that, for many, exposure to air pollutants is unavoidable.

Air pollution

Air pollution is a complex mixture of gases (*e.g.*, ozone, carbon monoxide, nitrogen oxides), volatile organic compounds, and particulate matter (PM). PM is generally divided into coarse (diameter <10 μm), fine (diameter <2.5 μm), and ultrafine (diameter <100 nm) fractions (43). Coarse PM is derived from numerous sources, including burning of natural materials, silica-based crustal particles, construction or road dust, and mechanical abrasion (*e.g.*, brake wear). Fine and ultrafine fractions of PM in urban environments chiefly arise from industrial burning of fossil fuels and from traffic-related sources (Fig. 1).

Epidemiological evidence suggests that while gaseous air pollutants, including carbon monoxide, ozone, and nitrogen oxides, can have serious health effects, the strongest associations for mortality and cardiovascular disease are with PM (21, 50, 55, 111, 117), particularly combustion-derived fine PM (100, 144). Indeed, in a prospective cohort study of 8111 adults residing across six major U.S. cities, an increased rate ratio of mortality (after adjustment for potential confounders) was found for combustion-derived fine PM, but not coarse PM or crustal fine PM (39, 84). This may reflect the ability of smaller particles to penetrate deep into the lung (44) and potentially translocate into the circulation, as well as present a larger reactive surface area with a host of adsorbed surface chemicals that include redox-active organic compounds and

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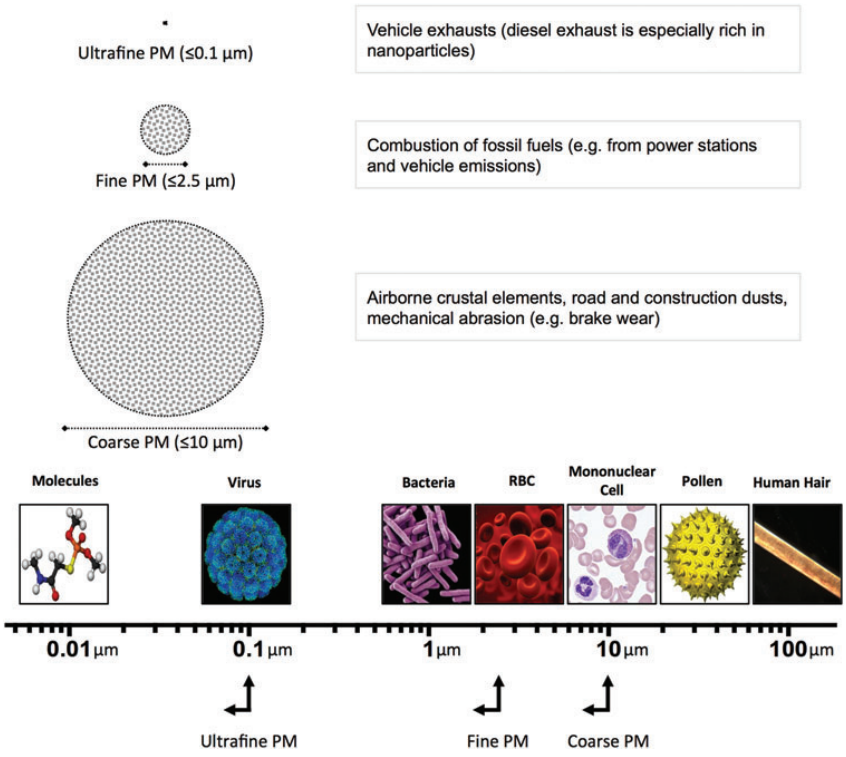

FIG. 1. The relative size of the three main fractions of PM and the principle sources in urban environments. Images from open source library: www.commons.wikimedia.org (virus, human hair); www.flickr.com (bacteria, mononuclear cell); www.vimeo.com (RBC); www.pixabay.com (pollen). PM, particulate matter. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

reactive metals (157). Vehicle exhaust emissions, and diesel exhaust in particular, are especially rich in the combustion-derived nanoparticles that make up a significant proportion of urban fine and ultrafine PM.

Diesel exhaust

In the late 20th century, a number of regulatory bodies advocated the use of diesel vehicles due to their seemingly greater environmental friendliness, including lower emissions of carbon dioxide, hydrocarbons, and lead, compared to petrol cars. Government incentives and superior fuel economy led to a marked increase in the popularity of diesel engine cars, particularly in the European and Indian markets. However, emissions from diesel exhaust can be ~100-fold higher in particle numbers than gasoline engines (147, 148, 163) due to the greater proportion of smaller particles emitted per unit mass of PM (84, 131). Diesel exhaust emissions, therefore, represent a major source of harmful combustion-derived PM, and despite the greater recognition of their potential health effects, levels of these pollutants are expected to increase in urban environments of many countries over the next few years.

At present, the precise mechanisms underlying exposure to combustion-derived PM and increased cardiovascular morbidity remain incompletely understood although there is increasing evidence that oxidative stress may have a key role. While it can be argued that much can (and should) be done to clean the air without knowing these mechanisms, such an approach risks unnecessary resource expenditure as a consequence of under- or overtargeting specific pollutants. Furthermore, even at air pollution levels below current international guidelines, associations between fine PM exposure and cardiovascular events have been found, particularly in sensitive individuals (13, 21, 24, 156, 184). A greater understanding of the pathways linking air pollution exposure to cardiovascular disease may allow the development of specific therapies for individuals at greatest risk and permit a more directed and cost-effective adjunct to future global environmental strategies.

This review examines the evidence for the associations between PM and cardiovascular dysfunction. We focus on the findings from controlled exposure studies, which provide a unique insight into the means by which diesel exhaust and other combustion-derived PM can induce cardiovascular impairment. The potential for diesel exhaust to generate oxidative stress is examined with emphasis and then given to evidence linking PM-induced oxidative stress to adverse cardiovascular responses.

PM and Cardiovascular Disease

Epidemiological evidence

Acute exposure. Meta-analyses and multicity studies of daily changes in exposure to PM have demonstrated a 0.5–2% increase in cardiovascular mortality with each 10–20 $\mu g/m^3$ increase in PM air pollution (3, 41, 77, 85, 122). Acute exposure to traffic-derived PM has been linked to the triggering of acute myocardial infarction (16, 128). Indeed, due to the ubiquitous nature of air pollution, traffic exposure was

identified as carrying the highest population attributable risk for precipitating an acute coronary syndrome event in a recent meta-regression analysis (114).

