MONASH University

Defining the role of protease-activated receptor 4 during thrombosis

Shauna Louise French

BAppSci (Medical Science) (Hons)

A thesis submitted for the degree of *Doctor of Philosophy* at

Monash University in 2017

Australian Centre for Blood Diseases

Faculty of Medicine, Nursing and Health Sciences

Monash University

Table of Contents

Copyright notice

Abstract

Background: Thrombin-induced platelet activation is a major contributor to the formation of the arterial thrombi that cause two of the most lethal manifestations of cardiovascular disease – acute myocardial infarction and occlusive stroke. Thrombin activates human platelets via protease-activated receptors 1 (PAR1) and 4 (PAR4). Much is known about the function of the higher affinity receptor, PAR1, and a PAR1 antagonist is in clinical use for the prevention of myocardial infarction. However, PAR1 inhibition increases the rate of intracranial haemorrhage, suggesting there remains much to learn regarding how to best manipulate thrombin signalling in platelets for safe and effective antithrombotic therapy. As a result, there has been much recent interest in examining PAR4 as an antiplatelet drug target. However, in contrast to PAR1, far less is known about the function of PAR4 during platelet activation and thrombus formation.

Aim: To examine the function of PAR4 during thrombosis in order to determine whether targeting PAR4 represents a valid antithrombotic approach.

Key Findings: First, the function of PAR4 on human platelets during thrombosis was determined. To achieve this, a function-blocking PAR4 polyclonal antibody was developed and used to probe for platelet activation events dependent on PAR4. This approach determined that thrombin-induced production of procoagulant platelets is mediated predominantly by PAR4, and is largely independent of PAR1. This effect translated into impaired thrombin production and fibrin formation in an *ex vivo* human thrombosis model, rationalising PAR4 as a novel antithrombotic target. Second, the effects of targeting PAR4 were examined *in vivo* using mouse genetic models. Previous studies have shown that mice genetically deficient in PAR4 are protected against thrombosis but do not bleed spontaneously. However, here, PAR4-deficient mice exhibited a subtle but significant spontaneous bleeding phenotype in the

perinatal period, suggesting PAR4 inhibition in humans may cause unwanted bleeding effects. However, in contrast to human platelets, mouse platelets do not express PAR1 and so thrombin signalling in mouse platelets is reliant entirely on PAR4. These *in vivo* studies therefore highlighted the need for an animal model of platelet-PAR expression that would more accurately predict the impact of PAR4 inhibition in humans. Therefore, third, the production of a mouse that expresses human PAR1 on the platelet surface was attempted. This approach failed to provide platelet PAR1 expression and suggests alternative animal models will be required for preclinical evaluation of PAR4 antagonism. Finally, the first human monoclonal inhibitory antibody targeting PAR4 was developed as a potential therapeutic strategy in humans. This antibody specifically and effectively inhibited thrombin-induced cleavage and activation of PAR4 and provided marked antithrombotic effects in human blood. Furthermore, this antibody-based approach to inhibiting PAR4 was equally effective against a commonly expressed and clinically significant sequence variant of PAR4 that is resistant to an existing PAR4 antagonist.

Conclusion: The findings from this thesis suggest that targeting PAR4 is valid antithrombotic approach. These studies have uncovered a distinct role for PAR4 in the setting of human thrombosis, developed an antibody against PAR4 with therapeutic potential, and shown that antibody-based inhibition of PAR4 provides an antithrombotic effect distinct from all current antiplatelet drugs and that may have advanced clinical utility over other PAR4 antagonists. Together, these studies provide strong rationale for the pursuit of PAR4 antagonists for the prevention of arterial thrombosis.

Publications during enrolment

French SL and Hamilton JR. (2017) Drugs targeting PAR4 improve the antithrombotic therapeutic window. *Ann Trans Med*. doi: 10.21037/atm.2017.09.10

French SL and Hamilton JR. (2017) Perinatal lethality of *Par4*$^{-/-}$ mice delivered by primiparous dams reveals spontaneous bleeding in mice without platelet thrombin receptor function. *Platelets*. doi: 10.1080/09537104.2017.1349310.

French SL, Paramitha AC, Moon MJ, Dickins RA and Hamilton JR. (2016) Humanizing the protease-activated receptor (PAR) expression profile in mouse platelets by knocking PAR1 into the Par3 locus reveals PAR1 expression is not tolerated in mouse platelets. *PLoS One*. 11 (10): e016556.

French SL, Arthur JF, Lee H, Nesbitt WS, Gardiner EE, Andrews RK and Hamilton JR. (2016) Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood. *J Thromb Haemost.* 14(8):1642-54

French SL and Hamilton JR (2016). Protease-activated receptor-4: from structure to function and back again. *Brit J Pharmacol*. 173(20):2952-65

French SL, Arthur JF, Tran HA and Hamilton JR (2015) Approval of the first protease-activated receptor antagonist: rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Reviews*. 29: 179-89.

Declaration: Thesis including published works

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original research papers and 3 reviews published in peer reviewed journals plus 1 submitted manuscript. The core theme of the thesis is 'Protease-activated receptor 4 during thrombosis'. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Australian Centre for Blood Diseases under the supervision of Justin Hamilton. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of *Chapters 2-5* my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood	Published	57%. Writing of manuscript, performed all experiments and data analysis for Figures 1, 3 and 4. Contributions to Figures 2 and 5.	1) Jane F Arthur, performed some experiments in Figure 5, 10%. 2) Hannah Lee, performed some experiments in Figure 2, 10%. 3) Warwick S Nesbitt, supervision, contribution of tools and reagents, 1%. 4) Robert K Andrews, supervision, contribution of tools and reagents, 1%. 5) Elizabeth E Gardiner, supervision, contribution of tools and reagents, 1%. 6) Justin R Hamilton, design of research, supervision and writing of manuscript, 20%	No Yes No No No No
3	Perinatal lethality of Par4-/- mice delivered by primiparous dams reveals	Published	70%. Performed data collection and analysis and writing of manuscript.	1) Justin R Hamilton, design of research, supervision and writing of manuscript, 30%	No

	spontaneous bleeding in mice without platelet thrombin receptor function				
4	Humanizing the protease-activated receptor (PAR) expression profile in mouse platelets by knocking PAR1 into the Par3 locus reveals PAR1 expression is not tolerated in mouse platelets	Published	60%. Writing of manuscript, performed all experiments and data analysis for Figures 1, 3, 4, and 5. Contribution and supervision of experiments and data analysis for Figures 2, 6 and 7.	2) Antonia Christa Paramitha, performed some experiments in Figures 2, 6 and 7, 15%. 3) Mitchell J Moon, technical support, 2.5% 4) Ross A Dickins, supervision and contribution of reagents and tools, 2.5% 5) Justin R Hamilton, design of research, supervision and writing of manuscript, 20%	Yes Yes No No
5	Inhibition of protease-activated receptor 4 (PAR4) with function blocking antibodies provides equivalent antithrombotic activity in the face of the hyper-reactive Thr120 PAR4 variant	Submitted	65%. Writing of manuscript, performed all experiments and data analysis with the exception of Figures 1B and 1C.	1) Claudia Thalmann, performed the experiments in Figure 1B and 1C, 5%. 2) Mark A Sleeman, supervision, contribution of reagents, 10%. 3) Justin R Hamilton, design of research, supervision and writing of manuscript, 20%	No No No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  **Date: 24/10/2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:  **Date: 24/10/2017**

Acknowledgements

I would firstly like to acknowledge and thank my supervisor, Justin Hamilton, for his encouragement, guidance, and friendship throughout this degree. The achievements contained within are a reflection of his unwavering support, for which I am eternally grateful. I would like to thank Robert Andrews, my second supervisor and who I am also lucky to call a friend, for his continual advice and academic inspiration. I also wish to thank all members past and present of the Hamilton Lab, who have been friends and co-workers throughout the duration of this PhD.

I am grateful for the opportunity to have completed my PhD within the Australian Centre for Blood Diseases, and to learn from the many talented scientists I have worked with. I would especially like to thank all the senior academics and co-authors who have provided invaluable insights over the duration of this project, most notably, Elizabeth Gardiner, Jane Arthur and Ross Dickins. I also wish to acknowledge those who have been of exceptional technical and organisational support - Core Facilities staff from Monash Micro Imaging and AMREP Flow Core, AMREP AS staff for animal care, the ACBD administration and operational officers, and, the AMREP cleaning staff for their timely presence on many a late night. I would also like to thank the Faculty of Nursing, Medicine and Health Sciences, Monash University, for providing my scholarship and general student support.

Finally, I would like to dedicate this thesis to my family and friends – those from the past who have been enduring throughout this process, the new ones I have made in Melbourne, and the special someone who was lost along the way.

List of Abbreviations

AA	Arachidonic acid
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
APTT	Activated partial thromboplastin time
Ca^{2+}	Calcium
CHO	Chinese hamster ovary
COX	Cyclooxygenase
DAG	Diacylglycerol
DRG	Dorsal root ganglion
ERK	Extracellular signal-related kinase
FDA	Food and Drug Administration
GEF	Guanine exchange factors
GP	Glycoprotein
GPCR	G-protein coupled receptor
HEK	Human embryonic kidney
MAP	Mitogen-activated protein
MI	Myocardial infarction
MLC	Myosin light chain
MTSP1	Membrane type serine protease 1
PAD	Peripheral artery disease
PAR	Protease-activated receptor
PAR-AP	Protease-activated receptor activating peptide
PI3	Phosphoinositide 3-kinase
PLC	Phospholipase C
PS	Phosphatidylserine
PT	Prothrombin time
TBD	Thrombin binding domain
TIA	Transient ischaemic attack
TLS	Tethered ligand site

TMPRSS2	Transmembrane protease, serine 2
TP	Thromboxane receptors
TT	Thrombin time
TXA2	Thromboxane A2
VWF	von Willebrand factor

Chapter 1 - Introduction

1.1 Cardiovascular disease and antithrombotic therapy

1.1.1 There is a significant clinical need for improved antithrombotic therapy

Cardiovascular disease, manifesting predominantly as ischaemic heart disease and ischaemic stroke, is by far the most common cause of death and disability in the world, accounting for approximately 30% of all deaths[1]. Strikingly, despite increased awareness and improved management, and in contrast to most other high-impact communicable and non-communicable diseases, the rates of cardiovascular disease rose over the past decade[1]. The increasing burden of conditions such as diabetes, obesity, and depression, as well as an overall ageing population, are likely to ensure that cardiovascular disease rates continue to rise into the foreseeable future, with each of these conditions having increased prevalence of cardiovascular-related morbidity and mortality.

Arterial thrombosis precipitates the most prevalent cardiovascular disease manifestations, most notably acute myocardial infarction (AMI), ischaemic stroke, and peripheral artery disease (PAD). Activated platelets are the main cellular component of arterial thrombi. Therefore, current therapies for the prevention of arterial thrombosis are predominantly antiplatelet agents, which prevent platelet activation. The current clinical recommendation for the prevention of primary or secondary cardiovascular events in patients with acute coronary syndrome (ACS) is antiplatelet therapy alone[2]. Yet current standard-of-care treatments have limited efficacy and are tempered by the attendant bleeding risk, meaning that improved antiplatelet approaches are sought.

1.1.2 Current antiplatelet agents as antithrombotics

The processes controlling platelet function in the setting of arterial thrombosis have been extensively studied for the purpose of rationalising the development of improved antiplatelet therapies. Platelet behaviour during thrombus formation is generally agreed to involve cell adhesion, activation, and aggregation[3] (Figure 1). Understanding these processes has been highly informative in predicting the success of antiplatelet approaches.

i) Adhesion: In response to vascular damage, such as atherosclerotic plaque rupture in the case of ACS, platelets adhere to the damaged vessel wall through a complex between sub-endothelial proteins and cognate receptors on the platelet surface. Rapid initial adhesion is mediated by von Willebrand factor (VWF) binding to the glycoprotein (GP) Ib-IX-V receptor complex[4], although this interaction does not support stable adhesion and a second adhesive step between collagen and GPVI and/or the integrin $\alpha_2\beta_1$, as well as fibrinogen and the integrin $\alpha_{IIb}\beta_3$, is required for firm platelet adhesion to the vessel wall[3].

ii) Activation: following adhesion, the captured platelets are activated and undergo a series of morphological and functional changes including shape change, spreading, release of granular contents, and the local generation of thrombin at the platelet surface as an endpoint of coagulation[5]. This platelet activation is primarily driven by the triumvirate of thrombin, ADP, and thromboxane A_2 (TxA_2).

iii) Aggregation: regardless of the adhesion and activation mechanism, the final common result is the activation of the integrin $\alpha_{IIb}\beta_3$, which engages fibrinogen (also VWF and fibronectin) to mediate platelet aggregation and ultimately stable thrombus formation, via thrombin-induced fibrin production[5].

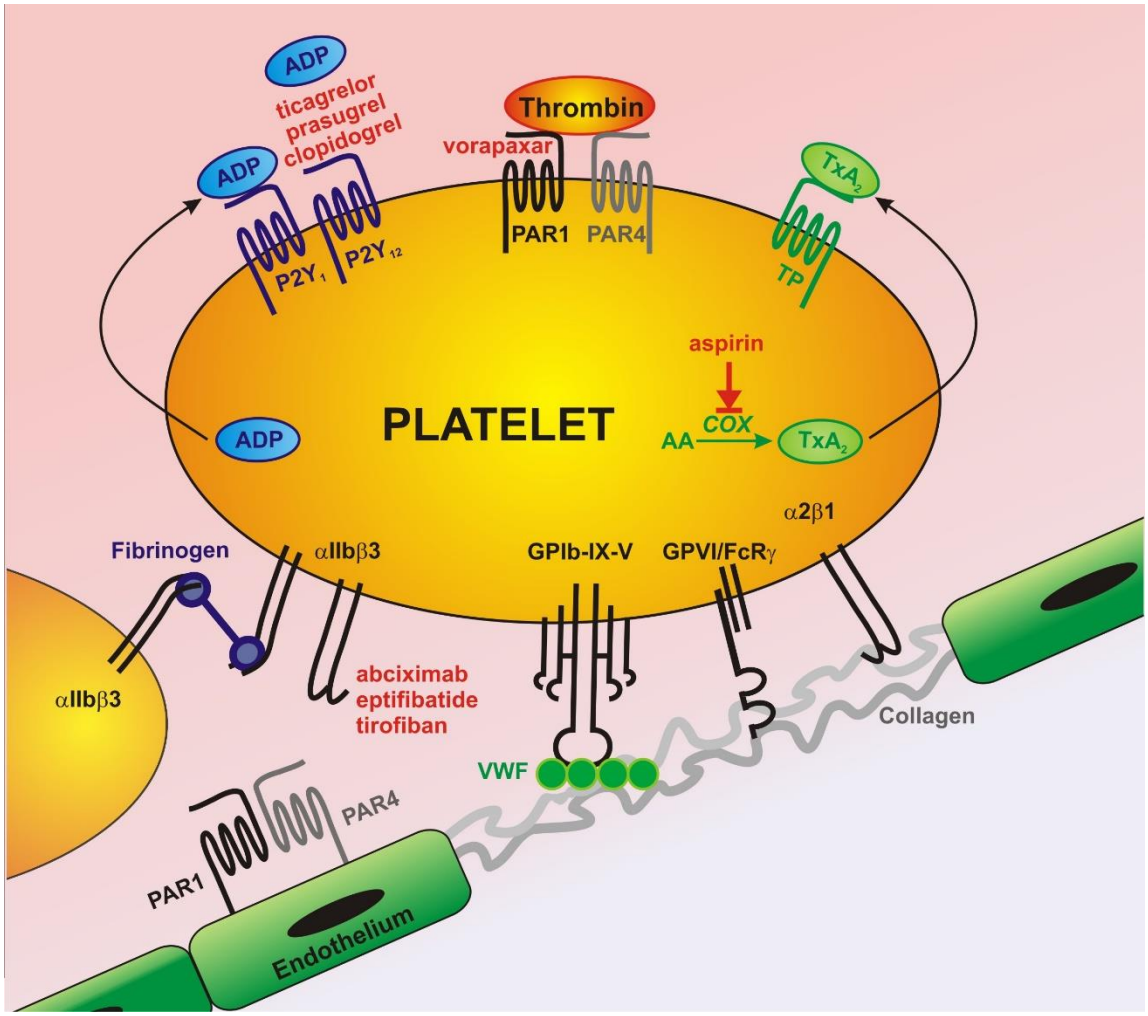


Figure 1. Activation and adhesion receptors on the platelet.

In response to vascular damage, platelets rapidly adhere to the vessel wall via the interaction of subendothelial von Willebrand factor (VWF, green spheres) with the platelet glycoprotein (GP) Ib-IX-V complex, and collagen (grey strands) with GPVI and integrin $\alpha_2\beta_1$. Adherent platelets become activated, and this activation is enhanced by signalling pathways initiated by: thromboxane receptors (TP) activated by thromboxane A_2 (TxA_2) generated from arachidonic acid (AA) by cyclooxygenase (COX); $P2Y_1$ and $P2Y_{12}$ receptors activated by ADP; and PAR1 and PAR4 activated by thrombin. Platelet activation culminates in the activation of integrin $\alpha_{IIb}\beta_3$ which binds fibrinogen (blue dumbbell), VWF and fibronectin (not shown) to mediate platelet aggregation. Current antiplatelet agents (in red) include the $P2Y_{12}$ inhibitors clopidogrel, prasugrel and ticagrelor; the COX inhibitor, aspirin; $\alpha_{IIb}\beta_3$ inhibitors, abciximab, eptifibatide and tirofiban; and the most recent addition, vorapaxar, which inhibits PAR1.

(Source: French et al., 2015, Appendix I)

The current guidelines for treatment of ACS and associated diseases are dual therapy with aspirin (an inhibitor of TxA_2 synthesis) and a $P2Y_{12}$ receptor antagonist, such as the thienopyridines, clopidogrel or prasugrel, or the cyclopentyl-triazolo-pyrimidine, ticagrelor (American guidelines tend to favour the use of clopidogrel, whereas European guidelines favour either ticagrelor or prasugrel, with clopidogrel as an alternative)[2]. However, aspirin and clopidogrel prevent just 15 and 17% of lethal cardiovascular events respectively and are only marginally more effective in combination[6-8]. In addition, an increasing number of patients are being reported as resistant to these agents[9, 10]. Large scale clinical trials assessing the efficacy of aspirin and clopidogrel (together and separately) in reducing thrombotic risk in cardiovascular disease (CAPRIE[7] and CHARISMA[8]) have ultimately determined greater efficacy when used in combination, with an associated minor increase in risk of bleeding complications. Antagonists of the major platelet integrin, $\alpha_{IIb}\beta_3$, such as tirofiban, eptifibatide, and abciximab, are by far the most potent platelet inhibitors as they inhibit platelet aggregation regardless of the activating pathway. However, these drugs all cause significant bleeding complications[11, 12] due to their disruption of the haemostatic function of platelets also dependent on this pathway. Therefore, existing antiplatelet therapies have limitations in one or both of safety and efficacy, with no current agent (or combination of agents) affording sufficiently potent, safe, and orally active prevention of arterial thrombosis. As a result, improved antiplatelet approaches are required to meet the significant clinical need for the safe and effective prevention of arterial thrombosis.

1.2 Targeting protease-activated receptors as an antiplatelet approach

1.2.1 Thrombin-induced platelet activation is important during thrombosis

Thrombin is a multifunctional allosteric serine protease which is by far the most critical non-cellular component of haemostasis. Thrombin interacts with a series of substrates via it's

activate site, located in the centre of the enzyme, and two exosites (exosite I and exosite II) located on either side of the active site. Generation of thrombin occurs via the coagulation cascade – a series of serine protease activation steps which ultimately produce and further amplify thrombin generation. In flowing blood, activated platelets are the main cellular contributors to thrombin amplification, via providing a phospholipid membrane surface for coagulation factor assembly. Once generated, thrombin is able to mediate platelet activation, while also engaging in the proteolysis of fibrinogen to facilitate stable thrombus formation.