Hospital rates for all major cardiovascular admissions have been shown to increase in parallel with short-term levels of ambient fine PM, with the largest association for heart failure (1.28% increase in risk per 10 $\mu g/m^3$ increase in same-day fine PM (95% confidence interval [CI]: 0.78–1.78) (42). It was estimated that for every 10 $\mu g/m^3$ decrease in fine PM, annual hospital admissions for heart failure and stroke would be reduced by 1.3% and 0.8%, respectively. A similar association between short-term (7 day) levels of ambient PM and incidence of stroke was demonstrated in a comprehensive meta-analysis of 94 studies (152). Elevated fine PM was shown to predict a 1.1% rise in event rate ([95% CI: 1.1–1.2] per 10 $\mu g/m^3$), while ambient coarse PM showed a weaker association (0.3% increase per 10 $\mu g/m^3$ [95% CI: 0.3–0.4%]).

Long-term exposure. The long-term effects of air pollution on mortality are mostly associated with fine PM and have been shown to be generally greater than for short-term exposure (21). In a recent meta-analysis, the pooled effect estimate was 6% (95% CI: 4–8) for all-cause and 11% (95% CI: 6–16) for cardiovascular mortality per 10 $\mu g/m^3$ increase in fine PM (68). Miller *et al*. in a study of 65,893 postmenopausal women without previous cardiovascular disease found a 21% increased risk for coronary heart disease events (RR 1.21, 95% CI: 1.04–1.42) and a 35% increased risk for cerebrovascular disease events (RR 1.35, 95% CI: 1.08–1.68) for every 10 $\mu g/m^3$ increment in fine PM over a median follow-up of 6 years (100). The results for mortality were more striking with a relative risk of death of 2.21 (95% CI: 1.17–4.16) from coronary heart disease (definite diagnosis) and 1.83 (95% CI: 1.11–3.00) from cerebrovascular disease per 10 $\mu g/m^3$ increment of fine PM. Large European cohort studies have shown similar long-term associations between cardiovascular events and background level air pollution, even in high-income nations where fine PM concentrations meet international guidelines (13, 24, 156).

There is also substantial evidence that long-term exposure to PM promotes atherogenesis. After correction for potential confounders, increased ambient levels of fine PM or traffic-related air pollution were associated with both a greater extent and rate of progression of coronary atherosclerosis, as determined by coronary artery calcium scores (70, 78). Studies using carotid intima-medial thickness as a surrogate marker for atheroma have demonstrated analogous results (12, 78, 82, 83). In older individuals, small increases in short- and long-term exposure to fine PM were associated with narrower retinal arteriolar diameters suggestive of impaired microvasculature function (2).

Controlled exposure studies

Epidemiological data have afforded strong evidence of associations between PM exposure and cardiovascular disease. Controlled exposure studies in man provide an extremely useful means to examine the associations derived from epidemiological studies in the absence of many confounders present in the real world. By exposing volunteers to relevant levels of specific pollutants and using sensitive experimental techniques to measure targeted endpoints, they can help evaluate the possible underlying pathophysiological mechanisms responsible for the acute cardiovascular effects of air pollutants and provide greater certainty of causality.

Arterial tone and blood pressure. Panel studies have demonstrated an association between short-term increases in ambient fine PM and elevated blood pressure (6, 19, 45, 90). Controlled exposure studies suggest that this may relate to alterations in arterial tone. In healthy volunteers (20, 129) and patients with metabolic syndrome (129), inhalation of fine PM resulted in acute brachial vasoconstriction (129). Diesel exhaust exposure has been shown to increase central arterial stiffness (Fig. 2) (96) and produce a small but consistent rise in diastolic blood pressure (20).

Cardiac autonomic function. Short-term increases in ambient air pollution, particularly fine PM, are associated with

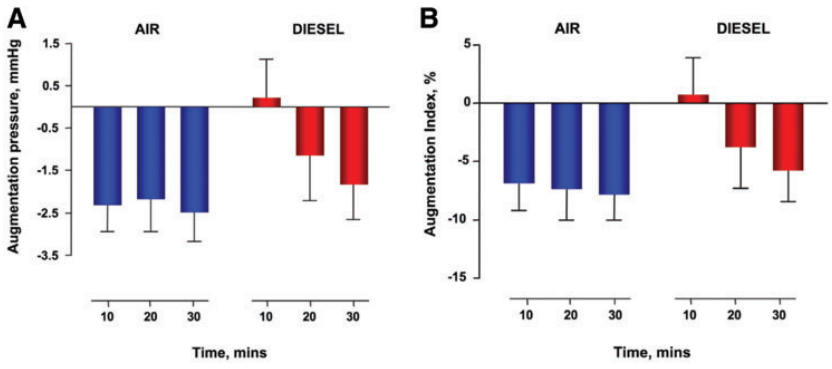

FIG. 2. The effect of dilute diesel exhaust on central arterial stiffness in healthy volunteers. (A) Following exposure to dilute diesel exhaust, augmentation pressure increased immediately compared to filtered air and normalized over 30 min ($p = 0.01$, diesel exhaust *vs.* filtered air, repeated-measures ANOVA, $n = 12$). **(B)** The augmentation index demonstrated a similar response ($p = 0.02$, respectively, diesel exhaust *vs.* filtered air, repeated-measures ANOVA, $n = 12$). Reproduced with permission from Lundback *et al.* (96). ANOVA, analysis of variance. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

a higher incidence of arrhythmia and cardiovascular death (21, 40, 126, 137). Heart rate variability (HRV) is a noninvasive measure of cardiac autonomic dysfunction with reduced HRV predicting an increased risk of adverse cardiovascular events in both healthy individuals (169) and vulnerable patients (80). Several panel studies have reported a decrease in HRV with elevated levels of ambient fine PM (56, 88, 97, 133), suggesting a potential role for cardiac autonomic dysfunction in the cardiovascular mortality associated with PM. However, the direction and magnitude of change across all available studies are variable and inconsistent (22, 107). Results from controlled exposures have been more consistent with reductions in HRV following inhalation of concentrated ambient particles (CAPs) (37, 57, 58) but not dilute diesel exhaust (107, 130). Thus, the composition and source of PM appear to be an important determination of the effects of air pollution on HRV and it remains to be established if diesel exhaust induces alterations in cardiac autonomic function.

Endothelial dysfunction. The vascular endothelium controls barrier function and through the synthesis and release of local mediators, such as nitric oxide and tissue plasminogen activator (tPA), helps regulate vascular tone and maintain an atheroprotective environment. Endothelial dysfunction is one of the earliest pathological processes involved in the development of atherosclerosis (176) as well as being an important determinant of subsequent cardiovascular events (62, 64).

Mills *et al*. demonstrated that short-term exposure to diesel exhaust impairs resistance vessel responses to both endothelium-dependent and endothelium-independent vasodilatation (Fig. 3) (106). Subsequent studies have exhibited similar results (172) with altered vasomotor function occurring soon after exposure (within 2 h) and remaining evident 24 later (167). Inhalation of diesel exhaust has been shown to reduce the ability of the endothelium to release tPA (a marker of endogenous fibrinolysis) in both healthy volunteers (11, 92, 106) and patients with stable ischemic heart disease (108). Lucking *et al*. demonstrated that impaired vasomotor and endothelial responses following diesel exhaust exposure were nearly abolished by the use of a particle trap (92). This suggests these effects were driven by the PM fraction of diesel exhaust as opposed to the gaseous copollutants and

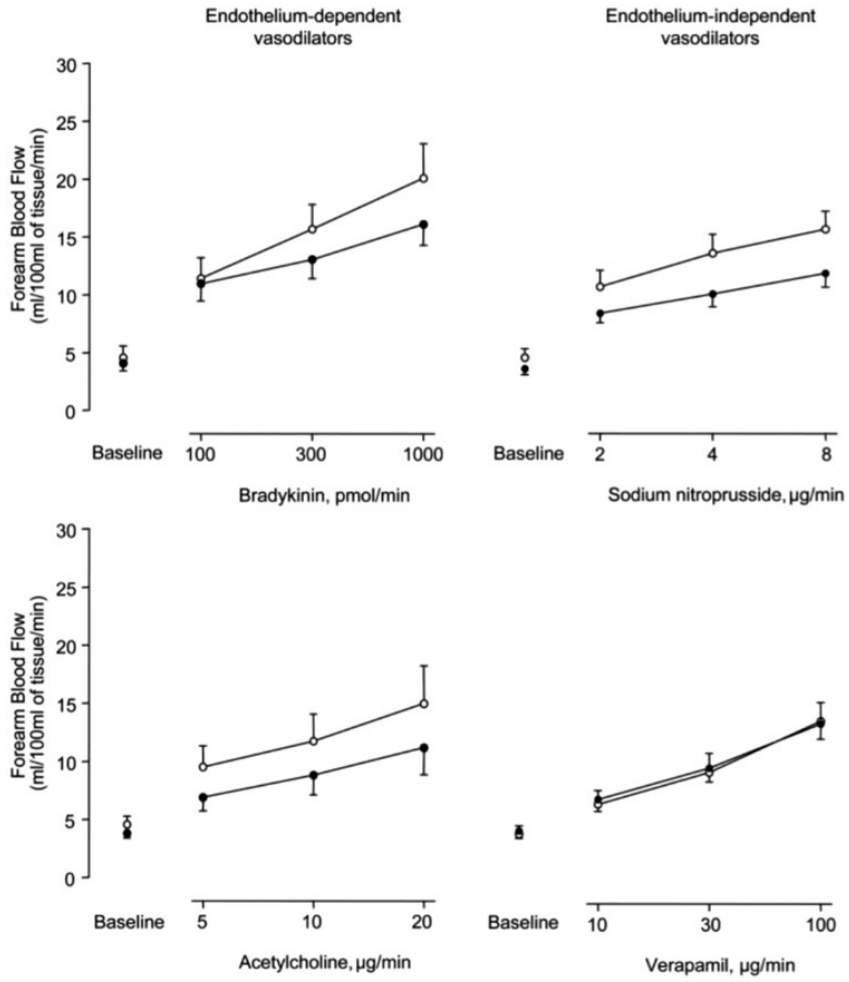

FIG. 3. The effect of dilute diesel exhaust on forearm blood flow in healthy volunteers. Infused forearm blood flow 2–4 h after diesel exposure (●) and air (○) during intrabrachial infusion of bradykinin, acetylcholine, sodium nitroprusside, and verapamil. For all dose–responses, $p < 0.0001$. For diesel exposure (●) *versus* air (○), bradykinin $p < 0.05$, acetylcholine $p < 0.05$, sodium nitroprusside $p < 0.001$, and verapamil $p =$ NS. Reproduced with permission from Mills *et al.* (106).

have been linked to the surface constituents of the particles rather than the carbon core (105).

Systemic inflammation. Atherosclerosis is inherently a chronic inflammatory condition and elevated systemic markers of inflammation, such as C-reactive protein (CRP) (138), white cell counts (175), and myeloperoxidase (66, 146), are strong predictors of cardiovascular death, myocardial infarction, and stroke. Evidence that diesel exhaust inhalation can invoke systemic inflammation (demonstrated by elevated circulating leukocytes, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and acute-phase response proteins) is strongest in animal models (113, 139, 181) and longitudinal studies (35, 69, 168, 171). In contrast, results from controlled exposure studies in man are less consistent (167), with several studies demonstrating little or no observable effect (65, 91, 106).

Cardiac ischemia. ST-segment depression on exercise testing or ambulatory electrocardiography is used as a noninvasive marker of myocardial ischemia and predicts an increased risk of adverse cardiovascular events in patients with established ischemic heart disease (30, 159). Panel studies of elderly subjects (36) and those with a history of myocardial infarction (28, 125) found that higher ambient levels of PM air pollution were associated with an increased occurrence of ST-segment depression during submaximal exercise stress testing or on ambulatory electrocardiography. During a 1-h controlled exposure to diesel exhaust, Mills *et al.* demonstrated an increase in exercise-related ST-segment depression in patients with stable ischemic heart disease, compared to filtered air (Fig. 4) (108). In a similar study by the same group, a small, but nonsignificant increase in maximal ST-segment depression was observed immediately postexposure during a formal exercise treadmill test (−185 μV [−115 to −203] *vs.* −217 μV [−139 to −245]; $p = 0.07$; unpublished data).