The rationale for targeting thrombin-induced platelet activation as an antithrombotic approach has long held appeal. First, thrombin functions at a time and place that is predicted to provide safe and effective antiplatelet activity. As outlined above, thrombin, in combination with ADP and TxA_2, mediates the platelet activation required for thrombus growth that follows initial cell adhesion. Second, thrombin is the most potent endogenous activator of platelets. This suggests that targeting thrombin-induced platelet activation may provide greater efficacy over existing mechanisms that block the platelet activating functions of TxA_2 (aspirin) and ADP (clopidogrel and co.). Third, existing drugs that block the production (e.g. rivaroxaban) or activity (e.g. dabigatran) of thrombin are effective as antithrombotics. However these agents provide global inhibition of thrombin's actions and, consequently, incur a significant bleeding risk – particularly at the high doses used for the prevention of platelet-rich arterial thrombi. Specifically targeting thrombin-induced platelet activation whilst leaving the other functions of thrombin intact may mitigate the bleeding risk and provide a more selective effect for arterial thrombosis prevention. In combination, these factors provided the impetus to develop inhibitors of platelet thrombin receptors.

1.2.2 Thrombin activates platelets via protease-activated receptors

Platelet responses to thrombin are predominately mediated by protease-activated receptors (PARs)[13, 14]. PARs are G-coupled protein receptors (GPCRs) which are expressed on the surface of numerous cell types. In the cardiovascular system this includes platelets[14-17], but also leukocytes[18], vascular endothelial and smooth muscle cells[19, 20], cardiomyocytes[21], and cardiac fibroblasts[22]. Humans express four PARs, with PAR1, PAR3 and PAR4 being activated by thrombin[15-17], and PAR2 by trypsin, tryptase, coagulation factors VIIa and Xa, and membrane-bound serine proteases MTSP1 and TMPRSS2[23-25]. The cleavage-based PAR activation mechanism is unique. PARs are all activated the same way, whereby the protease agonist cleaves the amino-terminus of the receptor to reveal a cryptic neo-amino terminal sequence known as the "tethered ligand"[26]. The newly exposed tethered ligand then activates the receptor by binding intramolecularly to the second extracellular loop[26]. This self-activation prompts the conformational change of the receptor that allows interactions with G proteins of the G_q, $G_{12/13}$ and $G_{i/z}$ families and consequent intracellular signalling events[13, 27]. The interactions with $G_{12/13}$ drive Rho-dependent cytoskeletal responses involved in platelet shape change[28], whereas G_q-mediated signalling facilitates the processes important for platelet granule release, activation of cell surface integrins, and platelet aggregation[29]. $G_{i/z}$ proteins are involved in inhibition of adenylyl cyclase, thereby removing a brake on platelet activation, while the G_i protein family is involved in modifying activities of enzymes such as PI3 kinase[27].

Human platelets express two thrombin-sensitive PARs, PAR1 and PAR4, and activation of either receptor is capable of inducing platelet activation[14-16]. Thrombin-induced platelet activation initiates platelet shape change, promotes platelet aggregation, and provides the procoagulant lipid surface that facilitates secondary coagulation reactions[13, 30]. Although often described as a dual platelet thrombin receptor system, some distinctions between PAR1 and PAR4 have been shown. PAR1 is a "high affinity" thrombin receptor, as it contains a high

affinity thrombin binding domain (TBD) ($K^{51}YEPF^{55}$) which binds exosite I of thrombin and aligns the active site with the specific cleavage site in the receptor[31, 32]. PAR1 is activated by trace amounts of thrombin (sub-nM range) and is responsible for the initial and rapid rise in intracellular calcium (Ca^{2+}) induced in platelets by the coagulation protease[33]. However, the PAR1-induced signal is transient and requires additional support from other platelet agonists such as ADP[34]. In contrast to PAR1, the interaction between PAR4 and thrombin occurs primarily at the active site due to the absence of a high affinity TBD in the amino-terminal of PAR4. Despite the lack of interaction between PAR4 and exosite I of thrombin, the receptor does bind at the active site with high affinity due to two optimally-positioned proline residues immediately amino-terminal to the thrombin cleavage site[35]. This interaction facilitates slowed dissociation of thrombin from the receptor and results in a slower but more sustained intracellular signalling profile[35]. As a result of these distinct receptor activation and signalling kinetics, PAR4, in contrast to PAR1, is capable of inducing irreversible platelet aggregation in the absence of additional agonist activation[34]. It has therefore been suggested that PAR1 and PAR4 complement each other during thrombin-induced platelet aggregation. However, historically, the major clinical focus has been on inhibiting PAR1 for antithrombotic activity due to the significantly greater sensitivity of this receptor to thrombin.

1.2.3 Mice lacking platelet-thrombin receptor function are protected against thrombosis

As is the case in many other systems, mouse genetic models and other small animal *in vivo* thrombosis experiments provided pre-clinical proof-of-concept studies. However the interpretation of these studies has always been hampered by the differing platelet PAR profile in these traditional model systems. Of the most commonly used animal models, only non-human primates appear to have an identical platelet PAR profile to humans (PAR1 and PAR4)[36]. In contrast, platelets from mice[37], rats[38], and rabbits[39] express PAR3 and PAR4, while platelets from guinea-pigs express PAR1, PAR3 and PAR4[40]. Despite these limitations,

significant insights have been gained from mouse genetic experiments. Although mouse PAR4 functions in an analogous manner to human PAR4, mouse PAR3 is incapable of mediating transmembrane signalling by itself, instead functioning as a cofactor that facilitates cleavage and activation of PAR4 at low thrombin concentrations[41, 42]. As this model predicts, platelets from PAR4-/- mice are unresponsive to thrombin[37, 43] and provide a clean genetic model to examine the overall contribution of thrombin-induced platelet activation in (patho)physiology.

The first phenotype reported in PAR4-/- mice was protection against thrombosis, with mild associated bleeding[37]. Initially, it was shown that after a ferric chloride-induced injury of mouse mesenteric arterioles, the time to vessel occlusion was prolonged ~ 3 times in PAR4-/- compared to wild type mice[37]. Subsequent studies have shown a similar protection against thromboplastin-induced pulmonary embolism[41], laser-induced endothelial cell ablation in mesenteric arterioles[44] as well as electrolytic-[45] and trauma-induced[46] injury of the carotid artery in PAR4-/- mice. Bone marrow transplantation studies confirmed that the antithrombotic effects observed in PAR4-/- mice are due to deficiency of PAR4, and therefore thrombin signalling, specifically in platelets[43]. Intriguingly, it appears that the protection against thrombosis associated with PAR4 deficiency leaves other haemostatic responses intact[44]. PAR4 deficiency in mice is also associated with a mild bleeding phenotype. This haemostatic effect has been most commonly assessed via tail bleeding time, where PAR4-/- mice are consistently shown to have prolonged tail bleeding times compared with wild type mice[37, 41]. Despite this commonly-reported effect upon active limb trauma, there has been no evidence for spontaneous bleeding reported in PAR4-/- mice. PAR4 deficiency also has protective benefits in a mouse transient middle cerebral artery occlusion model of stroke, in which PAR4-/- mice exhibited lower cerebral infarct volume, improved neurologic and motor function, and reduced blood brain barrier disruption and cerebral oedema, compared with wild type animals[47]. Despite the limitations of using mice as a model of human platelet PAR function,

these early proof-of-concept studies provided insight into the relative importance of thrombin-induced platelet activation in the setting of *in vivo* thrombosis, and in large part drove the development of PAR antagonists as a novel antiplatelet approach.

1.2.4 Considerations of the first clinically approved PAR1 antagonist

In May 2014 the US FDA approved the first clinical PAR1 antagonist, vorapaxar, for use in the prevention of thrombotic cardiovascular events in patients with a history of myocardial infarction or peripheral artery disease. After showing promising results in phase 2 trials, vorapaxar was assessed in two large scale phase 3 trials in patients with ACS (TRACER[48]) and stable atherosclerosis (TRA 2^{o}P – TIMI 50[49]). The overall findings of these trials showed that vorapaxar, when combined with standard-of-care therapy, reduced the risk of cardiovascular events at the cost of increased bleeding[48, 49]. However, several key issues and a narrow therapeutic window have limited the clinical utility of vorapaxar.

The major concerns associated with vorapaxar use were increased rates of intracranial bleeding and poor compatibility with standard-of-care antiplatelet agents. During the TRACER trial, intracranial haemorrhage increased more than 5-fold in the vorapaxar group[48]. This effect was especially noted in patients with a prior history of stroke or transient ischaemic attack (TIA) and increased incrementally over time[48]. As a result, patients with a history of stroke or TIA were removed from the TRAP 2^{o}P – TIMI 50 trial, however intracranial haemorrhage was still increased two-fold in the vorapaxar group[49]. The trials showed that these bleeding rates increased in patients receiving vorapaxar in combination with a thienopyridine compared to those receiving a thienopyridine alone[48]. Further, in patients receiving high dose aspirin (> 300 mg daily at both baseline and time of discharge), a consistent, albeit non-statistically significant, trend toward higher bleeding and ischaemic outcomes was recorded[50]. It remains unknown whether the bleeding complications observed in these trials were due to PAR1 inhibition per se, the concurrent use of three antiplatelet drugs, or to a particular drug

combination. Regardless, overall, in patients with no history of stroke or TIA, and with a body weight above 60 kg, the data from TRA 2^{o}P – TIMI 50 translated into 6 fewer cardiovascular deaths at the cost of two intracranial haemorrhages for every 1000 patients treated with vorapaxar[51].

Despite these setbacks, PAR1 inhibition did demonstrate consistent efficacy in certain pathological conditions where thrombin-mediated platelet activation is known to play a significant role. For example, PAR1 inhibition demonstrated a consistent reduction in the rate of type 1 (spontaneous) MI in vorapaxar-treated patients across the phase 2 and phase 3 studies[52]. This is perhaps unsurprising given the well-known role of thrombin generation in acute MI, particularly in patients with a background of unstable angina and/or coronary artery disease[53-55]. Vorapaxar was also shown to reduce the complications associated with PAD, notably limb ischemia and the requirement for peripheral vascularisation[56]. One explanation for this effect is recent work which has shown that thrombin-dependent platelet activation (via PARs) is more prevalent at lower blood shear rates[46], presumably due to limited assembly of blood borne coagulation factors on the surface of activated platelets at higher blood flow/shear rates. Understanding the relative contribution of thrombin-mediated platelet activation to thrombus formation in particular pathologies will likely be of significant use in predicting the clinical success of PAR inhibitors. Further, the limitations of PAR1 antagonism (i.e. increased bleeding, poor compatibility with other agents) have led researchers to revisit PAR4 antagonism as a novel antithrombotic approach.

1.3 Is selectively targeting PAR4 a viable antiplatelet strategy?

1.3.1 PAR4 structure and biology

Despite being traditionally thought of as a “back-up” receptor to PAR1, PAR4 has several structural and biologically distinct characteristics to the rest of the PAR family. PAR4 was first

cloned in 1998[14, 16] and is the most recently cloned member of the PAR family. It is a 300 amino acid seven transmembrane-spanning domain GPCR (Figure 2) that retains most of the core features of the other PARs despite its genetic departure from the family[14, 16]. The human PAR4 gene is remarkably smaller than those of the other PARs, and it resides at a distinct location on chromosome 19p12, with the genes for PARs 1-3 located in tandem on chromosome 5q13[16, 57]. This genetic divergence of PAR4 from the other PARs is thought to have arisen from a remote gene duplication and subsequent translocation event which gave rise to ancestral PAR4 and PAR1/2/3 genes[57]. More recently, two gene duplication events have occurred to separate PAR1 and PAR2 from PAR3; and then PAR1 from PAR2[57]. These genetic differences may underlie some of the key differences in receptor structure and function within the PAR family.

The N-terminus of PAR4 contains a hydrophobic signal peptide sequence, with a signal peptidase cleavage site present at S^{17}/G^{18}. The extracellular amino-terminus also contains a serine protease cleavage site at R^{47}/G^{48} that is essential for proteolytic receptor activation. Mutation of the serine protease cleavage site ($R^{47} \rightarrow A$) renders the receptor completely unresponsive to proteolytic activation. Site-specific receptor cleavage unmasks a cryptic tethered ligand sequence (GYPGQV)[16] which then binds intra-molecularly to a defined region in the second extracellular loop of the receptor (Figure 2), resulting in conformational change of the receptor and subsequent coupling to intracellular effectors, as per other GPCR family members. While this proteolytic activation mechanism is common amongst PARs, there are several notable differences in the structure of PAR4 versus the other receptors of the family. First, both the extracellular amino terminus and intracellular carboxy terminus have little sequence similarity to the corresponding regions of other PARs[16]. Second, the tethered ligand binding site of PAR4 contains only three core amino acids (CHD; Figure 2) of the consensus

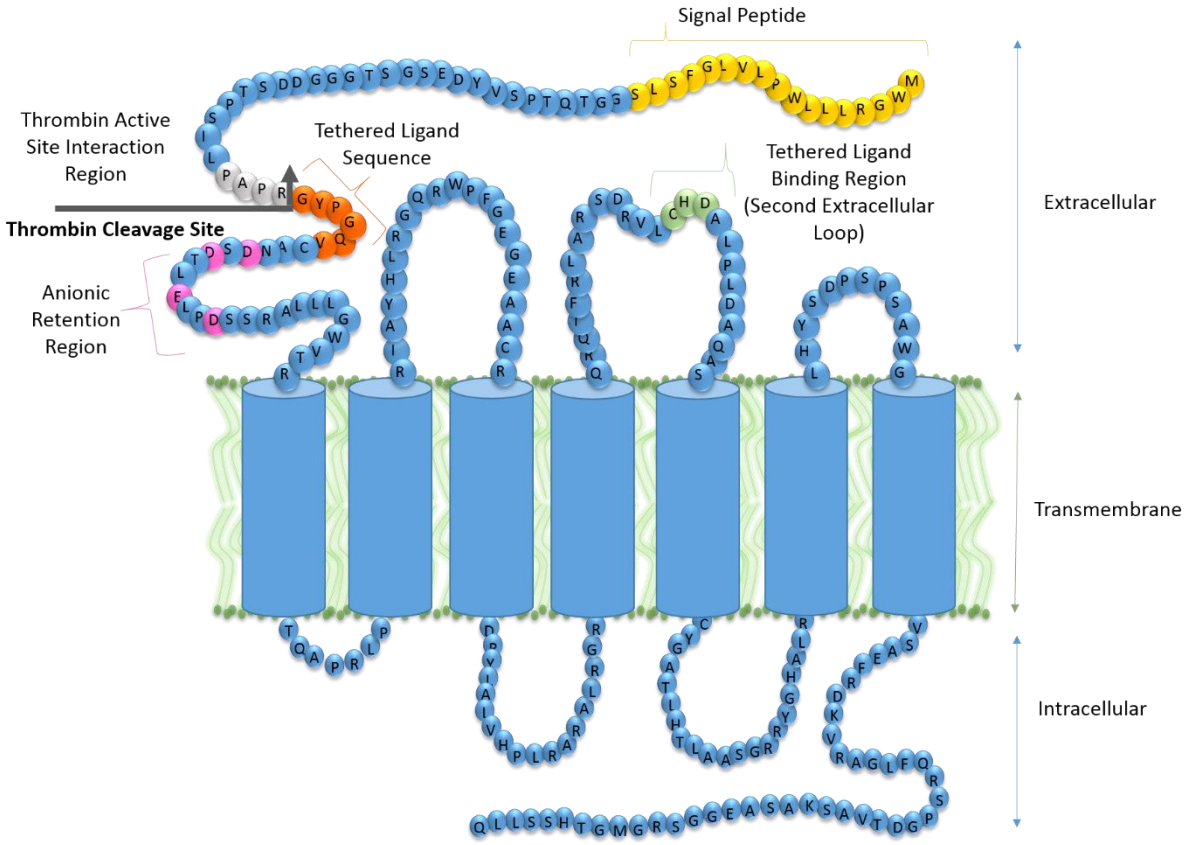


Figure 2. PAR4 structure. Depicted is a cartoon of the proposed structure of the 300 amino acid GPCR, PAR4. Site-specific proteolytic cleavage of the receptor's amino-terminus (thrombin cleavage site, R^{47}/G^{48}; black arrow) reveals a neo-amino-terminus (tethered ligand sequence; G^{48}YPGQV; orange) which binds intra-molecularly to the second extracellular loop of the receptor (tethered ligand binding region; green). PAR4 contains two regions for enhanced thrombin interaction – the anionic retention region (pink) which interacts with thrombin's exosite I; and the PAPR sequence (grey) which binds at the active site of thrombin with high affinity.

(Source: French and Hamilton, 2016, Appendix II)

sequence conserved in PARs 1-3 (ITTCHDV)[16]. PAR4 also lacks the high affinity thrombin binding domain that is present in the other two thrombin receptors, PAR1 and PAR3[14, 17, 58, 59].

PAR4 is broadly expressed, with gene expression by Northern blot most readily detected in the lungs, pancreas, thyroid, testis and small intestine[14, 16]. Moderate expression has also been detected in placenta, skeletal muscle, lymph nodes, adrenal gland, prostate, uterus and colon[14, 16]. In the nervous system, PAR4 protein and mRNA have been detected in rat dorsal root ganglion (DRG) non-neuronal cells (conversely, PARs 1-3 were detected in DRG neurons)[60]. Despite this vast expression profile of PAR4, most research has focussed on the physiological functions of this receptor in cardiovascular and inflammatory settings. In humans, key vascular cell types which express PAR4 include platelets[14, 16], leukocytes[61], endothelial cells[62], and smooth muscle cells[63-65]. In addition to the well-known pro-thrombotic and pro-inflammatory actions of PAR4-activating proteases, perhaps one reason for this is that several important animal models, including mice, rats, guinea pigs, rabbits, dogs and monkeys have similar PAR4 expression to humans throughout the vasculature (platelets are one example of such conserved PAR4 expression). Despite this relatively conserved expression of PAR4 in vascular cells, there is some evidence to suggest that PAR4 may function differently between species. Specifically, it is known that PAR4 contributes to thrombin responses of mouse endothelial cells[62], and, that PAR4 activation causes endothelium-dependent relaxation of rat aorta [63]. However, in human artery preparations as well as lung fibroblasts, these responses are only elicited when treated with additional inflammatory mediators[64, 66]. This distinct regulation of PAR4 expression and function between species remains an important consideration for interpretation of animal studies.

1.3.2 PAR4 activation and signalling: distinctions from PAR1

The setbacks of clinical PAR1 antagonists have sparked research interest into whether selective PAR4 antagonism may be an alternative approach to inhibiting thrombin-induced platelet

activation. Indeed, a significant line of evidence suggests that PAR4 signals differently and independently of PAR1 activation, and therefore potentially regulates distinct platelet activation mechanisms.