Atherothrombosis. Acute cardiovascular events, including myocardial infarction and stroke, are primarily the result of occlusive arterial thrombosis following rupture of atherosclerotic plaques. Studies in mice have demonstrated that PM exposure potentiates atherosclerosis (161) and is associated with increased markers of plaque instability (9, 23). In humans, short-term diesel exhaust exposure increased *ex vivo* thrombus formation (91), driven by the particulate constituents of the exhaust (92). Plausible mechanisms for enhanced thrombogenicity include increased platelet hyper-reactivity (31, 49, 61, 74, 134), loss of vascular endothelium-derived nitric oxide, and alterations in coagulation, including increased fibrinogen (31, 54, 151) and plasminogen activator inhibitor-1 (31), and decreased release of tPA (106) and tissue factor pathway inhibitor (31).

Biological Pathways

PM inhalation leads to cardiovascular disease

The pathophysiological mechanism(s) underlying the association between pulmonary exposure to combustion-derived PM and cardiovascular disease remain incompletely

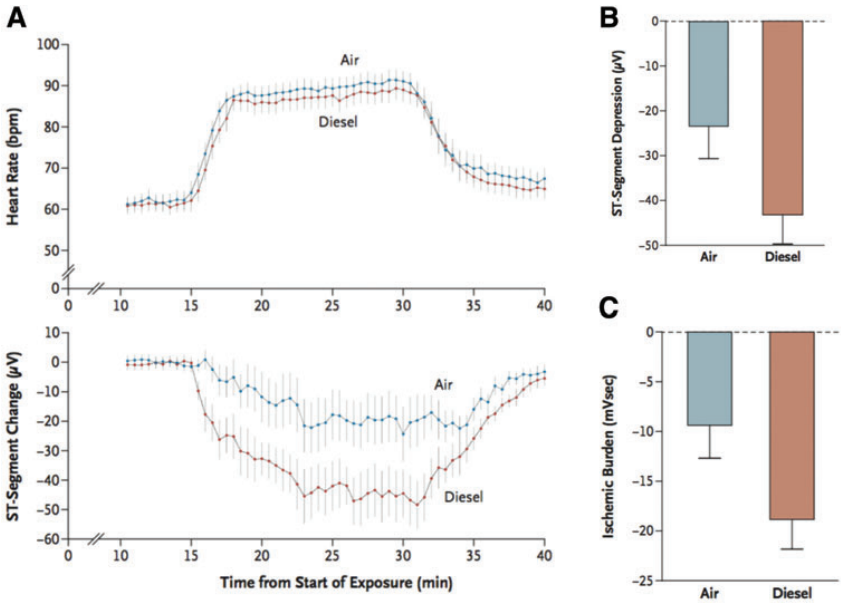

FIG. 4. Myocardial ischemia during 15-min intervals of exercise-induced stress and exposure to dilute diesel exhaust or filtered air in men with prior myocardial infarction. (A) Shows the average change in the heart rate and in the ST-segment in lead II. **(B)** Shows the maximum ST-segment depression during inhalation of dilute diesel exhaust compared with filtered air ($p = 0.003$), and **(C)** shows the total ischemic burden during inhalation of diesel exhaust compared with filtered air ($p < 0.001$); the values in **(B)** and **(C)** are averages of the values in leads II, V2, and V5. In all three *panels*, *red* indicates exposure to diesel exhaust, and *blue* exposure to filtered air. T *bars* denote standard errors. From Mills *et al.* (107) Copyright © (2007) Massachusetts Medical Society. Reprinted with permission. mVsec, millivolt seconds. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

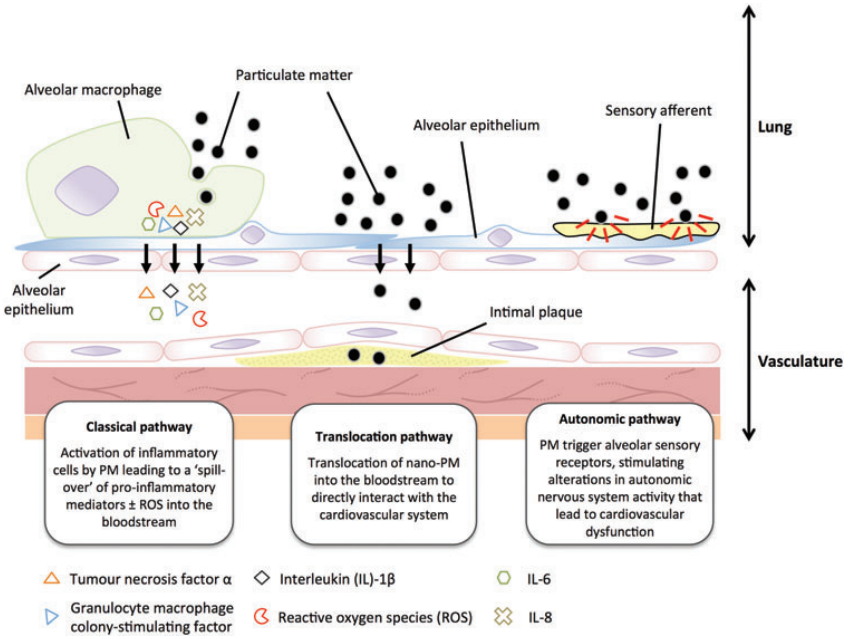

FIG. 5. Hypothetical pathways through which inhaled PM determines effects on the cardiovascular system. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

understood (Fig. 5). The classical hypothesis is that inhaled particles deposited deep in the alveoli of the lung activate local inflammatory cells leading to a ''spill-over'' of proinflammatory mediators into the bloodstream, which then impair cardiovascular function (5, 67, 150). Alternatively, inhaled particles may trigger sensory receptors on the alveolar surface, stimulating alterations in autonomic nervous system activity, which in turn promote cardiovascular dysfunction (132, 133).

More recently, it has been proposed that nanosized combustion-derived particles are able to translocate across the alveolar membrane into the bloodstream to directly interact with the cardiovascular system (46, 51, 52, 149). Other mechanisms have been suggested that may build on these pathways, including stimulation of sensory afferents in the upper respiratory or gastrointestinal tract (following mucociliary clearance) and accumulation of PM in nonvascular tissues with subsequent amplification of local inflammatory responses. While it remains unclear which, if any, of these predominate, there is a potential role for oxidative stress at numerous points in all these pathways (101). Oxidative stress has therefore emerged as one of the possible major pathobiological mechanisms underlying the cardiovascular effects of diesel exhaust inhalation (103).