The serine proteases capable of cleaving PAR4 are generally key regulators of coagulation (e.g. thrombin and coagulation factor Xa) and/or inflammation (e.g. trypsin and cathepsin G released from neutrophils, and bacterial proteases such as gingipains)[14, 16, 37, 67]. Additional serine proteases that are specific for arginine or lysine cleavage include coagulation factors VIIa, IXa, Xa, XIa, urokinase, and plasmin; however, none of these have significant activity on PAR4, with the exception of factor Xa which showed small effects at non-physiological concentrations[16]. Initial studies indicated that PAR4 is activated by thrombin and trypsin at similar concentrations, with an EC_{50} of ~ 5 nM each[16] – significantly higher than the EC_{50} for thrombin at either PAR1 or PAR3 (~ 0.2 nM). The comparatively lower affinity of PAR4 for thrombin is a reflection of differences in the macromolecular association between the receptor and enzyme. As previously described, PAR1 is a high affinity thrombin receptor due to the presence of a thrombin binding domain (TBD) in its N-terminal exodomain[59]. The TBD sequence, $K^{51}YEPF^{55}$, interacts with exosite I of thrombin and upon binding causes significant allosteric effects essential for rapid association of thrombin[33]. PAR4 does not contain this TBD[16]. In fact, evidence indicates that PAR4 appears to have only limited interaction with exosite I of thrombin, since γ-thrombin (which lacks a functional exosite I) activates PAR4 as effectively as α-thrombin[16] and mutations in the exosite I of thrombin have significantly less effect on cleavage of PAR4 than of PAR1[68]. Instead, PAR4 primarily interacts with the active site of thrombin via two optimally positioned proline residues (P^{44} and P^{46}) in the receptor[35], just upstream of the thrombin cleavage site at R^{47}/G^{48}. A possible role for L^{43} in the thrombin-PAR4 interaction has also been identified, and believed to be in facilitating high-affinity binding of the PAR4 amino-terminal to thrombin's active site[35]. PAR4 also contains an anionic

cluster, $D^{57}…D^{59}...E^{62}…D^{65}$, just C-terminal of the thrombin cleavage site that is thought to interact with cationic residues that border exosite I of thrombin[68] and slow the dissociation rate of thrombin from PAR4[35]. This is reflected in the more sustained intracellular signals elicited by PAR4 activation compared with the more transient signals in response to PAR1 activation[69]. These differences in receptor structure and activation kinetics between PAR1 and PAR4 indicate the two receptors have the potential to initiate distinct intracellular signalling events.

Cleavage and activation of PARs prompts a conformational change in the receptor that allows G protein coupling and initiates multiple intracellular signalling events. G_q, G_i, and $G_{12/13}$ have all been demonstrated to mediate signals in response to activation of PARs, with PAR4 specifically being shown to couple to both G_q and $G_{12/13}$ family proteins[13, 70], but not $G_{i/o}$[71] (Figure 3). PAR4 coupling to $G_{12/13}$ initiates binding of RhoGEFs (guanine-nucleotide exchange factors that activate Rho) to the α-subunit. Consequent Rho activation induces a series of Rho-dependent cytoskeletal responses and PLC activation (Figure 3). For example, in platelets, activation of Rho-kinase dependent cytoskeletal responses via such $G_{12/13}$-mediated PAR4 signalling triggers platelet shape change[13, 72, 73] – one of the key initial events in platelet aggregation during thrombosis. Evidence also exists for PAR4 activation eliciting a prolonged signal via $G_{12/13}$, which is thought to involve the regulation of MLC phosphorylation and RhoA[74, 75]. PAR4 coupling to G_q causes intracellular Ca^{2+} mobilisation via activation of phospholipase Cβ and consequent phosphoinositide hydrolysis (Figure 3). PAR4-induced Ca^{2+} mobilisation promotes the activity of several Ca^{2+}-regulated kinases and phosphatases (e.g. MAP kinases, protein kinase Cs, phospholipase A2, and calpain)[13], with wide-ranging effects. For example, PAR4 activation mediates several key platelet responses that are induced by G_q-dependent Ca^{2+} mobilisation, including the secretion of platelet storage granules and integrin activation – both of which are critical for effective platelet aggregation[13, 76].

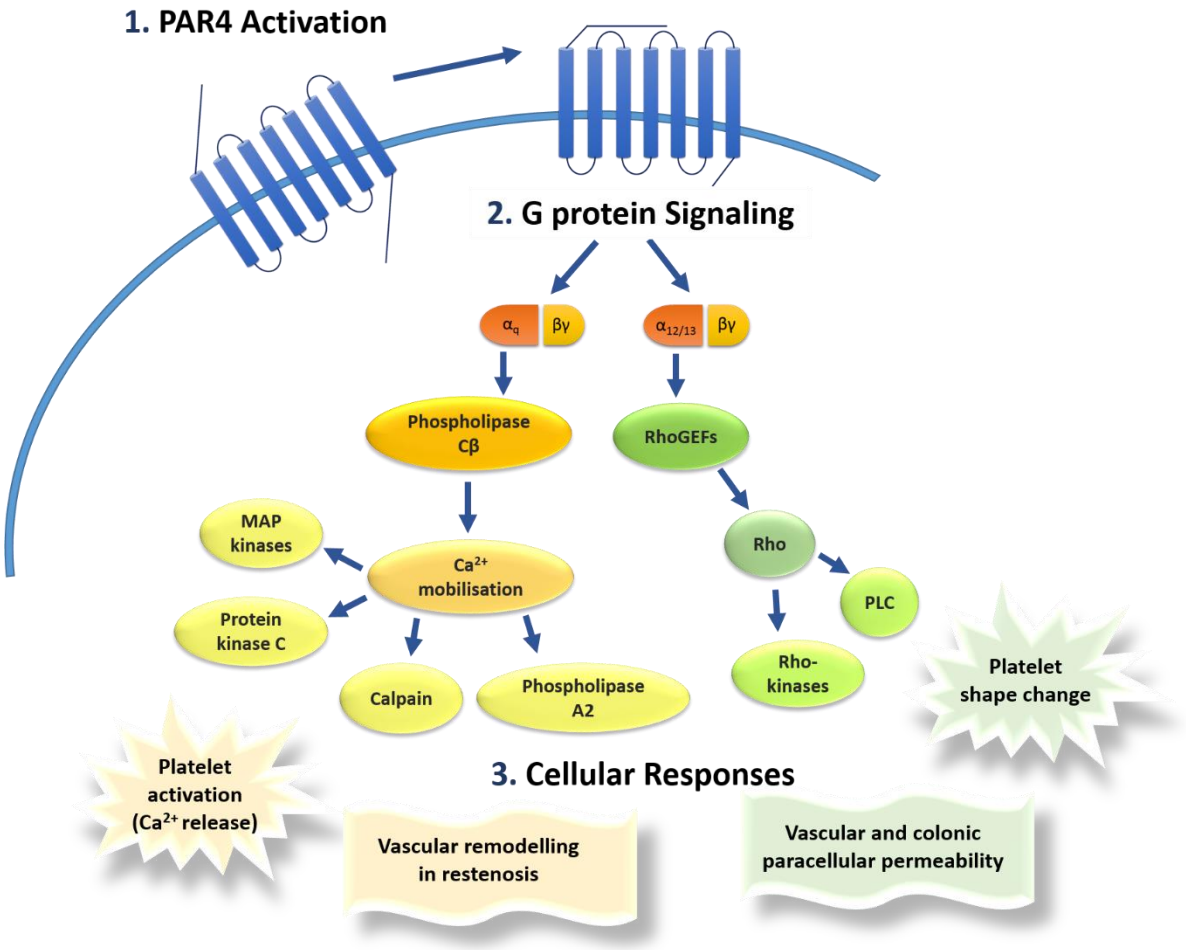


Figure 3. PAR4 signalling. Following cleavage and activation of the receptor, PAR4 signals via coupling to G_q and/or $G_{12/13}$ family members. The most well characterised signalling downstream of G_q is via phospholipase Cβ-mediated phosphoinositide hydrolysis and resultant intracellular Ca^{2+} mobilisation. This promotes the activity of several Ca^{2+}-regulated kinases and phosphatases (e.g. MAP kinases, protein kinase C, phospholipase A2, and calpain) which underlie PAR4-induced cellular responses such as platelet activation and vascular remodelling. The most well characterised signalling downstream of PAR4-$G_{12/13}$ coupling is via RhoGEF and consequent Rho activation, which underlie PAR4-induced cellular responses such as cytoskeletal responses in platelets and vascular smooth muscle and endothelial cells.

(Source: French and Hamilton, 2016, Appendix II)

As indicated above, one key difference between PAR4 and PAR1 is the kinetics of receptor activation and signalling. Regarding G_q-mediated signalling, it is known that PAR1 activation stimulates a rapid burst of intracellular Ca^{2+} mobilisation, whereas PAR4 activation elicits a slower rise which is much more sustained over time[69]. This is likely a result of the slower cleavage of PAR4 allowing prolonged G protein signalling. Given that mice deficient in G_q lack thrombin-dependent IP_3 and Ca^{2+} responses[29, 77] and that PAR4 activation stimulates ongoing IP_3 and DAG, differences in Ca^{2+} mobilisation are likely a result of prolonged G_q signalling through PAR4[34, 78]. It is thought that this may be important for ongoing cell signalling under conditions of prolonged agonist exposure. In platelets, PAR1-mediated Ca^{2+} signalling undergoes rapid desensitisation but can be rescued by subsequent PAR4 activation[79]. Receptor desensitisation is of particular importance for PARs due to the irreversible, cleavage-based, endogenous activation of these receptors, with continued responsiveness to agonists requiring new receptors to be trafficked to the cell surface. After receptor cleavage and activation, PARs are rapidly internalised on a phosphorylation signal in regions of the C-terminus of the receptor[80]. Yet PAR4 has a shorter C-terminus than PAR1 and does not have many of the phosphorylation sites shown to be necessary for desensitisation of these receptors[81]. As a result, agonist-triggered phosphorylation and consequent receptor internalisation is significantly slower for PAR4 than for PAR1[81], providing a further mechanism for the comparatively prolonged intracellular signalling downstream of PAR4 activation.

1.3.3 PAR4 antagonists

The unique structure and activation mechanism of PARs has long posed a problem for the development of inhibitors. In particular, activation by proteolytic cleavage is a highly efficient and irreversible system, while the tethered ligand-based activation mechanism requires antagonism of the binding of an agonist intrinsic to the receptor and presumably with

considerable steric advantage. These issues have hindered the development of PAR4 antagonists, although recent efforts have begun to overcome these challenges, with several distinct antagonist classes emerging (Table 1). This diverse set of PAR4 antagonists developed over recent years have been invaluable for investigations into the physiological roles of PAR4, and have also served to promote the idea of targeting this receptor as a novel therapeutic approach. However, there remains a lack of PAR4 antagonists that are able to demonstrate the necessary levels of specificity and efficacy required for clinical evaluation.

The earlier classes of PAR4 antagonists include peptidomimetics, pepducins and function-blocking antibodies. These inhibitors have been predominately research-focused. The first approach taken to identify potential inhibitors of PAR4, was the peptidomimetic approach which resulted in the generation of numerous peptide analogues based on the tethered ligand sequences of human, mouse, and rat PAR4. The resulting compound, tc-YPGKF-NH_2, appeared to bind but not activate PAR4 and was shown to abolish PAR4-activating peptide (PAR4-AP)-induced aggregation of rat platelets and significantly reduce thrombin-induced platelet aggregation, at very high concentrations (400 μM)[38]. tc-YPGKF-NH_2 has also been reported to inhibit thrombin-induced platelet aggregation in human platelets[82], although there is limited other evidence of this agent inhibiting human PAR4[83]. Pepducins are a distinct class of PAR4 inhibitors that mimic the region of the receptor that binds G proteins and essentially work by absorbing the interactions between the receptor and effector G protein[84]. The anti-PAR4 pepducins, P4pal-10 (pal-SGRRYGHALR-NH_2) and P4pal-i1[85], have been shown to inhibit ~85% of thrombin-induced aggregation of both human and mouse platelets. However, the specificity of these remain debatable, as they reportedly display a level of cross-reactivity with PAR1-AP-induced platelet activation[92] as well as collagen and TxA_2 [95]. Several function-blocking anti-PAR4 antibodies have also been developed and proven to be useful PAR4 antagonists. These include rabbit polyclonal antibodies against the thrombin cleavage site of

Table 1. Properties of experimental PAR4 antagonists.

Class	**Compound**	**Target**	**IC50**[Ŧ]	**Species**	**Cell Type**	**Reference**
Peptidomi -metics	tc-YPGKF-NH_2	TLS binding site	100 μM	Rat, human	Platelets	Hollenberg, 2001 [38] Hollenberg, 2004 [83] Ma, 2005 [82]
Small molecules	YD-3	TLS binding site	28 μM	Rabbit, rat, human	Platelets, smooth muscle cells	Wu, 2002 [86] Wu, 2003 [87] Peng, 2004 [88]
	19, 25, 31	TLS binding site	>36 μM	Human	Platelets	Huang, 2006 [89]
	ML354	TLS binding site	140 nM[#]	Human	Platelets	Young, 2013 [90] Wen, 2014 [91]
Pepducins	P4pal-10	Third-intracellular loop	1 μM	Mouse, human	Platelets	Covic, 2002a [84] Covic, 2002b [92]
	P4pal-i1	First-intracellular loop	5 μmol/L*	Guinea pigs, human	Platelets	Leger, 2006 [85]
Function-blocking antibodies	Rabbit polyclonal	Thrombin cleavage site	1 mg/mL*	Human, rat	Platelets, fibroblasts	Kahn, 1999 [30]
	Rabbit polyclonal (Can12)	Anionic region	10 ng/mL^{-1}	Mouse, human	Platelets	Mumaw, 2014 [93]
	Mouse monoclonal (14H6, 5F10)	Thrombin cleavage site	50 μg/mL* partial inhibition	Human	Platelets, HEK-293s	Mumaw, 2015 [94]

TLS = tethered ligand binding site
[Ŧ] IC_{50} values correspond to inhibition of thrombin-induced PAR4 activation
[#] IC_{50} values correspond to inhibition of PAR4 agonist peptide-induced activation
*Values are concentration reported to inhibit thrombin-induced PAR4 activation

(Source: Adapted from French and Hamilton, 2017, Appendix III)

PAR4[30] and anionic region of the receptor (C^{54}ANDSDTLTLPD), just downstream of the thrombin cleavage site[93]. However, despite being valuable research tools, these antibodies have also struggled to demonstrate efficacy[30] and specificity[93].

To date, the most widely used and desired inhibitor class are the small molecule PAR4 antagonists which have been developed via extensive screening of various heterocyclic structures. The first compound identified to selectively inhibit PAR4 was the indazole derivative YD-3 [1-benzyl-3(ethoxycarbonylphenyl)-indazole][86, 96]. YD-3 has been shown to inhibit thrombin-induced platelet aggregation in rabbits with an IC_{50} of 28 μM; however in humans, it was shown to only partially inhibit platelet aggregation in response to thrombin concentrations lower than 0.5 nM[86, 87]. Several studies have aimed to increase the efficacy of YD-3, and have produced several derivatives[89, 97], including the *N*^{2}-(substituted benzyl)-3-(4-methylphenyl)-*2H*-indazoles, compounds 19, 25, and 31[89], and ML354, a substituted indole derivative[90, 91]. However, these compounds have been shown to inhibit PAR1-AP induced activation with an IC_{50} of 10 μM, and demonstrate limited efficacy in inhibiting thrombin-induced PAR4 activation. Despite the challenges associated with developing suitable compounds for both research and therapeutic use, there remains a strong interest in continuing development of PAR4 antagonists.

1.3.3 Targeting PAR4 as an antithrombotic approach

During the course of this thesis, a number of major advances were made regarding the clinical significance of platelet PAR4 and the utility of PAR4 antagonists during arterial thrombosis. Firstly, the pharmaceutical sector developed a clinically viable small molecule PAR4 antagonist. In a recent study by Wong and colleagues[98], a library of over 1 million compounds was screened to identify a lead candidate that was then subject to iterative rounds of medicinal chemistry and testing to result in BMS-986120 – a potent and selective PAR4 antagonist with impressive oral bioavailability and antithrombotic efficacy (Table 2). In contrast to previously

generated antagonists, BMS-986120 demonstrated specificity in human platelets, and effectively suppressed platelet aggregation in response to thrombin with concomitant PAR1 inhibition. Further, binding studies revealed that BMS-986120 is a high affinity and reversible binder of PAR4, which translated to normalised aggregation 24 h after a single dose of 0.2 mg/kg. Most importantly though, BMS-986120 appeared to provide an impressive therapeutic window, with a single oral dose of BMS-986120 providing marked antithrombotic effects and a low bleeding profile in a series of *in vivo* models in the cynomolgus monkey. Importantly, when compared directly with clopidogrel, at doses of these two agents that caused equivalent antithrombotic effects, markedly more bleeding was observed with clopidogrel compared with BMS-986120. This work indicates that PAR4 antagonism exhibits a markedly improved therapeutic window over at least one of the standard antiplatelet drugs.

The second major advance to occur during this thesis regarding the clinical utility of targeting PAR4 for arterial thrombosis prevention was the identification of a sequence variant in PAR4 that affects the receptor's pharmacology[99-101]. Specifically, platelets from healthy North American black subjects were shown to be hyper-responsive to PAR4 activation when compared with platelets from non-blacks. This increased sensitivity persisted in the face of PAR1 antagonism (vorapaxar) or aspirin treatment and led to the identification of a genetic variant of PAR4, Ala^{120}Thr, which is associated with greater PAR4 reactivity and occurs at a much higher frequency in blacks versus non-blacks. This Thr^{120} PAR4 variant, expressed in 20 – 80% of people depending on the population, markedly impacts PAR4 pharmacology by rendering the receptor hyper-sensitive to agonists and hypo-sensitive to antagonists[100, 101]. The mechanism behind this change in PAR4 pharmacology remains unknown, as does whether all PAR4 antagonists will be similarly affected and whether this sequence variant is associated with poorer cardiovascular outcomes. Studies directly addressing these points will be critical

Table 2. Pharmacological characteristics of a leading PAR4 antagonist, BMS-986120.

	Cell	*Parameter measured*	*BMS-986120*
Binding studies	PAR4-HEK293T membranes	K_D	0.098 nM
		K_{on}	0.12 nM
		K_{off}	0.0082 nM
Selectivity/specificity studies	HEK293-PAR4 + PAR4-AP	Ca^{2+} mobilisation (PAR4-AP)	0.56 nM
	HEK293-PAR1 + PAR1-AP	Ca^{2+} mobilisation (PAR1-AP)	>5000 nM
	CHO-PAR2 + PAR2-AP	Ca^{2+} mobilisation (PAR2-AP)	>42000 nM
Inhibition of PAR4 signaling	PAR4-HEK293T	G-protein activation	Gα11 – 3.4 nM Gαq – 3.9 nM Gα14 – 31 nM
		β-arrestin 2 recruitment	7.2 nM
		ERK 1/2 activation	47 nM
Platelet aggregation	Platelet-rich plasma (human)	γ-thrombin	7.3 nM
	Whole blood (human)	PAR4-AP	9.5 nM
	Whole blood (monkey; *ex vivo*)	PAR4-AP	2.1 nM
		PAR1-AP	No effect (1 mg/kg)
		Collagen	No effect (1 mg/kg)
		ADP	No effect (1 mg/kg)
Coagulation studies	Whole blood (monkey; *ex vivo*)	APTT	No effect (0.2-1 mg/kg)
		PT	No effect (0.2-1 mg/kg)
		TT	No effect (0.2-1 mg/kg)
In vivo *thrombosis*	Whole blood (monkey)	Occlusion time	0.2mg/kg - ~75 min 0.5mg/kg - ~75 min 1mg/kg – did not occlude
		Thrombus weight (% reduction from vehicle)	0.2mg/kg - -36% 0.5mg/kg - -50% 1mg/kg – -82%
In vivo *haemostasis*	Whole blood (monkey)	Kidney bleeding time (fold above vehicle control)	0.2mg/kg – 1.4X 0.5mg/kg – 1.9X 1mg/kg – 2.2X
		Mesenteric bleeding time	0.2mg/kg – 1.4X 0.5mg/kg – 1.7X 1mg/kg – 1.8X

PAR-AP = protease-activated receptor-activating peptide; HEK = human embryonic kidney; CHO = Chinese hamster ovary; ERK = Extracellular signal-related kinase; ADP = adenosine diphosphate; APTT = activated partial thromboplastin time; prothrombin time; TT = thrombin time.

(Source: Adapted from French and Hamilton, 2017, Appendix III)

in determining the approach to PAR4 antagonism that will afford consistent antithrombotic benefit across the population.