Oxidative Stress

What is oxidative stress?

Reactive oxygen species (ROS) is a term used to describe a variety of oxidizing molecules that include both free radicals (molecules with one unpaired electron such as superoxide anion [$O_2^{-\bullet}$] and hydroxyl free radical [$OH^{\bullet}$]) and nonradicals (*e.g.*, hydrogen peroxide [H_2O_2] and peroxynitrite [$ONOO^-$]). ROS are a by-product of normal cellular metabolism but can also be produced from exogenous sources (*e.g.*, particulates themselves). They have the potential to cause deleterious effects and their levels are finely regulated by antioxidants to maintain redox homeostasis and cellular function. Oxidative stress refers to a net excess of ROS, through an increase in ROS, a decrease in cellular antioxidant capacity, or combination of both. This can lead to indiscriminate oxidation of a wide range of cellular molecules, resulting in dysregulation of cellular function, cell death, and tissue damage. ROS generation and oxidative stress have been implicated in the pathogenesis of many disease processes (73, 135).

Evidence for the role of oxidative stress in cardiovascular disease

Oxidative modification of low-density lipoprotein (LDL) has been shown to be an important step in the pathogenesis of atherosclerosis. Within the vessel wall, oxidized LDL activates macrophages and other inflammatory cells leading to the accumulation of lipid-laden foam cells and plaque formation (47). Antioxidant therapies have been shown to attenuate plaque progression in animal and observational studies, although the results from randomized controlled trials have largely been negative (27). Oxidative stress may promote hypertension through vascular smooth muscle cell proliferation and hypertrophy (145), quenching of nitric oxide by superoxide and other ROS (98), generation of vasoconstrictor products, including F2-isoprostanes (32) and upregulation of the Rho/Rho-associated kinase pathway (160). Increased markers of vascular oxidative stress have been demonstrated in animal models of hypertension (118, 164, 179) although less reliably in human studies (33, 124, 142, 174).

Oxidative stress plays an important role in the pathogenesis of heart failure. Lipid peroxidation refers to the oxidative

degeneration of lipids by ROS and can lead to decreased cell membrane integrity and function. Markers of lipid peroxidation, including exhaled breath pentane and circulating malondialdehyde, have been shown to be elevated in patients with congestive heart failure (38, 154) and following myocardial infarction (177). Exogenous generators of ROS result in increased resting tension, structural damage to myocytes, and contractile failure in isolated perfused rat hearts; effects that are reduced by coadministration of antioxidants (59, 180). Belch *et al*. demonstrated that plasma levels of free radicals and antioxidants (thiols) were both significantly, although weakly, correlated with the severity of left ventricular systolic impairment (15). Reperfusion injury following myocardial ischemia is believed, at least in part, to be mediated by a burst of oxidative stress (63, 185). Accordingly, attenuation of free radical production has been shown to enhance contractile recovery in animal models (17, 18).

Evidence that diesel exhaust induces oxidative stress

Combustion-derived PM is highly oxidizing and is capable of initiating free redox cycling reactions in physiological solutions and biological environments (1, 79, 102, 121, 158). Increased markers of ROS (*e.g.*, superoxide, H_2O_2) and oxidative stress (*e.g.*, superoxide dismutase-1, heme oxygenase-1, tetrahydrobiopterin, thiobarbituric acid reactive substances, 15-F2t-isoprostane, 8-hydroxy-2-deoxyguanosine) have been demonstrated within the vascular wall (9, 93–95, 119, 161, 173), heart (10, 34, 53, 60), lung (60, 116), and plasma (9, 93, 116) of animals following pulmonary exposure to PM or vehicle exhaust. Other investigators have found these pollutants to induce an increased expression of inflammatory cytokines (*e.g.*, intracellular adhesion molecule-1, IL-6, TNF-α) that are under the regulatory control of redox-sensitive transcription factors (75, 76, 86, 87).

Diesel exhaust particles and urban air pollution have been shown to oxidize LDL *in vitro* (72) and *in vivo* (153), respectively. Exposure of Apo $E^{-/-}$ mice to diesel exhaust increased the expression of oxidative stress markers (inducible nitric oxide synthase [iNOS], CD36, and nitrotyrosine) in aortic plaque (9) and led to upregulation of lectin-like oxidized LDL receptors (94). Fine PM was found to decrease the ability of high-density lipoprotein to protect LDL from oxidation (5).

In human studies, higher concentrations of ambient fine PM concentrations were associated with increased urinary levels of 8-hydroxy-2-deoxyguanosine and malondialdehyde (both markers of oxidative stress) (8, 136, 140). Interestingly, Sørensen *et al*. reported that plasma malondialdehyde was increased by fine PM exposure in women, but not in men, suggesting that sex differences may add to the complexity of systemic PM responses (155). Controlled exposure studies have shown that vehicle exhaust precipitated increased levels of malondialdehyde in exhaled breath condensate (141), urate and reduced glutathione (antioxidants) in airway lavage (14, 110), 8-hydroxy-2-deoxyguanosine in urine (162), and the expression of genes related to oxidative stress (*e.g.*, dual-specificity phosphatase-1, glutathione *S*-transferase M4, peroxiredoxin-1, and heme oxygenase-1) in peripheral blood monocytes (127). Finally, in a meta-analysis of cross-sectional, panel, and controlled exposure studies, inhalation of combustion-derived PM was found to be consistently associated with oxidatively damaged DNA and lipid peroxidation in blood, urine, and exhaled breath condensate (112).

Evidence that oxidative stress drives diesel exhaust-induced vascular dysfunction

To examine the role of oxidative stress in driving the pathological effects of diesel exhaust, several studies have assessed the effects of combustion-derived PM exposure with and without exogenous administration of either antioxidants or inhibitors of ROS generating pathways.

Animal studies. In rat aortic ring models, addition of superoxide dismutase and other free radical scavengers (*e.g.*, tiron) abolished the inhibitory effect of diesel exhaust on endothelium-dependent (25, 26, 71, 102) and endothelium-independent (25, 102) aortic relaxation (Fig. 6). Similarly, alterations in phenylephrine-stimulated vascular tone were inhibited by *N*-acetylcysteine (170) and the iNOS inhibitor, 1400W (10). Knuckles *et al*. demonstrated that L-nitro-arginine-methyl-ester, an endothelial nitric oxide synthase (eNOS) inhibitor, reversed the proconstrictor effects of diesel exhaust, suggesting that generation of superoxide from uncoupling of eNOS may be a mechanism for diesel exhaust-induced vascular dysfunction (81).

Intratracheal instillation of diesel exhaust particles in mice results in pulmonary inflammation, increased systemic oxidative stress (as determined by a decrease in plasma Trolox equivalent antioxidant capacity), and proaggregatory effects in pial cerebral venules (116). These effects were reversed by pretreatment with the antioxidant l–2-oxothiazolidine-4-carboxylic acid. Matrix metalloproteinases (MMPs) are important regulators of vascular remodeling. Lund *et al.* demonstrated that in mice, increases in expression of vascular MMP-2 and ROS in response to vehicle emissions (95) could be ameliorated by oral coadministration of the antioxidant, 4-hydroxy-TEMPO (Tempol) (95).

Human studies. Evidence directly linking PM-induced oxidative stress with vascular dysfunction in man is challenging due to the fewer techniques available for use in readily collectable biological samples (*i.e.*, blood and urine) and a more restricted set of antioxidants or administration routes. Nevertheless, there is emerging literature supporting the role of oxidative stress in cardiovascular effects of air pollution.

In a study of 76 students living in Taipei, small increases in background levels of PM were associated with a reduction in HRV, accompanied by an increase in plasma levels of 8-hydroxy-2-deoxyguanosine (29). Liu *et al*. demonstrated that increases in black carbon and fine PM exposure caused alterations in brachial artery diameter, blood pressure, and heart rate, which were associated with increased plasma thiobarbituric acid reactive substances (90). However, in both of these studies, concomitant increases in other biological markers, including CRP, fibrinogen, and endothelin-1, mean that oxidative stress alone cannot be assumed to be the cause of vascular impairment.

Sack *et al.* examined the effect of pretreatment with an antioxidant regimen (*N*-acetylcysteine and ascorbate) on diesel-induced brachial vasoconstriction in healthy volunteers. Surprisingly, antioxidant treatment augmented, rather