1.4 Aims of this thesis

As discussed above, thrombin-induced platelet activation is a key driver in the formation of the arterial thrombi that cause two of the most lethal manifestations of cardiovascular disease – acute myocardial infarction and occlusive stroke. However, there remains much to learn regarding how to best manipulate thrombin signalling in platelets for safe and effective antithrombotic therapy. Despite known differences in receptor structure and signalling, the specific function of PAR4 during platelet activation and thrombus formation in humans has gone largely unstudied, in large part due to several crucial limitations hindering research into PARs. The purpose of this project was to examine whether targeting PAR4 is a valid antithrombotic approach by examining the relative roles of platelet PAR1 vs PAR4 in the setting of thrombosis, and determining whether PAR4 antagonism is an effective *and* safe strategy for the prevention of arterial thrombosis. Specifically, this thesis aimed to:

1: Determine the relative roles of PAR1 and PAR4 on human platelets in the setting of thrombosis (Chapter 2).

2: Assess the safety and efficacy of PAR4 inhibition *in vivo* using animal models (Chapters 3 and 4).

3: Examine the utility of PAR4 antagonism for the prevention of human thrombosis (Chapter 5).

Sections of this chapter have been published as:

French SL, Arthur JF, Tran HA, Hamilton JR. Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Reviews*, 2015, 29 (3):179-89. (See Appendix I)

French SL, Hamilton JR. Protease-activated receptor 4: from structure to function and back again. *British Journal of Pharmacology*, 2016, 173:2952-65. (See Appendix II)

French SL and Hamilton JR. (2017) Drugs targeting PAR4 improve the antithrombotic therapeutic window. *Annals of Translational Medicine*, doi: 10.21037/atm.2017.09.10 (See Appendix III)

Chapter 2 – Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood

2.1 Introduction

The overall goal of this thesis was to examine the validity and utility of blocking PAR4 as a novel antithrombotic approach. As a first step, the specific functions of PAR4 on human platelets during thrombosis were determined. At the time of these studies, suitably specific and effective inhibitors of PAR4 were not freely available. Therefore this thesis began by developing a function-blocking anti-PAR4 antibody and then using it to probe for platelet activation events reliant on PAR4.

Defining the relative functions of the two platelet PARs is of patent interest for any rationalisation of PAR4 as an antiplatelet drug target. Whether PAR4 performs distinct functions to PAR1 on human platelets or simply acts in a redundant manner as a 'back-up' receptor has long been debated, although recent studies suggest the former alternative is more likely. As outlined in Chapter 1, one major distinction between the two platelet PARs relates to the kinetics of intracellular signalling, where PAR4 activation induces a slower and more sustained intracellular Ca^{2+} signal compared with PAR1 activation. This temporal difference in Ca^{2+} signalling was recently shown to prolong the thrombin-PAR4 interaction and facilitate persistent receptor activation. However, the cellular consequences of such sustained platelet activation downstream of PAR4 have not yet been completely characterised – most likely due to the limited availability of appropriate PAR4 antagonists required for such studies. The studies in this Chapter developed such an antagonist and then went on to directly address this question.

The findings from this Chapter demonstrate that thrombin-dependent platelet procoagulant activity – and consequent thrombin generation and fibrin formation – is mediated

predominantly by PAR4 and is largely independent of PAR1 in the setting of thrombus formation.

These findings suggest that PAR1 and PAR4 have distinct functions in platelets, and that the function of PAR4 may be important for thrombus formation and/or stabilisation. These findings suggest blocking PAR4 function may have utility in the prevention of arterial thrombosis and help rationalise the development of PAR4 antagonists for this purpose.

This chapter has been published as:

French SL, Arthur JF, Lee H, Nesbitt WS, Andrews RK, Gardiner EE, Hamilton JR. Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood. *Journal of Thrombosis and Haemostasis*, 2016, 14(8): 1642-54.

Journal of Thrombosis and Haemostasis, **14**: 1642–1654 DOI: 10.1111/jth.13293

IN FOCUS

Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood

S. L. FRENCH,* J. F. ARTHUR,* H. LEE,* W. S. NESBITT,*† R. K. ANDREWS,* E. E. GARDINER* and J. R. HAMILTON*

**Australian Centre for Blood Diseases, Monash University; and †Microplatforms Research Group, School of Engineering, RMIT University, Melbourne, Australia*

To cite this article: French SL, Arthur JF, Lee H, Nesbitt WS, Andrews RK, Gardiner EE, Hamilton JR. Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood. *J Thromb Haemost* 2016; **14**: 1642–54.

See also Lindahl TL, Macwan AS, Ramström S. PAR4 is more important than PAR1 for the thrombin-induced procoagulant effect on platelets. This issue, pp 1639–41.

Essentials

- The platelet thrombin receptor, PAR4, is an emerging anti-thrombotic drug target.
- We examined the anti-platelet & anti-thrombotic effects of PAR4 inhibition in human blood.
- PAR4 inhibition impaired platelet procoagulant activity in isolated cells and during thrombosis.
- Our study shows PAR4 is required for platelet procoagulant function & thrombosis in human blood.

Summary. *Background:* Thrombin-induced platelet activation is important for arterial thrombosis. Thrombin activates human platelets predominantly via protease-activated receptor (PAR)1 and PAR4. PAR1 has higher affinity for thrombin, and the first PAR1 antagonist, vorapaxar, was recently approved for use as an antiplatelet agent. However, vorapaxar is contraindicated in a significant number of patients, owing to adverse bleeding events. Consequently, there is renewed interest in the role of platelet PAR4 in the setting of thrombus formation. *Objectives:* To determine the specific antiplatelet effects of inhibiting PAR4 function during thrombus formation in human whole blood. *Methods and Results:* We developed a rabbit polyclonal antibody against the thrombin cleavage site of PAR4, and showed it to be a highly specific inhibitor of PAR4-mediated platelet function. This function-blocking anti-PAR4 antibody was used to probe for PAR4-dependent platelet functions in human isolated platelets in the absence and presence of concomitant PAR1 inhibition. The anti-PAR4 antibody alone was sufficient to abolish the sustained elevation of cytosolic calcium level and consequent phosphatidylserine exposure induced by thrombin, but did not significantly inhibit integrin $\alpha_{IIb}\beta_3$ activation, α-granule secretion, or aggregation. In accord with these *in vitro* experiments on isolated platelets, selective inhibition of PAR4, but not of PAR1, impaired thrombin activity (fluorescence resonance energy transfer-based thrombin sensor) and fibrin formation (anti-fibrin antibody) in an *ex vivo* whole blood flow thrombosis assay. *Conclusions:* These findings demonstrate that PAR4 is required for platelet procoagulant function during thrombus formation in human blood, and suggest PAR4 inhibition as a potential target for the prevention of arterial thrombosis.

Keywords: antiplatelet drugs; platelets; protease-activated receptors; thrombin; thrombosis.

Correspondence: Justin Hamilton, Australian Centre for Blood Diseases, Monash University, L2, AMREP Building, The Alfred Commercial Rd, Melbourne, Victoria, 3004, Australia.
Tel.: +61 3 9903 0122; fax: +61 3 9903 0228.
E-mail: justin.hamilton@monash.edu

Received 4 September 2015
Manuscript handled by: J. Heemskerk.
Final decision: P. H. Reitsma, 26 January 2016.

Introduction

Activated platelets are the key cellular components of arterial thrombosis – the most common cause of death and disability in the world [1,2]. Platelets are activated by a combination of endogenous agonists that trigger platelet aggregation and the promotion of coagulation, which together facilitate pathologic thrombus formation. Antiplatelet drugs therefore constitute the main pharmacotherapy for the prevention of arterial thrombosis. However, despite an array of such agents, limitations in safety and/or efficacy necessitate the rationalization of new antiplatelet drug targets.

Thrombin is the most potent known activator of human platelets, and is also the key effector protease of the coagulation cascade. Thrombin activates platelets predominantly via protease-activated receptors (PARs) [3–9]. Mice lacking all platelet PAR function ($PAR4^{-/-}$) are protected against thrombosis without showing spontaneous bleeding [10–15], indicating the potential of targeting these receptors for antithrombotic therapy. There are two PARs on human platelets, PAR1 and PAR4. Of these, PAR1 is the higher-affinity thrombin receptor and has been the focus of antiplatelet drug development with two PAR1 antagonists, atopaxar (E5555) [16] and vorapaxar [17,18], having been evaluated in clinical trials. Vorapaxar was recently granted Food and Drug Administration approval for use in the USA for the prevention of thrombotic events in patients with a history of myocardial infarction or peripheral artery disease [19]. However, vorapaxar in combination with single or dual antiplatelet therapy was also associated with significantly increased rates of intracranial bleeding, particularly in patients with a history of stroke or other predisposing factors [17,18]. These observations suggest that there remains much to learn regarding how to best manipulate thrombin signaling in platelets for safe and effective antithrombotic therapy. With this in mind, there has been renewed interest in the function of PAR4 on human platelets in the setting of thrombus formation.

Whether PAR4 performs distinct functions from PAR1 on human platelets or simply acts in a redundant manner as a 'back-up' receptor has long been debated, although recent studies suggest that the former alternative is more likely [20–25]. One major distinction between the two platelet PARs relates to the kinetics of intracellular signaling [20,21,26–28]. Both PAR1 and PAR4 signal via Gq to mobilize intracellular calcium and drive platelet functions, including integrin activation, granule secretion, and phosphatidylserine (PS) exposure. However, PAR4 activation induces a slower and more sustained intracellular calcium signal than PAR1 activation [26,29]. This temporal difference in calcium signaling may be attributable, in part, to an anionic sequence C-terminal to the PAR4 cleavage site that was recently shown to prolong the thrombin–PAR4 interaction and facilitate persistent receptor activation [29]. The cellular consequences of such sustained platelet activation downstream of PAR4 have not yet been completely characterized, but may involve sustained platelet-secretion kinetics [30] and platelet procoagulant function, given the reliance of these phenomena on sustained, elevated, intracellular calcium levels [31,32]. This hypothesis is supported by studies showing that selective activation of PAR4, but not of PAR1, results in the release of coagulation factor V from α-granules [22], microparticle shedding [22], and partially sustained Akt phosphorylation [33].

Despite this previous work, there have been no studies examining the contribution of PAR4 to procoagulant activity in the setting of human thrombus formation. This may be because of the limited availability of appropriate PAR4 antagonists required for such studies. The most commonly used PAR4 antagonists are the small molecule YD-3 [34], the peptidomimetic tc-YPGKF-NH_2 [35], and the pepducins P4pal-10 and P4pal-i1 [36–38]. However, these agents are either not widely available (YD-3) or have been reported to lack specificity (pepducins) and/or efficacy (tc-YPGKF-NH_2) in studies using human platelets [39–41].

In order to determine the impact of selectively inhibiting PAR4, we developed a function-blocking anti-PAR4 antibody, and used it to probe for platelet activation events that are reliant on PAR4. Selective inhibition of PAR4 with the anti-PAR4 antibody, but not selective inhibition of PAR1 with atopaxar, impaired platelet procoagulant activity in human isolated platelets and in a whole blood thrombosis assay. Defining the relative functions of the two platelet PARs is of patent interest for any rationalization of PAR4 as an antiplatelet drug target. Our findings demonstrate that thrombin-dependent platelet procoagulant activity – and consequent thrombin generation and fibrin formation – is mediated predominantly by PAR4 and is largely independent of PAR1 in the setting of thrombus formation. These findings suggest that PAR1 and PAR4 have divergent functions on platelets that may be important for thrombus formation and/or stabilization, and that PAR4 antagonists may have additional and/or distinct utility in the prevention of arterial thrombosis.

Materials and methods

Materials

A rabbit polyclonal anti-PAR4 antibody was raised against a 21-residue peptide with the sequence GGDDSTPSILPAPR$^{\downarrow}$GYPGQVC [42], matching residues 34–54 of human PAR4, which spans the thrombin cleavage site (indicated by $^{\downarrow}$ in the peptide sequence), as previously described [43]. All antibody production protocols were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Antibodies in pooled sera from immunized rabbits were affinity-purified with an AffiGel 10–15 column (BioRad, Hercules, CA, USA) conjugated with the antigen peptide. Preimmune antibody was isolated from sera with protein G–Sepharose (BioRad). The following reagents were stored according to the manufacturers' recommendations: E5555 (atopaxar, gift from P. Little, University of Queensland Brisbane, QLD, Australia), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Millipore, Lake Placid, NY, USA), FITC-conjugated PAC-1 antibody (BD Biosciences, San Jose, CA, USA), AK4 anti-P-selectin antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), FITC-conjugated anti-mouse antibody (Millipore),

Alexa Fluor 488-conjugated annexin-V (Sigma, St Louis, MO, USA), anti-CD9–phycoerythrin (PE) (BD Biosciences), anti-CD41a antibody (BD Biosciences), Alexa Fluor 488-conjugated anti-fibrin antibody (clone 59D8 [44,45]; generous gift from V. Chen, University of New South Wales, Sydney, NSW, Australia), Oregon Green BAPTA-1 AM, Fura Red AM, DM-BAPTA AM (all from ThermoFisher Scientific, Waltham, MA, USA), human α-thrombin (Sigma; activity expressed in NIH Units [U] obtained by direct comparison with NIH thrombin reference standard), PAR4-activating peptide (PAR4-AP; AYPGKF), PAR1-activating peptide (PAR1-AP; TFLLR) (both from Auspep, Melbourne, Victoria, Australia), ADP (Sigma), cross-linked collagen-related peptide (CRP) (from R. Farndale, University of Cambridge, Cambridge, UK), calcium ionophore A23187 (Sigma), bovine type 1 collagen (Sigma), and hirudin (lepirudin; Celgene, Melbourne, Victoria, Australia). A fluorescence resonance energy transfer (FRET)-based thrombin activity sensor (Thr-SP, a generous gift from S. Diamond, University of Pennsylvania, Philadelphia, PA, USA), was linked to the anti-CD41a antibody via CLICK chemistry as previously described [46], to examine thrombin activity in human whole blood under flow.

Human blood samples

All human studies were approved by the Monash University Human Research Ethics Committee. Blood was collected after informed consent had been obtained from healthy adults (aged 21–50 years, of both sexes) who had not taken antiplatelet medications in the past 10 days. Blood was drawn from the antecubital vein with a 19-gauge butterfly needle into syringes containing either one-seventh volume acid citrate dextrose (ACD) (7 : 1 v/v, final concentration) for platelet isolation, or one-tenth volume trisodium citrate (0.32% w/v, final concentration) for whole blood flow experiments, as previously described [47].

Mice

All mouse studies were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Mice in these studies were either PAR4-deficient ($PAR4^{-/-}$) [10] or wild-type ($PAR4^{+/+}$). Mouse whole blood was collected into ACD (7 : 1 v/v, final concentration), and platelets were isolated as previously described [47].

Detection of PAR4 via flow cytometry

Human or mouse washed platelets (5×10^7 mL^{-1}) were incubated with the anti-PAR4 antibody (0.1 mg mL^{-1}) for 30 min at 37 °C, and then fixed with paraformaldehyde (1% v/v, final concentration). The suspension was then centrifuged at 1000 × *g* for 2 min to obtain the platelet pellet, which was then resuspended in modified Tyrode's buffer (12 mM $NaHCO_3$, 10 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 5.5 mM D-glucose, 1 mM $CaCl_2$) containing a 1 : 50 dilution of an FITC-conjugated anti-rabbit IgG. After 30 min at room temperature, the samples were centrifuged again, and the platelet pellet was resuspended in modified Tyrode's buffer and analyzed by use of a flow cytometer (FACSCalibur; BD Biosciences).

Platelet aggregation

Human isolated platelets (3×10^8 mL^{-1}) were pretreated for 10 min at 37 °C with dimethylsulfoxide (DSMO) (0.1% v/v), the PAR1 antagonist E5555 (0.1 μM; minimum concentration required for inhibition of PAR1-AP [48]), the anti-PAR4 antibody (0.1 mg mL^{-1}), or a combination of E5555 and the anti-PAR4 antibody. Platelets were treated with one of thrombin (0.1 or 1 U mL^{-1}), PAR4-AP (100 μM), PAR1-AP (10 μM), ADP (10 μM), or CRP (10 μg mL^{-1}). Platelet aggregation was measured with a platelet aggregometer (Helena Laboratory, Beaumont, TX, USA).

Microplate-based platelet aggregometry

Human and mouse washed platelets were adjusted to a concentration of 2×10^8 platelets mL^{-1} in Tyrode's buffer supplemented with 1.8 mM $CaCl_2$, 10% bovine serum albumin, and 0.02 U mL^{-1} apyrase. Platelets (100 μL) were pipetted into each well of a clear, flat-bottomed 96-well tissue culture plate (BD Falcon (TM); BD Biosciences). Buffer alone was set to the maximum aggregation level, and unstimulated platelets were used set to the baseline light transmission (no aggregation). Agonists were added, and the plate was analyzed in a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) plate reader with a 595-nm excitation filter. After each reading cycle, the plate was subjected to a 5-min shake period (double orbital shake; shaking width of 4 mm), and this was repeated for a total of 10 cycles. Platelets were maintained at 37 °C throughout the experiment with the in-built incubator in the plate reader. Ability to aggregate was calculated as $\frac{OD(NoAgonist)-OD(Agonist)}{OD(NoAgonist)-OD(blank)\times 100}$ at the time point when aggregation was at a maximum.

Platelet calcium signaling dynamics

Calcium flux was measured in human isolated platelets with a dual-dye (ratiometric) microimaging assay, as previously described [49]. Briefly, isolated human platelets (3×10^8 mL^{-1}) were loaded with Oregon Green BAPTA-1 AM (0.625 μM) and Fura Red AM (0.625 μM) at 37 °C for 30 min, as in [49]. Dye-loaded platelets were subsequently treated with PAR antagonists (as above), and stimulated with thrombin (1 U mL^{-1}). Cells were monitored in real time, and emission at 500–570 nm and

600–710 nm was recorded every 10 s for 10 min (Nikon A1r). The increase in cytosolic calcium concentration (nM) was determined according to [49].

Platelet activation events

The platelet activation events measured were integrin activation (PAC-1 antibody binding), P-selectin expression (AK4 antibody binding), and PS exposure (annexin-V binding). Human isolated platelets (5×10^7 mL^{-1}) were pretreated with PAR antagonists as described above, and incubated with either FITC-conjugated PAC-1 antibody (1 : 100), AK4 anti-P-selectin antibody (1 : 100) followed by an FITC-conjugated anti-mouse antibody (1 : 10 000), or Alexa Fluor 488-conjugated annexin-V (1 : 100), prior to stimulation with either PAR1-AP, PAR4-AP, thrombin, CRP, or calcium ionophore A23187, as described above. After stimulation, platelets were resuspended in modified Tyrode's buffer for flow cytometry analysis (FACSCalibur; BD Biosciences).

Whole blood thrombosis assay

Human whole blood collected in citrate (3.2%) was preincubated for 15 min at 37 °C with both PE-conjugated anti-CD9 antibody (4 μg mL^{-1}) and Thr-SP (5 μM), and one of hirudin (800 U mL^{-1}), DMSO (1% v/v), PAR-1 antagonist E5555 (1 μM), anti-PAR4 antibody (0.2 mg mL^{-1}), or the combination of both PAR inhibitors. In parallel experiments, an Alexa Fluor 488-conjugated anti-fibrin antibody (5 μg mL^{-1}) was substituted for the thrombin sensor in order to analyze fibrin deposition. Whole blood was recalcified with 5–7.5 mM $CaCl_2$ (final concentration) to initiate coagulation, and drawn over glass microslides (1 × 0.1-mm internal diameter; Vitrotubes, Vitrocom, NJ, USA) coated with bovine type 1 collagen (250 μg mL) by use of a Harvard pump (Instech Laboratories, Plymouth Meeting, PA, USA) at a fixed flow rate of 0.06 mL min^{-1}, resulting in a wall shear rate of 600 s^{-1}. Dual-color confocal fluorescence images were recorded at excitation wavelengths of 488 nm and 561 nm, and collected through a × 40 water immersion objective. Confocal *Z*-stacks were continuously recorded (16-bit images of 512 × 512 pixels; 317 × 317 μm, *Z*-step 0.5 μm; Nikon A1r, with NIS software) for 2 min, and modified, calcium-free Tyrode's buffer was then passed over the thrombi, and *Z*-stacks encompassing the entire height of the thrombus field were recorded over a period of 10 min. Offline analysis of thrombi parameters was performed with NIS software. Image series were initially thresholded empirically, and the same threshold was then applied to all subsequent experiments, as identical experimental and confocal settings were used throughout. Platelet thrombi were defined by the use of anti-CD9–PE, and thrombin activity and fibrin volume were quantified according to the average fluorescence of the thrombus field. Data were normalized against the hirudin baseline, and expressed as a percentage of the control.