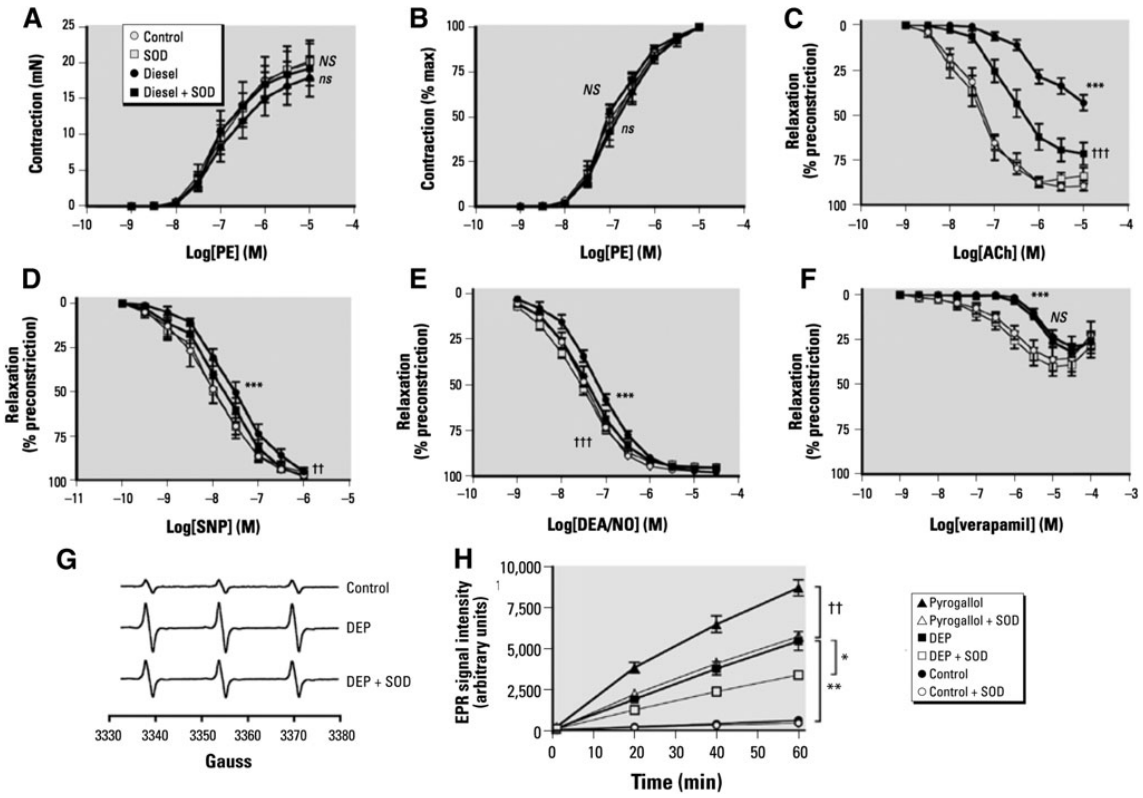

FIG. 6. The effects of diesel exhaust particulate with and without SOD on (A–F) contraction and relaxation in isolated rat aortic rings and (G, H) generation of oxygen-centered free radicals. (A, B) DEP had no effect on phenylephrine-induced vasoconstriction. **(C)** Acetylcholine-induced relaxation was attenuated by DEP (maximum relaxation reduced from 91 ± 4% to 49 ± 6% with 100 μg/ml DEP; $p < 0.001$) but was restored by SOD (maximum relaxation, 73 ± 6%; $p < 0.001$). **(D, E)** DEP caused a modest inhibition of relaxation to NO donor drugs [sodium nitroprusside, 2-(*N*,*N*-dimethylamino)-diazenolate-2-oxide], an effect that could be reversed by SOD ($p < 0.01$). **(F)** DEP inhibited verapamil-induced relaxation ($p < 0.001$) by a mechanism independent of SOD. Two-way repeated-measures ANOVA: $^{***}p < 0.001$ (ns, not significant), control *versus* DEP; $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ (NS, not significant), DEP *versus* DEP + SOD. **(G)** Sample spectra after 60 min showing the characteristic three-peaked spectrum produced by oxidation of the spin trap TEMPONE-H (1 m*M*); DEP (10 μg/ml) caused a large increase in peak amplitude, an effect that was partially reduced by coincubation with SOD (100 U/ml). **(H)** EPR intensity generated by auto-oxidation (control), DEP, or pyrogallol (100 μM) over a 1-h period, in the presence (*open symbols*) or absence of SOD (*solid symbols*). EPR intensity is calculated using the area under the curve of the first derivative of the first line of the three-line spectrum generated. Data are mean ± standard error ($n = 6$). Paired *t*-tests: $^{*}p < 0.05$, DEP *versus* DEP + SOD; $^{**}p < 0.01$, control *versus* DEP; $^{\dagger\dagger}p < 0.01$, pyrogallol *versus* pyrogallol + SOD. Reproduced with permission from Miller *et al*. (102). EPR, electron paramagnetic resonance; SOD, superoxide dismutase.

than reversed, the vasoconstrictor effects of diesel exhaust (143). Ultrafine PM can alter fibrin clot structure *in vitro* and this may contribute to the increased incidence of thromboembolic events related to combustion-derived air pollution. These alterations were inhibited by the presence of mannitol, a scavenger of $OH^{\bullet}$ (99). In a randomized, double-blind, controlled exposure study in middle-aged healthy volunteers, fish oil supplementation, which is high in the antioxidant omega-3 fatty acid (4), successfully attenuated reductions in HRV and acute increases in LDL and triglyceride concentrations in response to acute exposure to CAPs (166). In a similar study by Zhong *et al*., alterations in heart rate, HRV, and white blood cell counts following a 2-h controlled exposure to fine PM were almost completely mitigated by vitamin B supplementation (183). B vitamins have anti-inflammatory, antioxidant, and immunoepigenetic effects (7, 182). Finally, inhalation of diesel exhaust has frequently been shown to attenuate vasodilatation to endothelium-derived and nonendothelium-derived nitric oxide. While this is indirect evidence for a role of oxidative stress, these results are consistent with reduced nitric oxide bioavailability due to scavenging by superoxide free radicals.

Summary

Epidemiological evidence has repeatedly demonstrated that acute and chronic exposure to PM, especially combustion-derived fine and ultra-PM, is strongly associated

with adverse cardiovascular outcomes. Diesel exhaust emissions are of greatest concern in urban environments, where they are a major source of fine and ultrafine PM. Using controlled exposure studies, combustion-derived PM and diesel exhaust particles in particular have been shown to promote a wide array of adverse cardiovascular effects that may underlie the observed increases in cardiovascular morbidity and mortality. These include alterations in blood pressure, heart rate and vascular tone, endothelial dysfunction, myocardial ischemia, thrombosis, atherogenesis, and plaque instability (Fig. 7).

Our understanding of the biological pathways that link the lung response to inhaled pollutants to their cardiovascular effects has advanced significantly over the past decade but remains incomplete. The proinflammatory response to PM, both in the lung and (less consistently) systemically, provides a prominent mechanism through which inhalation of diesel exhaust could contribute to oxidative stress and cardiovascular impairment. However, PM-induced increases in markers of oxidative stress have been shown to be more pronounced than classical biomarkers of systemic inflammation (113), and multiple studies have demonstrated little or no increase in systemic inflammatory mediators despite observable cardiovascular dysfunction. Similar to epidemiological studies (22), controlled exposure studies have demonstrated mixed results with regard to induced alterations in autonomic function as determined by HRV (107). Further assessment of the effects of diesel exhaust inhalation on cardiac autonomic function is required, but alternative metrics of these neural reflexes can be challenging to perform and more difficult to interpret. On the contrary, recent studies in animal and man have provided convincing evidence that inhaled nanoparticles translocate into the circulation and accumulate at sites of vascular inflammation (104, 115, 120). This provides a mechanism through which environmental nanoparticles could directly impair cardiovascular function and exacerbate pre-existing disease. Precisely how combustion-derived PM translocates across the pulmonary alveolar membrane and the pathobiological consequences of uptake into vascular plaque will be an important area of future research.

Oxidative stress has emerged as a highly plausible and unifying pathobiological mechanism by which inhalation of diesel exhaust emissions leads to cardiovascular dysfunction. Three lines of evidence support this hypothesis. First, oxidative stress plays an important role in many cardiovascular disease processes, including vascular dysfunction, atherosclerosis, myocardial injury, and heart failure. Second, diesel exhaust is highly oxidizing and increased levels of ROS and markers of oxidative stress have been demonstrated in multiple cardiovascular compartments following exposure. Finally, an increasing number of studies have shown that coadministration of antioxidants can attenuate or reverse the cardiovascular effects of diesel exhaust on a number of cardiovascular endpoints, including alterations in endothelial function, vasomotor responses, thrombosis, and regulators of vascular remodeling (Fig. 8).

Exposure to diesel air pollution is ubiquitous and unavoidable in almost every urban environment. On a population level, interventions that reduce levels of air pollutants, especially combustion-derived PM, represent a clear strategy to limit the adverse health effects of air pollution. These include the use of particle traps fitted to exhaust pipes, and the modification of fuels and engine technology to ensure more complete combustion. For high-risk populations, targeted therapies to mitigate the effects of PM exposure may be