Statistical analyses

Statistical analyses were performed with GRAPHPAD PRISM (version 6.0, La Jolla, CA, USA). Significance was defined at $P < 0.05$ as determined with either an unpaired, two-tailed Student's *t*-test or one-way ANOVA with Fisher's LSD test for multiple comparisons, as advised and indicated in the relevant figure legends.

Results

Selective inhibition of PAR4-mediated platelet activation by an anti-PAR4 antibody

A rabbit polyclonal anti-PAR4 antibody was raised against a peptide with sequence matching a region spanning the thrombin cleavage site of human PAR4 (Fig. 1A). Affinity-purified anti-PAR4 antibody recognized both human and mouse PAR4 on platelets as assessed by flow cytometry. The anti-PAR4 antibody produced a consistent rightward shift in fluorescence intensity as compared with the same concentration of preimmune rabbit IgG in platelets isolated from either humans or wild-type mice (Fig. 1B). This signal was entirely absent in platelets isolated from PAR4$^{-/-}$ mice (Fig. 1B), indicating that the antibody selectively binds to PAR4 on the native platelet surface.

The inhibitory function of the anti-PAR4 antibody was first assessed via aggregation of human washed platelets. Such studies were confounded by the requirement to use thrombin as the agonist (the anti-PAR4 antibody is predicted to inhibit thrombin-induced but not PAR1-AP-induced or PAR4-AP-induced receptor activation). As thrombin activates human platelets via both PAR1 and PAR4, we first established conditions of thrombin-induced platelet aggregation that were largely PAR4-dependent (Fig. S1). To this end, 0.1 U mL^{-1} was the minimum thrombin concentration required to reliably induce maximum aggregation of human platelets (Fig. S1C). Responses to 0.1 U mL^{-1} thrombin were partially inhibited in a concentration-dependent manner by the PAR1 antagonist E5555, but remained largely intact in the presence of 0.1 μM E5555 (Fig. S1D). This residual response to 0.1 U mL^{-1} thrombin in the presence of 0.1 μM E5555 was inhibited in a concentration-dependent manner by the anti-PAR4 antibody, and was almost abolished with an antibody concentration of 0.1 mg mL^{-1} (Fig. S1E).

The specificity of this inhibitory effect of the anti-PAR4 antibody was then assessed under these conditions (Fig. 2). Here, aggregation of human platelets stimulated with thrombin (0.1 U mL^{-1}) was unaffected by pretreatment

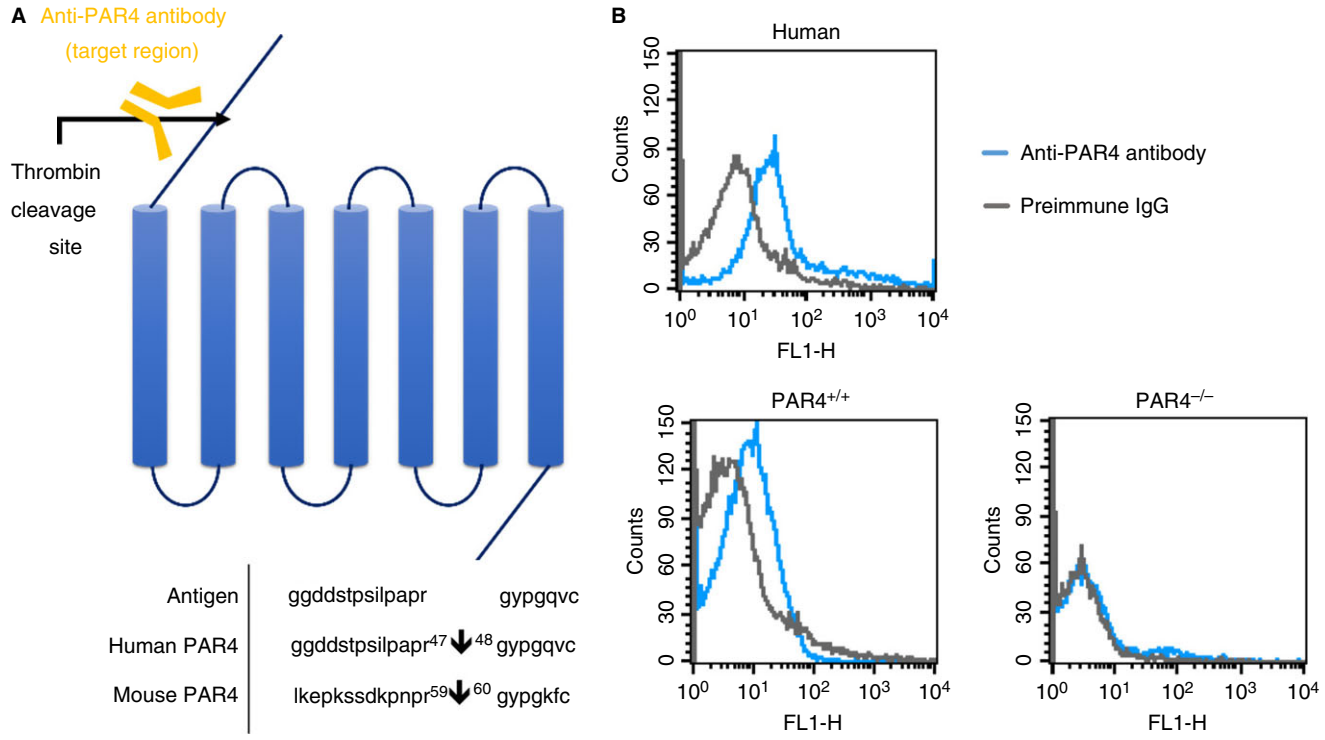


Fig. 1. The anti-protease-activated receptor (PAR)4 antibody selectively binds to PAR4 on the platelet surface. (A) Schematic of PAR4 showing the region corresponding to the antigen peptide sequence (yellow), which spans the thrombin cleavage site (gray arrow). Note the homology between the mouse and human PAR4 sequences in this region. (B) Representative (N = 3–6) flow cytometry traces showing binding of the anti-PAR4 antibody (blue line, 0.1 mg mL^{-1}) versus preimmune rabbit IgG (gray line, 0.1 mg mL^{-1}) to platelets isolated from a human or a wild-type (PAR4$^{+/+}$) or PAR4-deficient (PAR4$^{-/-}$) mouse. Note the complete loss of binding in platelets from the PAR4$^{-/-}$ mouse.

with either a PAR1 antagonist (E5555; 0.1 μM) or the anti-PAR4 antibody (0.1 mg mL^{-1}) alone, but was abolished in the presence of both inhibitors combined (Fig. 2A). This inhibitory effect was overcome by increased thrombin concentrations (Figs. 2B and S1F), indicating that the anti-PAR4 antibody functions as a competitive inhibitor of thrombin-induced platelet aggregation. The anti-PAR4 antibody had no effect on platelet aggregation induced by PAR4-AP (AYPGKF, 100 μM; Fig. 2C), demonstrating specificity of the anti-PAR4 antibody against thrombin cleavage of the receptor over other PAR4 activation mechanisms. Furthermore, the anti-PAR4 antibody had no effect on aggregation induced by any of PAR1-AP, ADP, or CRP (Fig. 2D–F). Importantly, PAR1-AP-induced aggregation was abolished by the PAR1 antagonist (E5555; 0.1 μM), confirming effective PAR1 antagonism under the conditions used here (Fig. 2D).

Selective inhibition of PAR4 abolishes sustained thrombin-induced calcium signals in human platelets

To confirm the inhibition of thrombin-induced platelet function by the anti-PAR4 antibody, we examined changes in cytosolic calcium flux. In accord with the well-characterized thrombin-induced calcium signaling profile in platelets and other cells [50–53], we observed that thrombin (1 U mL^{-1}) induced an initial transient increase in the cytosolic calcium concentration in human platelets that was followed by a sustained plateau (Fig. 3A). Inhibition of PAR1 alone (E5555; 0.1 μM) significantly attenuated the initial transient increase in the cytosolic calcium concentration (Fig. 3A,B) but had no effect on the sustained increase in the cytosolic calcium concentration (Fig. 3A,C). In contrast, inhibition of PAR4 alone (anti-PAR4 antibody; 0.1 mg mL^{-1}) significantly decreased the initial transient increase (Fig. 3A,B) and abolished the sustained plateau (Fig. 3A,C). Pretreatment of platelets with both PAR inhibitors essentially eliminated all thrombin-induced calcium signaling within the 10-min observation period (Fig. 3A–D). These studies are suggestive of a role for PAR4 in mediating platelet activation events that are reliant on sustained calcium signaling. Therefore, we next examined the effect of PAR4 inhibition on specific platelet activation readouts that are known to be dependent on either acute (integrin $\alpha_{IIb}\beta_3$ activation; α-granule release) or sustained (PS externalization) elevations in intracellular calcium.

Selective inhibition of PAR4 is sufficient to impair thrombin-induced PS exposure, but not $\alpha_{IIb}\beta_3$ activation or P-selectin expression, on human platelets

We examined the effect of PAR inhibition on three separate markers of platelet activation: $\alpha_{IIb}\beta_3$ activation

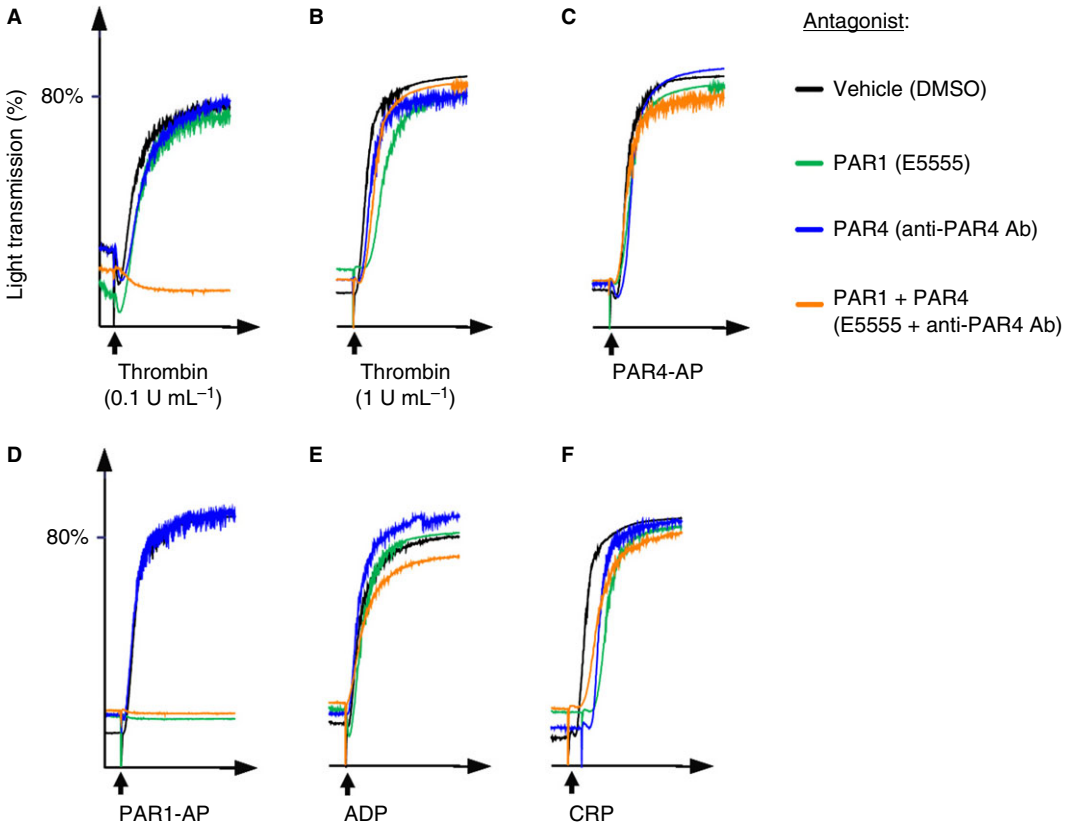


Fig. 2. The anti-protease-activated receptor (PAR)4 antibody specifically inhibits thrombin-induced PAR4 activation of human platelets. Shown are representative ($N \geq 3$) aggregation traces of human isolated platelets in the presence of a vehicle control (black; dimethylsulfoxide [DMSO], 0.1% v/v), a PAR1 inhibitor (green; E5555, 0.1 μM), the anti-PAR4 antibody (blue; 0.1 mg mL^{-1}), or both PAR inhibitors (orange). Platelets were stimulated with (A) 0.1 U mL^{-1} thrombin, (B) 1 U mL^{-1} thrombin, (C) 100 μM PAR4-activating peptide (PAR4-AP), (D) 10 μM PAR1-activating peptide (PAR1-AP), (E) 10 μM ADP, or (F) 10 μg mL^{-1} collagen-related peptide (CRP). Note that the anti-PAR4 antibody inhibits thrombin-induced (0.1 μM) platelet aggregation in the presence of concomitant PAR1 antagonism, but that no inhibition is observed at a higher thrombin concentration (1 U mL^{-1}) or with other platelet activation mechanisms. Arrows indicate the time point of agonist addition. Ab, antibody.

(PAC-1 antibody binding), α-granule release (AK4 antibody binding to P-selectin), and PS externalization (annexin-V binding). Inhibition of either PAR1 or PAR4 alone did not significantly affect $\alpha_{IIb}\beta_3$ activation (Fig. 4A) or P-selectin expression (Fig. 4B) in response to thrombin (0.1 U mL^{-1}), but concomitant inhibition of both receptors produced a non-significant decrease in both $\alpha_{IIb}\beta_3$ activation and P-selectin expression. In contrast, PS exposure in response to thrombin (1 U mL^{-1}) was significantly reduced in platelets pretreated with the anti-PAR4 antibody alone ($P > 0.05$; Fig. 4C). Pretreatment with the PAR1 inhibitor E5555 (0.1 μM) also decreased the number of PS-expressing platelets, although combined inhibition of PAR1 and PAR4 caused no further reduction over PAR4 inhibition alone (Fig. 4C). Further evidence of a role for PAR4 in mediating platelet PS exposure was provided by the observation that stimulation of human platelets with PAR4-AP (300 μM; minimum concentration required to elicit maximal platelet activation; Fig. S1) significantly increased the number of annexin-V-positive platelets ($P < 0.05$) but that stimulation with PAR1-AP (30 μM; 10-fold the concentration required to elicit maximal platelet activation; Fig. S1) did not (Fig. 4D).

As observed for aggregation (Fig. 2), the anti-PAR4 antibody had no effect on any of $\alpha_{IIb}\beta_3$ activation, α-granule release or PS externalization induced by higher concentrations of thrombin (1 U mL^{-1}), PAR4-AP, CRP, or, in the case of PS externalization, calcium ionophore (Fig. 4D–F). In addition, only platelets treated with a PAR1 inhibitor (E5555, 0.1 μM) were unresponsive to platelet activation with PAR1-AP (Fig. 4E–G), further confirming the specificity of PAR1 and PAR4 inhibition in these studies. Together, these findings suggest that selective inhibition of PAR4 is sufficient to impair thrombin-induced PS externalization in human platelets.

Selective inhibition of PAR4 impairs platelet procoagulant activity during thrombus formation

A human whole blood thrombosis assay was performed under conditions of coagulation and with a FRET-based

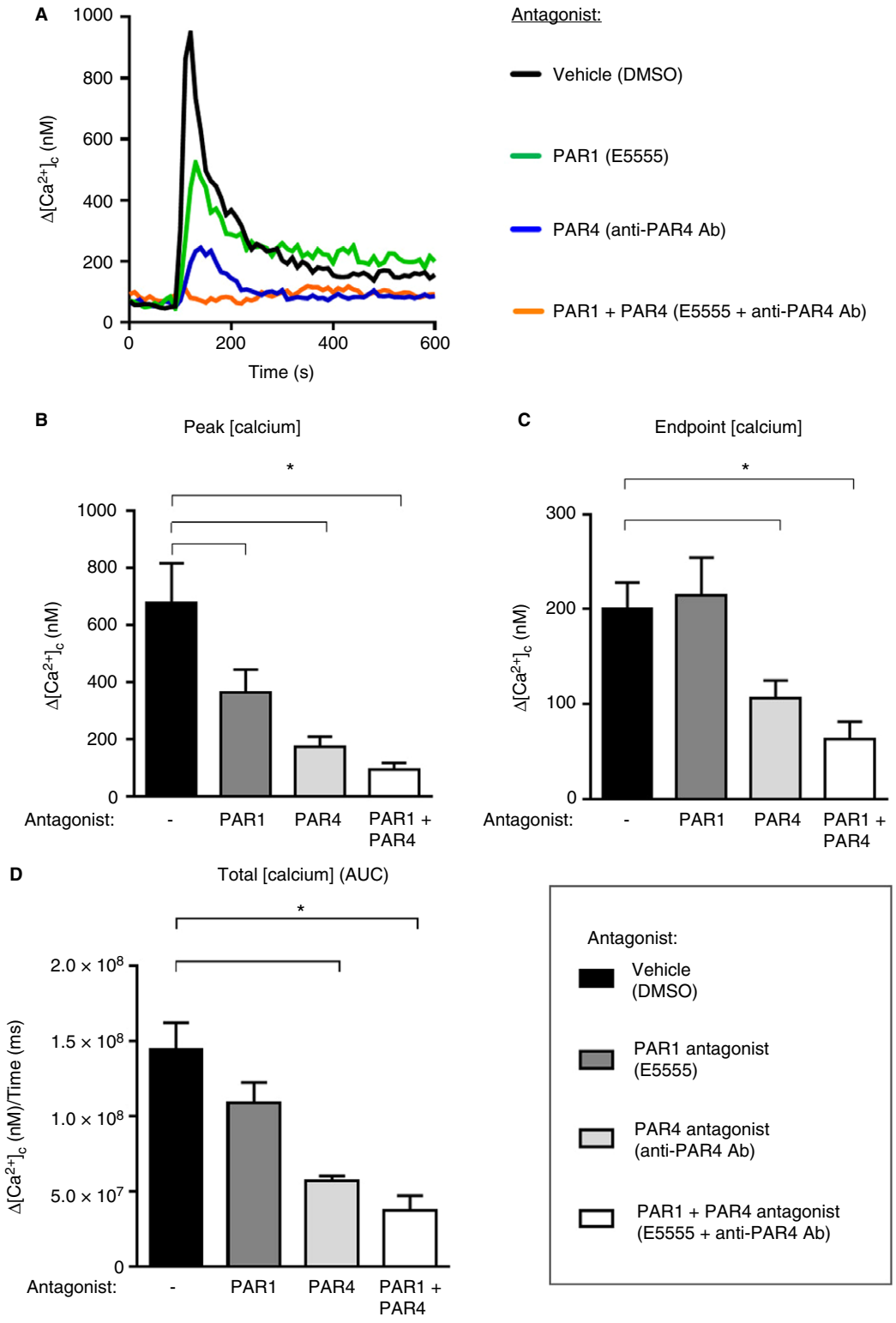


Fig. 3. Selective inhibition of protease-activated receptor (PAR)4 abolishes the sustained thrombin-induced calcium signals in human platelets. (A) Representative trace of cytosolic calcium flux in human platelets pretreated with vehicle (black; dimethylsulfoxide [DMSO], 0.1% v/v), a PAR1 inhibitor (green; E5555, 0.1 μM), the anti-PAR4 antibody (blue; 0.1 mg mL^{-1}), or both PAR inhibitors (orange), and stimulated with thrombin (1 U mL^{-1} at time = 60 s). (B–D) Real-time responses were analyzed for (B) the initial transient increase in cytosolic calcium concentration ($\Delta[Ca^{2+}]_c$) (peak), the (C) sustained plateau (endpoint), and (D) total calcium generated (expressed as area under the curve [AUC]). Note that inhibition of PAR4, but not of PAR1, inhibits the sustained plateau, and that inhibition of both receptors essentially abolishes the response. Data are mean ± standard error of the mean from three independent experiments (donors); $*P < 0.05$ versus control (one-way ANOVA with *post hoc* Fisher's LSD test). Ab, antibody.

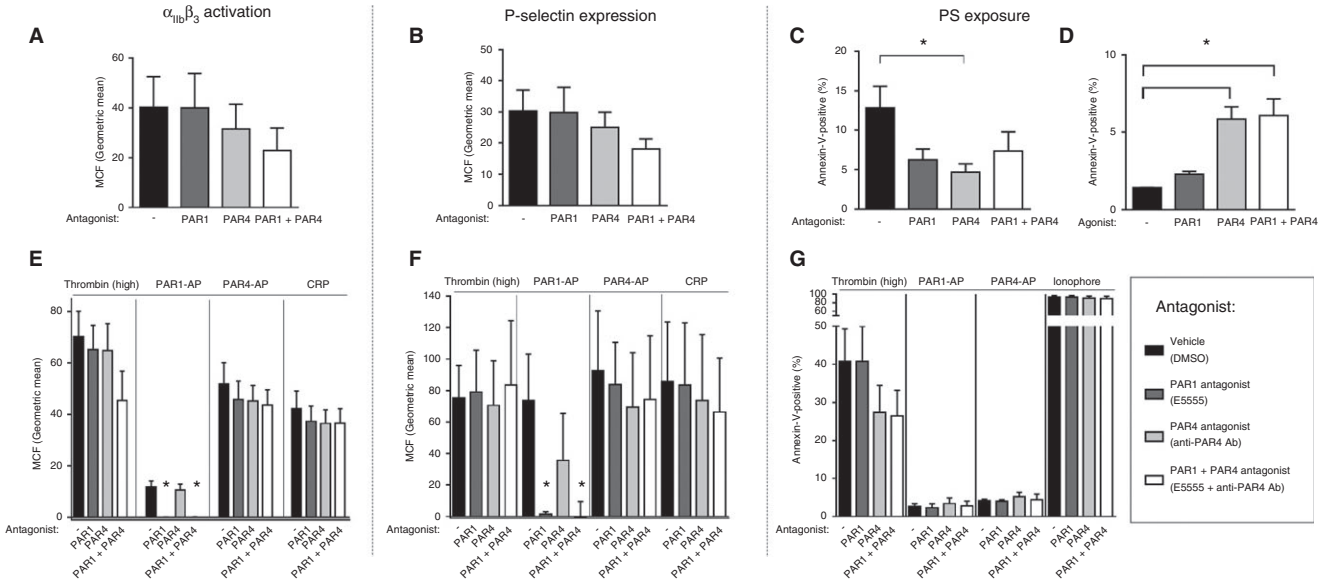


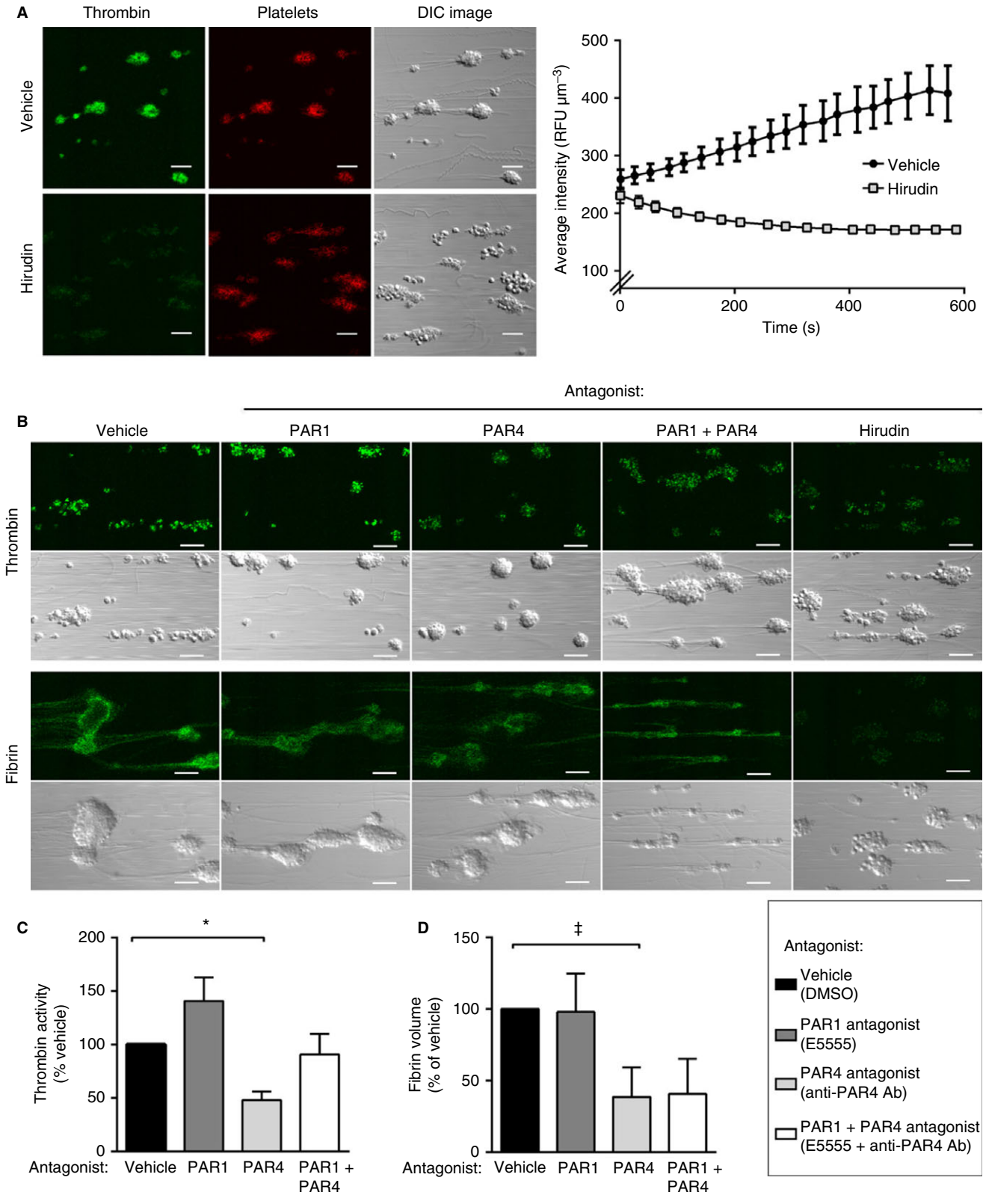
Fig. 4. Selective inhibition of protease-activated receptor (PAR)4 is sufficient to impair thrombin-induced phosphatidylserine (PS) exposure, but not $\alpha_{IIb}\beta_3$ activation or P-selectin expression, on human platelets. Thrombin-induced platelet activation events in human platelets were examined by measuring (A, E) $\alpha_{IIb}\beta_3$ activation (PAC-1), (B, F) α-granule release (P-selectin via AK4) or (C, D, G) PS exposure (annexin-V) via flow cytometry. Human washed platelets were pretreated with a vehicle control (dimethylsulfoxide [DMSO], 0.1% v/v), a PAR1 inhibitor (E5555, 0.1 μM), a PAR4 inhibitor (anti-PAR4 antibody, 0.1 mg mL^{-1}), or the combination of PAR inhibitors, before (A–C) thrombin stimulation (0.1 U mL^{-1} for 10 min for $\alpha_{IIb}\beta_3$ activation and α-granule release; 1 U mL^{-1} for 30 min for PS exposure). Note that only PS exposure is inhibited by the anti-PAR4 antibody alone. Note also (D) that PS exposure is increased in response to PAR4-activating peptide (PAR4-AP) (300 μM) but not PAR1-activating peptide (PAR1-AP) (30 μM). (E, F) $\alpha_{IIb}\beta_3$ activation and α-granule release were also examined in response to high thrombin (1 U mL^{-1}), PAR1-AP (10 μM), PAR4-AP (300 μM), and collagen-related peptide (CRP) (10 μg mL^{-1}). (G) PS exposure in response to thrombin plus CRP (1 U mL^{-1} and 10 μg mL^{-1}, respectively), PAR1-AP (30 μM), PAR4-AP (300 μM) or calcium ionophore (10 μM) was also examined. Data are mean ± standard error of the mean of either mean cell fluorescence (MCF, geometric mean) or percentage of positive population, as indicated. *$P < 0.05$ versus control (one-way ANOVA with *post hoc* Fisher's LSD test); $N = 6$–8. Ab, antibody.

sensor of thrombin activity (ThSP-Ab) [46]. We observed significant thrombin generation on platelet thrombi that was abolished in the presence of the direct thrombin inhibitor hirudin, despite platelet deposition that continued unabated (Fig. 5A). Selective inhibition of PAR4, but not of PAR1, significantly reduced the thrombin activity detected on platelet thrombi (Fig. 5B,C). Curiously, we observed that the PAR1 antagonist E5555 caused an increase in thrombin activity in these experiments (Fig. 5C). The cause of this effect remains unknown, but may relate to the recently reported effects of PAR1 antagonists in promoting platelet activation [48,54]. In similar experiments, selective inhibition of PAR4, but not of PAR1, also reduced the amount of fibrin generation as compared with the control (Fig. 5B,D). These findings indicate that selective inhibition of PAR4, but not of PAR1, is sufficient to decrease thrombin activity and consequent fibrin formation in the setting of human thrombus formation.

Discussion

The current study provides the first evidence that PAR4 is required for platelet procoagulant function during thrombus formation in human blood. Here, we used a strategy similar to that previously employed by others [42,55] to develop a function-blocking anti-PAR4 antibody that was an effective and highly specific inhibitor of

Fig. 5. Selective inhibition of protease-activated receptor (PAR)4 impairs platelet procoagulant activity during thrombus formation in whole blood. (A) Representative images and quantification of thrombin activity (green; ThSP-Ab) and platelet deposition (red; CD9–phycoerythrin) in a human whole blood thrombosis assay analyzed with confocal microscopy. Note that the direct thrombin inhibitor hirudin (800 U mL^{-1}) abolished thrombin activity despite continued platelet deposition. (B–D) Representative images (B) and quantification of (C) thrombin activity ($N = 7$) and (D) fibrin deposition ($N = 4$) at the 10-min time point of a human whole blood thrombosis assay performed in the presence of a vehicle control (dimethylsulfoxide, 0.1% v/v), a PAR1 inhibitor (E5555, 1 μM), a PAR4 inhibitor (anti-PAR4 antibody, 0.2 mg mL^{-1}), the combination of PAR inhibitors, or hirudin (800 U mL^{-1}). Data are mean ± standard error of the mean of four to seven individual donors, and are expressed as average fluorescence intensity of thrombi (RFU μm^{-3}) as a percentage of the vehicle control for each donor (C) or total fibrin as a percentage of the vehicle control for each donor (D). *$P < 0.05$ and ‡$P = 0.05$ versus vehicle control (one-way ANOVA with *post hoc* Fisher's LSD test). Scale bar: 10 μm. Ab, antibody; DIC, differential interference contrast microscopy.



thrombin-induced PAR4 activation on human platelets. We used this anti-PAR4 antibody to probe for platelet functions that were primarily driven by PAR4-mediated rather than PAR1-mediated signaling. We observed that selective PAR4 inhibition attenuated the sustained calcium signal and consequent PS exposure induced by thrombin in human isolated platelets, but not other commonly assessed platelet activation events. These effects

in vitro translated to a marked impact on platelet procoagulant activity, thrombin generation and subsequent fibrin formation in a human whole blood thrombosis assay, suggesting an important role for PAR4 during platelet-dependent thrombosis.

Whether PAR1 or PAR4 predominantly drives thrombin-induced platelet procoagulant activity has been the source of some controversy [22,24,56,57]. Here, we used selective antagonists in a series of *in vitro* and *ex vivo* assays to consistently identify PAR4 as the primary mediator of platelet-driven PS exposure. There are several explanations for the discrepancies in the conclusions drawn from our current work and earlier studies implicating PAR1 in this process. First, the initial studies in which selective PAR4 activation failed to induce platelet PS exposure were performed with a weak activator of PAR4 (GYPGKF [56]). We observed here that selective activation of PAR4 with a significantly more potent PAR4 agonist (AYPGFK [58]) was indeed capable of inducing PS exposure. Second, previous studies using inhibitors to implicate PAR1 in platelet procoagulant function have relied on pepducins to selectively inhibit PAR1 versus PAR4 [57]. We and others [39] have observed a lack of specificity of these reagents that was overcome in the current studies with the use of atopaxar and our anti-PAR4 antibody to selectively inhibit PAR1 and PAR4, respectively. Third, technical differences that have been shown to significantly impact on the annexin-V binding assay in isolated platelets [59] may underlie some of the discrepancies. Regardless of these differences, it is important to note that previous studies addressing this issue have been limited to *in vitro* experiments on isolated platelets and measuring surrogate markers of platelet procoagulant activity. On this point, we too observed that selective PAR1 inhibition impairs thrombin-induced PS exposure in human washed platelets. However, when we extended these *in vitro* observations to examine the physiologic consequences of platelet procoagulant activity, the impact of selective PAR4 inhibition was strongly retained, whereas PAR1 inhibition was clearly without effect.

Indeed, the current study is the first to examine the contribution of PAR4 to thrombin generation in a human whole blood thrombosis assay under physiologic flow conditions. Our findings show that PAR4 inhibition was sufficient to significantly impair thrombin generation on platelet thrombi and subsequent fibrin formation, independently of PAR1 inhibition. The extent of impairment in thrombin activity and fibrin production provided by PAR4 antagonism was notably similar, with an approximately 50% reduction in each case. Until very recently, thrombin generation in whole blood has been difficult to examine, with most techniques using closed system approaches in plasma, thus making platelet–thrombin interactions difficult to elucidate [60,61]. The effect of PAR-mediated platelet activation in human plasma has been examined previously, and it was shown that PAR4-induced activation resulted in more rapid thrombin generation than observed following PAR1-induced activation [22]. Conversely, thrombin generation has been shown to be delayed by PAR4 inhibition, although total amount of thrombin generated was unaffected [24]. Here, we directly measured thrombin generation on platelet thrombi in whole blood under flow conditions known to be optimal for enhanced coagulation [46,62]. Our findings obtained with this approach indicate that PAR4 mediates platelet-dependent thrombin generation and consequent fibrin formation, suggesting that PAR4 inhibition significantly impairs platelet procoagulant activity during thrombosis.

Together, we have used four measures of platelet procoagulant activity to show that inhibition of PAR4 alone is sufficient to impair this important function. It is well known that PAR4 activation generates a more prolonged intracellular calcium signal than PAR1 activation [21,26], and our current study supports previous work [27,63] showing that PAR4 inhibition supresses this sustained calcium signal. Given that the most notable platelet activation event dependent on a sustained rise in intracellular calcium levels is PS exposure [31,32], our findings build on recently published work suggesting that PAR4 is predominantly responsible for additional procoagulant effects of activated platelets [22,27], and extend this to the setting of human thrombus formation.

Our findings suggest that PAR4 inhibitors might provide a useful strategy for the prevention of arterial thrombosis that is distinct from existing approaches. Whether targeting platelet procoagulant activity for antithrombotic benefit is a viable option remains unknown, but it is worth noting that patients with Scott syndrome – a congenital disorder in which defective platelet PS exposure drastically diminishes the thrombin generation capacity – have a clinically significant bleeding phenotype [64]. There is also emerging evidence that targeting PAR4 might be a useful antithrombotic approach [65–67]. Indeed, recent reports have demonstrated increased expression and function of PAR4 in black Americans that might explain the increased resistance to current antiplatelet drugs and overall poorer cardiovascular outcomes in this population [67,68]. As patients treated with PAR1 antagonists have intact PAR4-mediated platelet responses [69,70], such clinical scenarios provide further impetus for the development of PAR4 antagonists as a distinct antithrombotic therapy.

Conclusion

The results from the present study demonstrate that PAR4 performs at least one important platelet activation function that is distinct from that of PAR1, providing a further rationale for PAR4 antagonism as a novel target for the prevention of pathologic thrombosis. Given the ongoing clinical demand for improved and varied antiplatelet agents for the safe and effective prevention of

arterial thrombosis, the importance of thrombin-induced platelet activation in this setting, and the clinical setbacks with PAR1 antagonists, further investigation of PAR4 as a suitable target appears to be warranted.

Addendum

S. L. French, J. F. Arthur, H. Lee, and W. S. Nesbitt performed experiments. S. L. French and J. R. Hamilton wrote the manuscript; J. R. Hamilton designed the research and supervised the study. All authors analyzed data and contributed to editing of the manuscript.

Acknowledgements

The authors wish to thank S. Cody and the Monash Micro Imaging staff for microscopy support, the AMREP AS staff for animal care, C. Berndt, J. Jing, T. Sidhu, H. Qayum, M. Moon and A. Paramitha for technical assistance, and S. Diamond and V. Chen for providing key reagents, and we appreciate the thoughts of C. L. Eniss. This work was supported by grants to J. R. Hamilton, E. E. Gardiner and R. K. Andrews from the National Health & Medical Research Council of Australia. J. R. Hamilton is a Future Fellow of the Australian Research Council.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Establishment of an assay to examine PAR4-dependent platelet activation by thrombin.

References

1 Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, *et al*. Heart disease and stroke statistics – 2015 update: a report from the American Heart Association. *Circulation* 2015; **131**: e29–322.

2 Rosendaal FR, Raskob GE. On World Thrombosis Day. *Lancet* 2014; **384**: 1653–4.

3 Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; **64**: 1057–68.

4 Coughlin SR, Vu TK, Hung DT, Wheaton VI. Characterization of a functional thrombin receptor. Issues and opportunities. *J Clin Invest* 1992; **89**: 351–5.

5 Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin–receptor interaction. *Nature* 1991; **353**: 674–7.

6 Hung DT, Vu TK, Wheaton VI, Ishii K, Coughlin SR. Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J Clin Invest* 1992; **89**: 1350–3.

7 Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; **386**: 502–6.

8 Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, Farese RV, Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. *Nature* 1998; **394**: 690–4.

9 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000; **407**: 258–64.

10 Sambrano GR, Weiss EJ, Zheng Y-W, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature* 2001; **413**: 74–8.

11 Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. *Blood* 2002; **100**: 3240–4.

12 Camerer E, Duong DN, Hamilton JR, Coughlin SR. Combined deficiency of protease-activated receptor-4 and fibrinogen recapitulates the hemostatic defect but not the embryonic lethality of prothrombin deficiency. *Blood* 2004; **103**: 152–4.

13 Hamilton J, Cornelissen I, Coughlin S. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. *J Thromb Haemost* 2004; **2**: 1429–35.

14 Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. *Proc Natl Acad Sci USA* 2007; **104**: 288–92.

15 Hamilton JR. Protease-activated receptors as targets for antiplatelet therapy. *Blood Rev* 2009; **23**: 61–5.

16 Goto S, Ogawa H, Takeuchi M, Flather MD, Bhatt DL. Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. *Eur Heart J* 2010; **31**: 2601–13.

17 Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van de Werf F, White HD, Aylward PE, Wallentin L, Chen E. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *New Engl J Med* 2012; **366**: 20–33.

18 Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, Fox KA, Lipka LJ, Liu X, Nicolau JC. Vorapaxar in the secondary prevention of atherothrombotic events. *New Engl J Med* 2012; **366**: 1404–13.

19 French SL, Arthur JF, Tran HA, Hamilton JR. Approval of the first protease-activated receptor antagonist: rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Rev* 2015; **29**: 179–89.