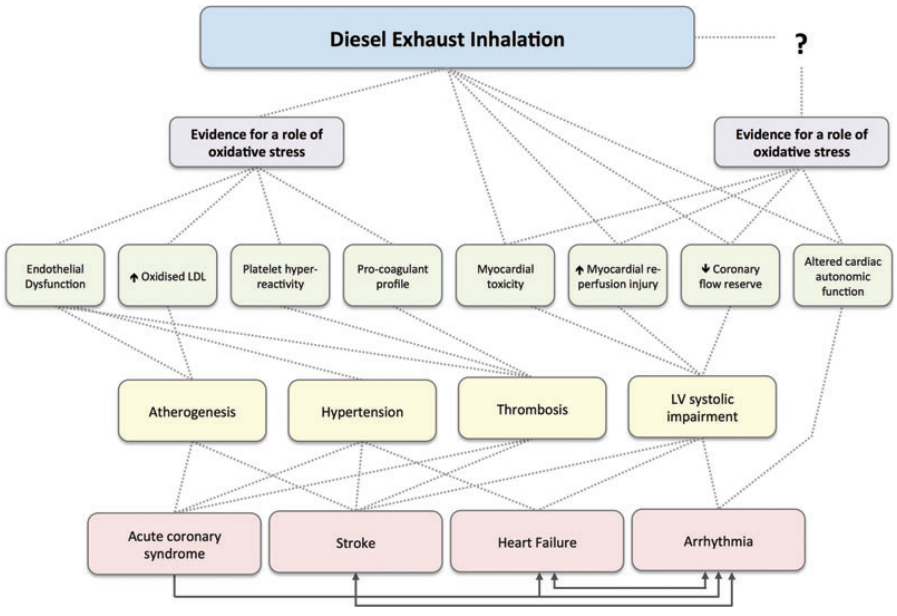

FIG. 7. Possible pathophysiological mechanisms responsible for the acute and chronic cardiovascular effects of diesel exhaust inhalation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

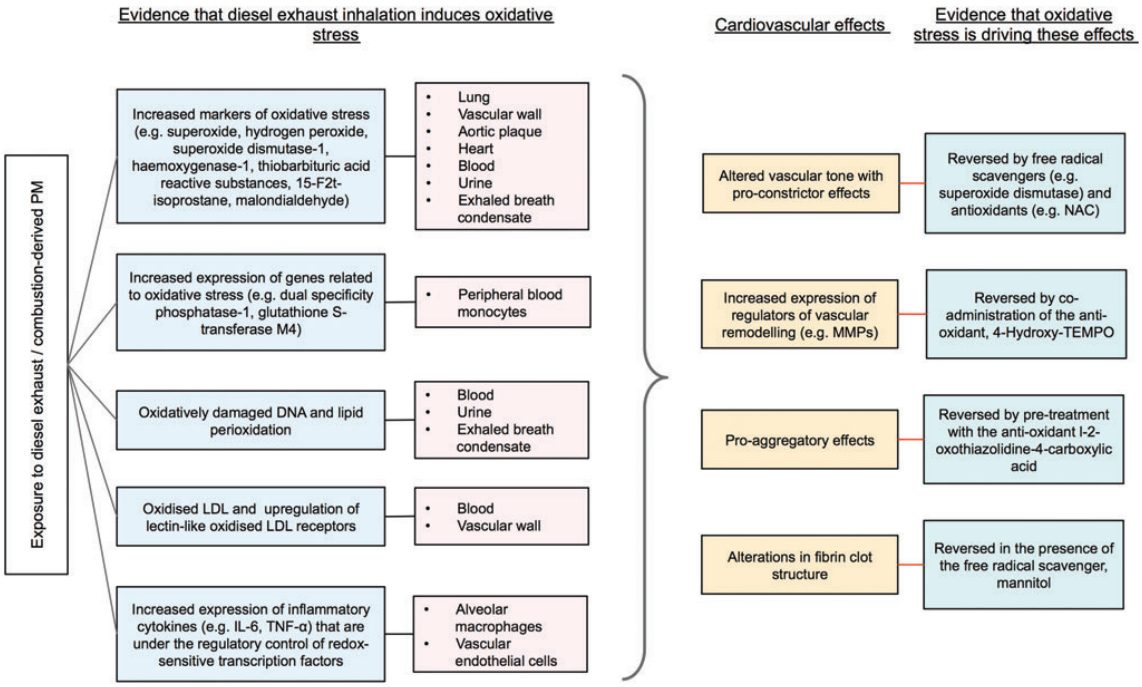

FIG. 8. Summarizing scheme of oxidative stress induction by diesel exhaust inhalation and the demonstrated cardiovascular effects. IL, interleukin; LDL, low-density lipoprotein; NAC, *N*-acetylcysteine; TNF-α, tumor necrosis factor alpha. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

useful, particularly as even at levels below current international guidelines, associations between fine PM exposure and increased cardiovascular risk have been demonstrated in sensitive individuals. In recent years, there have been an increasing number of studies examining whether various pharmacological agents can prevent the effects of air pollution. Given the apparent central role of oxidative stress in the action of combustion-derived PM, agents that scavenge oxygen-derived free radicals, inhibit enzymatic sources of these radicals [*e.g.*, NAD(P)H oxidase], or replenish the specific antioxidant stores consumed by PM would seem likely to be of most benefit. At present, however, most evidence relates to early experimental animal models with varying endpoints and pollutant exposure, while in humans, evidence that statins, B vitamins, omega-3 fatty acids, vitamin A, and vitamin C supplementation may mitigate some of the adverse cardiopulmonary effects of PM exposure is limited to observational or healthy volunteer studies only (48, 109, 123, 165, 166, 183). It therefore remains unclear which agents may offer an effective therapeutic profile and in view of the predominantly disappointing results of large clinical trials of antioxidants on cardiovascular disease in general, a considerable evidence base in man would be required before a therapeutic strategy could be proposed in addition to improvements in air quality.

Conclusions

Combustion-derived fine and ultrafine PM precipitates a diverse array of adverse effects within the cardiovascular system. Diesel exhaust is a prominent source of combustion-derived fine and ultrafine PM in urban environments and is likely to be a major contributor to the global excess of cardiovascular morbidity and mortality attributable to air pollution. Oxidative stress appears to be a prominent mechanism underlying the detrimental effects of diesel exhaust on many facets of the cardiovascular system. Further studies are required to determine if targeting oxidative stress pathways involved in the actions of PM could be of therapeutic value in addition to strategies to reduce air pollution.

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Abbreviations Used

ANOVA = analysis of variance
CAP = concentrated ambient particle
CI = confidence interval
CRP = C-reactive protein
eNOS = endothelial nitric oxide synthase
EPR = electron paramagnetic resonance
HRV = heart rate variability
IL = interleukin
iNOS = inducible nitric oxide synthase
LDL = low-density lipoprotein
MMP = matrix metalloproteinase
mVsec = millivolt seconds
NAC = *N*-acetylcysteine
PM = particulate matter
ROS = reactive oxygen species
SOD = superoxide dismutase
TNF-α = tumor necrosis factor alpha
tPA = tissue plasminogen activator