20 Italiano JE Jr, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 2008; **111**: 1227–33.

21 Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, Hamm HE. PAR4, but not PAR1, signals human platelet aggregation via Ca^{2+} mobilization and synergistic P2Y12 receptor activation. *J Biol Chem* 2006; **281**: 26665–74.

22 Duvernay M, Young S, Gailani D, Schoenecker J, Hamm HE. Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets. *Mol Pharmacol* 2013; **83**: 781–92.

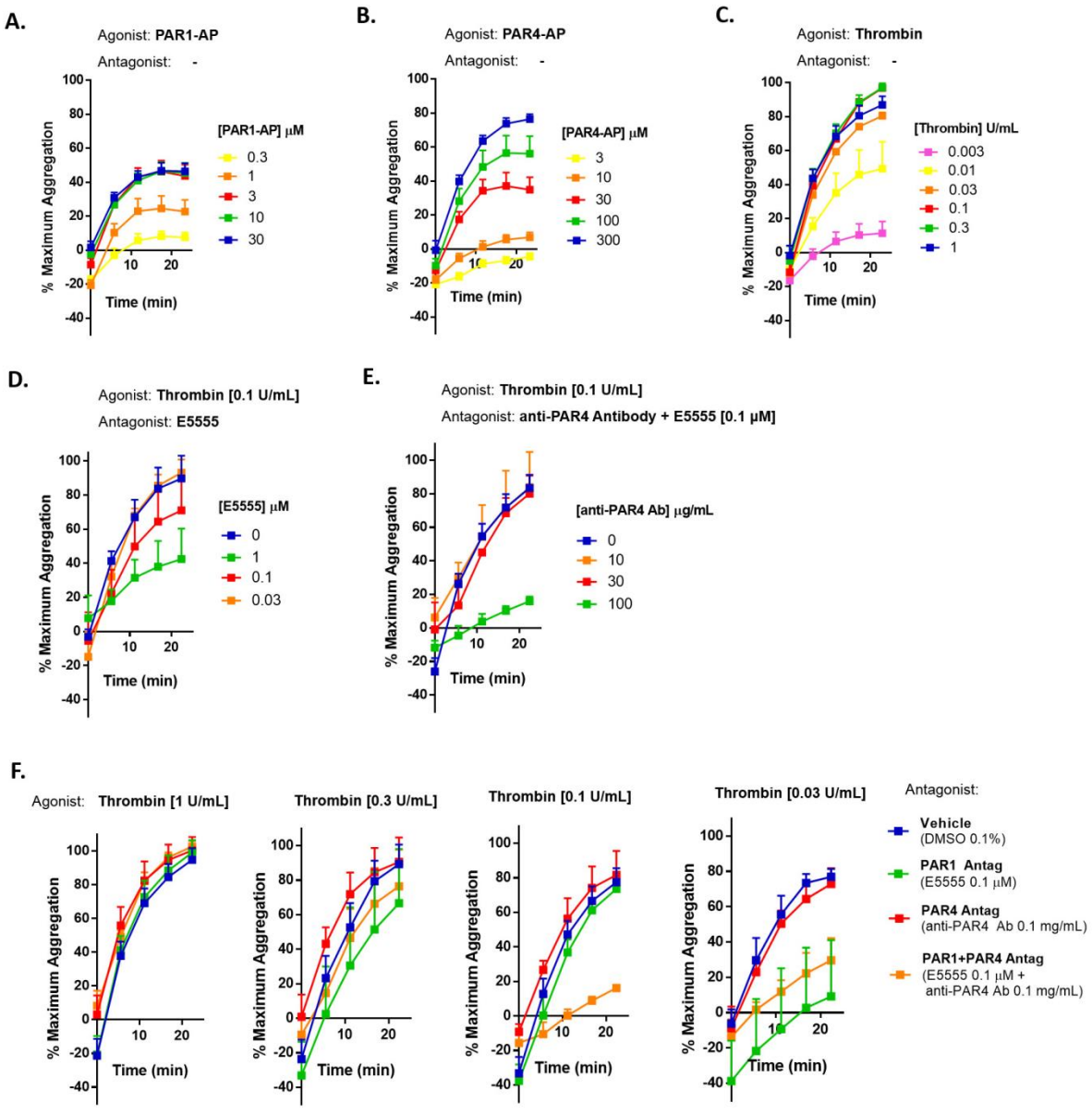
23 Covic L, Singh C, Smith H, Kuliopulos A. Role of the PAR4 thrombin receptor in stabilizing platelet–platelet aggregates as revealed by a patient with Hermansky–Pudlak syndrome. *Thromb Haemost* 2002; **87**: 722–7.

24 Vretenbrant K, Ramstrom S, Bjerke M, Lindahl TL. Platelet activation via PAR4 is involved in the initiation of thrombin

generation and in clot elasticity development. *Thromb Haemost* 2007; **97**: 417–24.
25 Wu CC, Wu SY, Liao CY, Teng CM, Wu YC, Kuo SC. The roles and mechanisms of PAR4 and P2Y12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. *Br J Pharmacol* 2010; **161**: 643–58.
26 Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* 2000; **39**: 5458–67.
27 Falker K, Haglund L, Gunnarsson P, Nylander M, Lindahl TL, Grenegard M. Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets. *Biochem J* 2011; **436**: 469–80.
28 Voss B, McLaughlin JN, Holinstat M, Zent R, Hamm HE. PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. *Mol Pharmacol* 2007; **71**: 1399–406.
29 Jacques S, Kuliopulos A. Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem J* 2003; **376**: 733–40.
30 Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. *Blood* 2012; **120**: 5209–16.
31 Williamson P, Bevers EM, Smeets EF, Comfurius P, Schlegel RA, Zwaal RF. Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. *Biochemistry* 1995; **34**: 10448–55.
32 Dachary-Prigent J, Pasquet JM, Freyssinet JM, Nurden AT. Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca^{2+}-ATPase inhibitors. *Biochemistry* 1995; **34**: 11625–34.
33 Resendiz JC, Kroll MH, Lassila R. Protease-activated receptor-induced Akt activation – regulation and possible function. *J Thromb Haemost* 2007; **5**: 2484–93.
34 Wu CC, Huang SW, Hwang TL, Kuo SC, Lee FY, Teng CM. YD-3, a novel inhibitor of protease-induced platelet activation. *Br J Pharmacol* 2000; **130**: 1289–96.
35 Hollenberg MD, Saifeddine M. Proteinase-activated receptor 4 (PAR4): activation and inhibition of rat platelet aggregation by PAR4-derived peptides. *Can J Physiol Pharmacol* 2001; **79**: 439–42.
36 Leger AJ, Jacques SL, Badar J, Kaneider NC, Derian CK, Andrade-Gordon P, Covic L, Kuliopulos A. Blocking the protease-activated receptor 1–4 heterodimer in platelet-mediated thrombosis. *Circulation* 2006; **113**: 1244–54.
37 Covic L, Gresser AL, Talavera J, Swift S, Kuliopulos A. Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci USA* 2002; **99**: 643–8.
38 Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med* 2002; **8**: 1161–5.
39 Stampfuss JJ, Schror K, Weber AA. Inhibition of platelet thromboxane receptor function by a thrombin receptor-targeted pepducin. *Nat Med* 2003; **9**: 1447; author reply 1447–8.
40 Hollenberg MD, Saifeddine M, Sandhu S, Houle S, Vergnolle N. Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists *in vivo*. *Br J Pharmacol* 2004; **143**: 443–54.
41 Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Lee CY, Teng CM. Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. *Thromb Haemost* 2002; **87**: 1026–33.
42 Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* 1999; **103**: 879–87.
43 Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Berndt MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb–IX–V by ADAM family metalloproteinases. *J Thromb Haemost* 2007; **5**: 1530–7.
44 Hui KY, Haber E, Matsueda GR. Monoclonal antibodies to a synthetic fibrin-like peptide bind to human fibrin but not fibrinogen. *Science* 1983; **222**: 1129–32.
45 Ivanciu L, Toso R, Margaritis P, Pavani G, Kim H, Schlachterman A, Liu JH, Clerin V, Pittman DD, Rose-Miranda R, Shields KM, Erbe DV, Tobin JF, Arruda VR, Camire RM. A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia. *Nat Biotechnol* 2011; **29**: 1028–33.
46 Welsh J, Colace T, Muthard R, Stalker T, Brass L, Diamond S. Platelet-targeting sensor reveals thrombin gradients within blood clots forming in microfluidic assays and in mouse. *J Thromb Haemost* 2012; **10**: 2344–53.
47 Mountford JK, Petitjean C, Putra HW, McCafferty JA, Setiabakti NM, Lee H, Tonnesen LL, McFadyen JD, Schoenwaelder SM, Eckly A, Gachet C, Ellis S, Voss AK, Dickins RA, Hamilton JR, Jackson SP. The class II PI 3-kinase, PI3KC2alpha, links platelet internal membrane structure to shear-dependent adhesive function. *Nat Commun* 2015; **6**: 6535.
48 Lee H, Hamilton JR. The PAR1 antagonist, SCH79797, alters platelet morphology and function independently of PARs. *Thromb Haemost* 2013; **109**: 164–7.
49 Nesbitt WS, Harper IS, Schoenwaelder SM, Yuan Y, Jackson SP. A live cell micro-imaging technique to examine platelet calcium signaling dynamics under blood flow. *Methods Mol Biol* 2012; **788**: 73–89.
50 Judge HM, Jennings LK, Moliterno DJ, Hord E, Ecob R, Tricoci P, Rorick T, Kotha J, Storey RF. PAR1 antagonists inhibit thrombin-induced platelet activation whilst leaving the PAR4-mediated response intact. *Platelets* 2015; **26**: 236–42.
51 Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem* 2000; **275**: 25216–21.
52 Kataoka H, Hamilton JR, McKemy DD, Camerer E, Zheng YW, Cheng A, Griffin C, Coughlin SR. Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* 2003; **102**: 3224–31.
53 Boknas N, Faxalv L, Sanchez Centellas D, Wallstedt M, Ramstrom S, Grenegard M, Lindahl TL. Thrombin-induced platelet activation via PAR4: pivotal role for exosite II. *Thromb Haemost* 2014; **112**: 558–65.
54 Aisiku O, Peters C, De Ceunynck K, Ghosh C, Dilks J, Fustolo-Gunnink S, Huang M, Dockendorff C, Parikh S, Flaumenhaft R. Parmodulins inhibit thrombus formation without inducing endothelial injury caused by vorapaxar. *Blood*. 2015; **125**: 1976–85.
55 Mumaw M, la Fuente M, Noble D, Nieman M. Targeting the anionic region of human protease activated receptor 4 (PAR4) inhibits platelet aggregation and thrombosis without interfering with hemostasis. *J Thromb Haemost* 2014; **12**: 1331–41.
56 Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci USA* 1999; **96**: 11189–93.
57 Keuren JF, Wielders SJ, Ulrichts H, Hackeng T, Heemskerk JW, Deckmyn H, Bevers EM, Lindhout T. Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1499–505.
58 Faruqi TR, Weiss EJ, Shapiro MJ, Huang W, Coughlin SR. Structure–function analysis of protease-activated receptor 4 tethered ligand peptides. Determinants of specificity and utility in assays of receptor function. *J Biol Chem* 2000; **275**: 19728–34.
59 Ramstrom S, O'Neill S, Dunne E, Kenny D. Annexin V binding to platelets is agonist, time and temperature dependent. *Platelets* 2010; **21**: 289–96.

60 Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Beguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb* 2003; **33**: 4–15.
61 Al Dieri R, de Laat B, Hemker HC. Thrombin generation: what have we learned? *Blood Rev* 2012; **26**: 197–203.
62 Lee H, Sturgeon SA, Jackson SP, Hamilton JR. The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. *Thromb Haemost* 2012; **107**: 328–37.
63 Wu CC, Wang WY, Wei CK, Teng CM. Combined blockade of thrombin anion binding exosite-1 and PAR4 produces synergistic antiplatelet effect in human platelets. *Thromb Haemost* 2011; **105**: 88–95.
64 Weiss HJ, Vicic WJ, Lages BA, Rogers J. Isolated deficiency of platelet procoagulant activity. *Am J Med* 1979; **67**: 206–13.
65 Lee H, Sturgeon S, Mountford J, Jackson S, Hamilton J. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. *Br J Pharmacol* 2012; **166**: 2188–97.
66 Mao Y, Zhang M, Tuma RF, Kunapuli SP. Deficiency of PAR4 attenuates cerebral ischemia/reperfusion injury in mice. *J Cereb Blood Flow Metab* 2010; **30**: 1044–52.
67 Edelstein LC, Simon LM, Lindsay CR, Kong X, Teruel-Montoya R, Tourdot BE, Chen ES, Ma L, Coughlin S, Nieman M, Holinstat M, Shaw CA, Bray PF. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood* 2014; **124**: 3450–8.
68 Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, Kong X, Nagalla S, Mohandas N, Cohen DE. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nat Med* 2013; **19**: 1609–16.
69 Judge HM, Jennings LK, Moliterno DJ, Hord E, Ecob R, Tricoci P, Rorick T, Kotha J, Storey RF. PAR1 antagonists inhibit thrombin-induced platelet activation whilst leaving the PAR4-mediated response intact. *Platelets* 2014; **26**: 1–7.
70 Knut F, Linda H, Peter G, Martina N, Tomas LL, Magnus G. Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets. *Biochem J* 2011; **436**: 469–80.

2.3 Supplementary Figures to French et al. "Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood" (J Thromb Haemost, 2016)



Supplementary Figure 1.

Supplementary Figure 1. Establishment of an assay to examine PAR4-dependent platelet activation by thrombin. Isolated human platelet aggregation concentration response curves to (A) PAR1 activating peptide (PAR1-AP; TFLLR; 0.3-30 µM), (B) PAR4 activating peptide (PAR4-AP; AYPGKF; 3-300 µM) and (C) thrombin (0.003-1 U/mL) were conducted in a plate reader. (D) A PAR1 antagonist (E5555, 0.1 µM (minimum concentration required to inhibit

PAR1-AP-induced platelet activation)) delays thrombin (0.1 U/mL) induced platelet aggregation but does not significantly affect maximal aggregation. (E) Platelet aggregation in response to thrombin (0.1 U/mL) and in the presence of the PAR1 antagonist, E5555 (0.1 μM), is inhibited in a concentration-dependent manner by the anti-PAR4 antibody. (F) The effect of PAR inhibition on thrombin-induced platelet activation is overcome at high thrombin concentrations (1 and 0.3 U/mL), whereas at low thrombin concentrations (0.03 U/mL) PAR1 inhibition alone is sufficient to inhibit platelet aggregation. Data are expressed as mean ± SEM; $n \geq 3$.

Chapter 3 - Perinatal lethality of PAR4-deficient mice delivered by primiparous dams reveals spontaneous bleeding in mice without platelet thrombin receptor function

3.1 Introduction

The studies of Chapter 2 generated a selective and effective experimental PAR4 antagonist and utilised it in a series of *in vitro* experiments to uncover a distinct role for PAR4 on human platelets during thrombosis. These findings suggest PAR4 blockade may be a useful anti-thrombotic strategy. Therefore, this thesis next extended on these *in vitro* studies by investigating the effects of targeting PAR4 *in vivo* by examining the impact of PAR4-deficiency in a mouse model.

Mouse platelets express PAR3 in place of the PAR1 on human platelets, but PAR3 in the mouse does not signal, meaning transduction of thrombin signalling in mouse platelets is entirely reliant on PAR4. Platelets from PAR4-/- mice fail to respond to thrombin and PAR4-/- mice allow examination of the overall importance of platelet activation by thrombin in (patho)physiology. PAR4-/- mice are protected against arterial thrombosis yet exhibit no evidence of spontaneous bleeding. This separation between haemostasis and thrombosis is perhaps surprising. Indeed, the lack of spontaneous bleeding in PAR4-/- mice contrasts with that experienced by mice with marked thrombocytopaenia, in which high rates of perinatal death due to haemorrhage are observed. Given the rationalisation of PAR4 as an anti-thrombotic drug target in Chapter 2, the studies of Chapter 3 examined in detail the potential for bleeding (perinatal haemorrhage) in PAR4-/- mice.

These studies indicate a subtle but significant effect of PAR4-deficiency on spontaneous bleeding in mice, suggesting careful consideration should be given to the safety of any PAR4

antagonists investigated. However, given the discrepancy in platelet PAR expression between mice and humans, these studies also suggest the suitability of any mouse model should be taken into consideration in assessing any *in vivo* studies relating to platelet PAR function.

This chapter has been published as:

French SL and Hamilton JR. Perinatal lethality of *Par4*$^{-/-}$ mice delivered by primiparous dams reveals spontaneous bleeding in mice without platelet thrombin receptor function. *Platelets*. 2017. doi: 10.1080/09537104.2017.1349310.

http://www.tandfonline.com/iplt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

platelets

Platelets, Early Online: 1–3
 DOI: https://doi.org/10.1080/09537104.2017.1349310

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Perinatal lethality of *Par4*$^{-/-}$ mice delivered by primiparous dams reveals spontaneous bleeding in mice without platelet thrombin receptor function

Shauna L. French & Justin R. Hamilton

Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

Abstract

Protease-activated receptor 4 (PAR4) is a cell surface G protein-coupled receptor for serine proteases, such as thrombin. *Par4*$^{-/-}$ mice have platelets that are unresponsive to thrombin and thereby allow examination of the importance of thrombin-induced platelet activation in (patho)physiology. *Par4*$^{-/-}$ mice are protected against arterial thrombosis but show no evidence of spontaneous bleeding. This contrasts with the bleeding experienced by mice with marked thrombocytopenia, such as those with genetic deficiency of the transcription factor, nuclear factor erythroid 2 (*Nfe2*$^{-/-}$), that have high rates of perinatal death due to hemorrhage. Given this discrepancy in spontaneous perinatal bleeding between mice without platelets and those without thrombin-induced platelet activation mechanisms, we examined in detail the immediate postnatal survival of *Par4*$^{-/-}$ pups. We observed significant postpartum loss of *Par4*$^{-/-}$ pups derived from *Par4*$^{+/-}$ intercrosses that was restricted to a dam's first litter; only 9% of surviving pups genotyped as *Par4*$^{-/-}$ in first litters and this normalized from the second litter onward (26%). A similar perinatal lethality in pups delivered by primiparous dams occurred in mice lacking platelets (*Nfe2*$^{-/-}$; 10%) but not in those lacking fibrinogen (*Fga*$^{-/-}$; 26%). These data,, provide the first evidence of spontaneous bleeding in *Par4*$^{-/-}$ mice, suggest that a dam's first litter provides a greater hemostatic challenge than subsequent litters, and uncovers an important role for platelets—and more specifically thrombin-induced platelet activation—in hemostasis during these more traumatic births.

Keywords

Hemostasis, perinatal hemorrhage, protease-activated receptors, thrombin

History

Received 19 May 2017
Revised 8 June 2017
Accepted 26 June 2017
Published online 28 September 2017

Introduction

Protease-activated receptors (PARs) are the target of the most recently-developed antiplatelet drugs [1]. PARs are attractive targets for antiplatelet agents because they mediate platelet activation by thrombin, the most potent endogenous activator. Human platelets have two PARs, PAR1, and PAR4. The first PAR1 antagonist was recently approved for the prevention of myocardial infarction and peripheral arterial disease [1] and PAR4 antagonists are in clinical trial for similar purposes [2]. Mouse platelets express non-signaling PAR3 in place of the PAR1 on human platelets, meaning transduction of thrombin signaling in mouse platelets is entirely reliant on PAR4. Platelets from *Par4*$^{-/-}$ mice fail to respond to thrombin [3,4] and *Par4*$^{-/-}$ mice allow examination of the overall importance of platelet activation by thrombin in (patho)physiology. Predictably, *Par4*$^{-/-}$ mice are protected against arterial thrombosis [3,5]. Yet despite this, *Par4*$^{-/-}$ mice exhibit no evidence of spontaneous bleeding; they are born at the expected frequency, have no obvious internal or external hemorrhage, and have a normal lifespan [4,6]. This lack of bleeding in *Par4*$^{-/-}$ mice contrasts with that observed in mice that have a marked thrombocytopenia, such as those with genetic deficiency of the transcription factor, nuclear factor erythroid 2 (*Nfe2*$^{-/-}$). *Nfe2*$^{-/-}$ mice have arrested megakaryocyte development that results in no (or very few) circulating platelets [7]. These mice with (near) absolute thrombocytopenia experience spontaneous bleeding, including significant levels of perinatal hemorrhage and subsequent death due to the hemostatic trauma of birth [7].

Given this discrepancy in the level of perinatal bleeding between mice without platelets and mice without thrombin-induced platelet activation mechanisms, we tracked in detail the genotypes of pups resulting from *Par4*$^{+/-}$ intercrosses. We observed significant loss of *Par4*$^{-/-}$ pups in the immediate postpartum period, but only in primiparous dams (i.e., the first litter from any given female). A similar perinatal lethality in pups delivered by primiparous dams occurred in mice lacking platelets (*Nfe2*$^{-/-}$) but not in those lacking fibrinogen (*Fga*$^{-/-}$), suggesting the effect was most likely primarily due to impaired thrombin-mediated platelet signaling. These data provide the first evidence of a spontaneous bleeding phenotype in *Par4*$^{-/-}$ mice.

Methods

Generation of mice deficient in PAR4 (*F2lr3* [4];), fibrinogen (*Fga* [8];), or platelets (*Nfe2* [7];) has been described previously. *Par4*$^{-/-}$ mice were either inbred ≥ 6 generations into C57BL/6 or were 50:50 C57BL/6:129/Sv as indicated. *Nfe2*$^{-/-}$ and *Fga*$^{-/-}$ were all 50:50 C57BL/6:129/Sv. All mice examined were derived from heterozygous intercrosses and all genotypes were confirmed by Southern blot analysis on biopsies taken from 10–14-day old pups. Genotypes of live

Correspondence: Justin Hamilton, Australian Centre for Blood Diseases, Monash University, L2, AMREP Building, The Alfred, Commercial Rd, Melbourne, Victoria 3004, Australia. E-mail: Justin.Hamilton@monash.edu

offspring were stratified by an individual female's litter number. Statistical significance was tested by χ^2 analysis (GraphPad Prism 7).

Results

In accord with previous observations [4,6], genotyping of all live births across a large number of *Par4*$^{+/-}$ intercrosses in an inbred C57BL/6 background revealed approximately the expected rate of production of *Par4*$^{-/-}$ mice (21%; 39 of 184; Table I). However, when births were stratified by the order of an individual female's litter, *Par4*$^{-/-}$ mice surviving to P10 were markedly reduced in the first litter (9%; 5 of 57; Table I; P = 0.01). This significant reduction in the number of *Par4*$^{-/-}$ mice surviving their first 10 days was completely corrected from the second litter onward (Table I). Analysis of the number of pups born at P1-3 revealed almost no loss of mice (4 of 184) between P3 and P10. Further, the average size of the surviving first litter from *Par4*$^{+/-}$ intercrosses (6.3 pups per litter) was less than in subsequent litters (7.5) (Table I), closely accounting for the absent *Par4*$^{-/-}$ pups. Our data, when combined with the observation that *Par4*$^{-/-}$ embryos are present at the expected rate throughout the entirety of embryonic development [6], strongly suggest that a significant fraction of *Par4*$^{-/-}$ mice are selectively lost from a mother's first litter in the immediate postnatal period.

Since such an observation has not been previously reported, we next directly compared this loss of *Par4*$^{-/-}$ pups from primiparous dams to that experienced by mice deficient in platelets (*Nfe2*$^{-/-}$) or fibrinogen (*Fga*$^{-/-}$). Since neither *Nfe2*$^{-/-}$ nor *Fga*$^{-/-}$ mice survive at high rates in an inbred C57BL/6 background [7,8], we performed this head-to-head comparison in the outbred (50:50) C57BL/6:129Sv strain, where markedly improved survival has been observed [7,8]. *Par4*$^{+/-}$ intercrosses in this mixed strain produced a similar loss of *Par4*$^{-/-}$ pups from primiparous dams (14%; 14 from 98; P < 0.05; Table II) that again immediately corrected from the same mothers' second litter (Table II). Again, the average surviving litter size in the first litter of *Par4*$^{+/-}$ intercrosses was less than in subsequent litters (6.1 vs 7.3; Table II). A similar trend was observed in pups produced from *Nfe2*$^{+/-}$ intercrosses; *Nfe2*$^{-/-}$ pups represented only 10% of a mother's first litter (5 of 51; P < 0.05; Table II) but 20% in subsequent litters (23 of 113; Table II). In contrast, the rate of *Fga*$^{-/-}$ pups from *Fga*$^{+/-}$ intercrosses was not different across the mother's litter number, with 26% in first litters (14 of 53; Table II) and 21% in subsequent litters (23 of 107; Table II).

Table I. Live offspring produced from *Par4*$^{+/-}$ intercrosses (C57BL/6).

Mother's litter no.	Genotype: observed (expected)			Total pups (litters)
	+/+	**+/–**	**–/–**	
1	**20 (14.25)**	**32 (38.5)**	**5 (14.25) ***	**57 (9)**
2	12 (13.5)	29 (27)	13 (13.5)	54 (7)
3	12 (12.75)	23 (25.5)	16 (12.75)	51 (6)
4	5 (5.5)	12 (11)	5 (5.5)	22 (4)
≥2 combined	**29 (31.75)**	**64 (63.5)**	**34 (31.75)**	**127 (17)**
Total	**49 (46)**	**96 (92)**	**39 (46)**	**184 (26)**

*Overall distribution is significantly different from expected by chi-square analysis (P < 0.05).

Discussion

This study provides the first evidence of a spontaneous bleeding phenotype in *Par4*$^{-/-}$ mice. Here, *Par4*$^{-/-}$ pups were selectively lost from litters delivered by primiparous dams. This effect was mirrored in mice deficient in functional platelets (*Nfe2*$^{-/-}$) but not fibrinogen (*Fga*$^{-/-}$). The specificity of this loss of knockout mice to the first litter of *Par4* and *Nfe2* mice supports the hypothesis that the first litter delivered by a given female endures a more significant hemostatic challenge during birth, and that the postnatal lethality observed here is due to impaired platelet function.

Par4$^{-/-}$ mice have platelets that do not respond to thrombin. Numerous studies have reported protection against thrombosis in *Par4*$^{-/-}$ mice accompanied by prolonged bleeding upon hemostatic challenge (e.g., tail transection [3]). Yet, perhaps surprisingly, there is no report of spontaneous bleeding in *Par4*$^{-/-}$ mice. When combined with a number of previous observations, our data indicate postnatal loss of *Par4*$^{-/-}$ pups rapidly after birth—a time of major hemostatic challenge. First, *Par4*$^{-/-}$ embryos are present at the expected rate throughout the entirety of embryonic development [6]. Second, newborn *Nfe2*$^{-/-}$ pups, as well as those with a combined deficiency of fibrinogen and PAR4, that do not survive the immediate postnatal period, exhibit marked intraperitoneal hemorrhage [6]. Therefore, while we did not directly measure hemostasis in the present study, it appears most likely that the loss of *Par4*$^{-/-}$ pups observed here in the immediate postnatal period is the result of internal hemorrhage. That this death of neonatal

Table II. Live offspring produced from *Par4*$^{+/-}$, *Nfe2*$^{+/-}$, *or Fga*$^{+/-}$ intercrosses (C57BL/6 x 129/Sv).

Mother's litter no.	Genotype: observed (expected)			Total pups (litters)
Par4	+/+	+/–	–/–	
1	29 (24.5)	55 (49)	14 (24.5) *	98 (16)
≥2	45 (48.25)	102 (96.5)	44 (48.25)	191 (26)
Total	74 (72.25)	157 (144.5)	58 (72.25)	289 (42)
Nfe2	+/+	+/–	–/–	
1	13 (12.75)	33 (25.5)	5 (12.75) *	51 (9)
≥2	29 (28.25)	61 (56.5)	23 (28.25)	113 (17)
Total	41 (41)	94 (82)	29 (41)	164 (26)
Fga	+/+	+/–	–/–	
1	15 (13.25)	24 (26.5)	14 (13.25)	53 (9)
≥2	33 (26.75)	51 (53.5)	23 (26.75)	107 (16)
Total	48 (40)	75 (80)	37 (40)	160 (25)

*Overall distribution is significantly different from expected by chi-square analysis (P < 0.05).

Par4$^{-/-}$ pups is restricted to those in a mother's first litter suggests the impact of PAR4-deficiency is heavily dependent on the strength of the hemostatic challenge, with the hemostatic trauma of birth presumably greater during a primiparous delivery than in subsequent deliveries.

The observed perinatal loss of *Par4*$^{-/-}$ pups appears most likely platelet-dependent given that a similar observation was noted with *Nfe2*$^{-/-}$ pups. However, as PAR4 is expressed on a number of other cell types, including some that may contribute to hemostasis such as vascular endothelial cells, we cannot formally rule out a contribution from cells other than platelets to the bleeding observed here. On this point, it is worth noting that a previous study used bone marrow transplantation to show that the impaired hemostasis in *Par4*$^{-/-}$ mice after injury (prolonged bleeding after tail clip) is fully attributable to PAR4 deficiency in platelets [3]. Therefore, the present study is highly suggestive of an important role for platelets —and more specifically thrombin-induced platelet activation—in hemostasis during traumatic births. Such a phenotype has not been previously described, and whether or not this observation extends to other commonly-used mouse lines with major platelet defects remains unknown. For example, PAR3 is a thrombin co-factor receptor on mouse platelets. Platelets from *Par3*$^{-/-}$ mice exhibit reduced sensitivity to thrombin that is sufficient to impair hemostasis—but this impairment is notably less than that experienced by *Par4*$^{-/-}$ mice [9]. It will therefore be of interest to determine if the more subtle impairment in platelet function found in, for example, *Par3*$^{-/-}$ mice is sufficient to cause a similar perinatal hemorrhage and death in pups from primiparous dams to that observed here in *Par4*$^{-/-}$ mice.

Finally, mice without thrombin (prothrombin-deficient mice; *F2*$^{-/-}$) exhibit 100% embryonic lethality—approximately half of which occurs during mid-gestation due to a PAR1-dependent defect in vascular patterning [10] and the remainder due to hemorrhage at birth [11]. This latter effect is presumably due to the combined input of thrombin to platelet activation and fibrin formation, since both *Fga*$^{-/-}$ and *Par4*$^{-/-}$ mice are unaffected to term but combined genetic deficiency recapitulates the hemostatic effect of prothrombin-deficiency with almost all *Par4*$^{-/-}$*:Fga*$^{-/-}$ pups experiencing fatal postnatal internal hemorrhage [6]. The more detailed analysis of hemostasis at birth performed here indicates thrombin-induced platelet activation may be more relevant to birth trauma hemostasis than thrombin-induced fibrin formation. With this in mind, our study suggests that any impact of PAR inhibition on spontaneous bleeding may be heavily dependent on the strength of the hemostatic challenge.

Acknowledgements

The authors thank AMREP AS staff for animal care and Christopher J Boyle for his invaluable legacy.

Declaration of interest

The authors report no declarations of interest.

Funding

This work was supported by grants to JRH from the National Health & Medical Research Council of Australia (#1047295), the Australian Research Council (FT130100540), and the CASS Foundation (SM16/7334).

ORCID

Justin R. Hamilton http://orcid.org/0000-0001-7554-6727

References

1. French SL, Arthur JF, Tran HA, Hamilton JR. Approval of the first protease-activated receptor antagonist: rationale, development, significance, and considerations of a novel anti-platelet agent. Blood Rev 2015;29:179–189.
2. Wong PC, Seiffert D, Bird JE, Watson CA, Bostwick JS, Giancarli M, Allegretto N, Hua J, Harden D, Guay J, Callejo M, Miller MM, Lawrence RM, Banville J, Guy J, Maxwell BD, Priestley ES, Marinier A, Wexler RR, Bouvier M, Gordon DA, Schumacher WA, Yang J. Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding. Sci Transl Med 2017;9 (371):eaaf5294.
3. Hamilton JR, Cornelissen I, Coughlin SR. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. J Thromb Haemost 2004;2:1429–1435.
4. Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 2001;413:74–78.
5. Lee H, Sturgeon SA, Mountford JK, Jackson SP, Hamilton JR. Safety and efficacy of targeting platelet protease-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. Br J Pharmacol 2012;166:2188–2197.
6. Camerer E, Duong DN, Hamilton JR, Coughlin SR. Combined deficiency of protease-activated receptor-4 and fibrinogen recapitulates the hemostatic defect but not the embryonic lethality of prothrombin deficiency. Blood 2004;103:152–154.
7. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, Jackson CW, Hunt P, Saris CJM, Orkin SH. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell 1995;81:695–704.
8. Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter SS, Degen JL. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 1995;9:2020–2033.
9. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. Blood 2002;100:3240–3244.
10. Griffin CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR. A role for thrombin receptor signaling in endothelial cells during embryonic development. Science 2001;293:1666–1670.
11. Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJ. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci U S A 1998;95:7597–7602.

Chapter 4 - Humanising the protease-activated receptor (PAR) expression profile in mouse platelets by knocking PAR1 into the Par3 locus reveals PAR1 expression is not tolerated in mouse platelets

4.1 Introduction

The studies of Chapter 2 provide rationale for the preclinical evaluation of PAR4 antagonism as an antithrombotic approach. However the studies in Chapter 3 highlight the need for an animal model mimicking human platelet PAR expression, so that appropriate safety and efficacy studies can be performed *in vivo*. Since only primate platelets express the PAR1/PAR4 profile, there are significant limits on preclinical *in vivo* research models allowing the requisite investigation into the utility of PAR4 antagonists. The studies of Chapter 4 attempted to address this. Specifically, these studies sought to overcome the limitation of current mouse models by 'humanising' the platelet PAR expression profile of the mouse, essentially by replacing PAR3 with PAR1. Here, it was theorised that 'knock-in' of PAR1 into the endogenous mouse *Par3* locus in a manner allowing platelet-specific expression would create a mouse with PAR1 and PAR4 on platelets but without ectopic PAR1 expression. However, for reasons that are not yet clear, this approach failed. Taken together with earlier studies using distinct approaches, this study indicates forced expression of PAR1 in mouse platelets is difficult to achieve and suggests other options are required for reliable preclinical screening of PAR antagonists.

This chapter has been published as:

French SL, Paramitha AC, Moon MJ, Dickins RA, Hamilton JR. Humanizing the Protease-Activated Receptor (PAR) Expression Profile in Mouse Platelets by Knocking PAR1 into the Par3 Locus Reveals PAR1 Expression Is Not Tolerated in Mouse Platelets. *PloS One*, 2016. 11 (10): e016556.

PLOS | ONE

RESEARCH ARTICLE

Humanizing the Protease-Activated Receptor (PAR) Expression Profile in Mouse Platelets by Knocking *PAR1* into the *Par3* Locus Reveals PAR1 Expression Is Not Tolerated in Mouse Platelets

Shauna L. French, Antonia C. Paramitha, Mitchell J. Moon, Ross A. Dickins, Justin R. Hamilton*

Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

* Justin.Hamilton@monash.edu

OPEN ACCESS

Citation: French SL, Paramitha AC, Moon MJ, Dickins RA, Hamilton JR (2016) Humanizing the Protease-Activated Receptor (PAR) Expression Profile in Mouse Platelets by Knocking *PAR1* into the *Par3* Locus Reveals PAR1 Expression Is Not Tolerated in Mouse Platelets. PLoS ONE 11(10): e0165565. doi:10.1371/journal.pone.0165565

Editor: Zhenyu Li, University of Kentucky, UNITED STATES

Received: July 17, 2016

Accepted: October 13, 2016

Published: October 27, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by a grant to JRH from the National Health & Medical Research Council of Australia (1047295) and a Future Fellowship from the Australian Research Council (130100540). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Anti-platelet drugs are the mainstay of pharmacotherapy for heart attack and stroke prevention, yet improvements are continually sought. Thrombin is the most potent activator of platelets and targeting platelet thrombin receptors (protease-activated receptors; PARs) is an emerging anti-thrombotic approach. Humans express two PARs on their platelets–PAR1 and PAR4. The first PAR1 antagonist was recently approved for clinical use and PAR4 antagonists are in early clinical development. However, pre-clinical studies examining platelet PAR function are challenging because the platelets of non-primates do not accurately reflect the PAR expression profile of human platelets. Mice, for example, express Par3 and Par4. To address this limitation, we aimed to develop a genetically modified mouse that would express the same repertoire of platelet PARs as humans. Here, human *PAR1* preceded by a lox-stop-lox was knocked into the mouse *Par3* locus, and then expressed in a platelet-specific manner (hPAR1-KI mice). Despite correct targeting and the predicted loss of Par3 expression and function in platelets from hPAR1-KI mice, no PAR1 expression or function was detected. Specifically, PAR1 was not detected on the platelet surface nor internally by flow cytometry nor in whole cell lysates by Western blot, while a PAR1-activating peptide failed to induce platelet activation assessed by either aggregation or surface P-selectin expression. Platelets from hPAR1-KI mice did display significantly diminished responsiveness to thrombin stimulation in both assays, consistent with a *Par3-/-* phenotype. In contrast to the observations in hPAR1-KI mouse platelets, the PAR1 construct used here was successfully expressed in HEK293T cells. Together, these data suggest ectopic PAR1 expression is not tolerated in mouse platelets and indicate a different approach is required to develop a small animal model for the purpose of any future preclinical testing of PAR antagonists as anti-platelet drugs.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Anti-platelet drugs are the primary therapy for heart attack and stroke prevention, yet improvements are continually sought. Thrombin is by far the most potent endogenous platelet activator, which it achieves via cell surface protease-activated receptors (PARs). Due to the importance of thrombin-induced platelet activation during thrombosis, targeting platelet thrombin receptors has received significant clinical attention, and PAR antagonists are one of the most promising of the emerging anti-thrombotic approaches [1–3]. Humans express two PARs on their platelets, PAR1 and PAR4, both of which are cleaved and activated by thrombin, and both of which are capable of inducing robust platelet activation [4, 5]. PAR1 has a higher affinity for thrombin and was therefore the target of initial drug development. The first PAR1 antagonist, vorapaxar, was recently approved by the FDA for clinical use in the USA [2]. So far, however, vorapaxar has limited clinical utility due to an increase in major bleeding events associated with its use [6, 7]. These limitations on the first PAR1 inhibitor have ignited interest in evaluating the clinical potential of PAR4 antagonists, and these agents are now in early clinical development (NCT02208882) [8].

However, a major limitation in examining platelet PAR function in detail is the absence of a small animal model that accurately reflects the PAR expression profile in human platelets: only primate platelets express the PAR1/PAR4 profile [9]. This places significant limits on preclinical research into the utility of PAR antagonists *in vivo*. Of the commonly-used small animals, for example, platelets from mice, rats and rabbits express PAR3 and PAR4 [10–13], while guinea-pig platelets express PAR1, PAR3 and PAR4 [14]. *Par4-/-* mice have been crucial for early proof-of-concept studies into the overall role of PARs in thrombosis [15–17], but are unsuitable for elucidating, for example, the relative anti-thrombotic effects of PAR1 vs PAR4 inhibition.

One way to overcome this limitation is to 'humanize' the platelet PAR expression profile of the mouse, essentially replacing mouse PAR3 (Par3) with human PAR1 (PAR1). Initial attempts used platelet-specific expression of a PAR1 transgene in *Par3-/-* mice, but were unsuccessful [18]. Possible explanations for the failure of this transgene-based approach include insufficient expression levels or unpredicted gene silencing consequences as a result of nonspecific transgene insertion [18]. We theorized that 'knock-in' of PAR1 into the endogenous mouse *Par3* locus in a manner that would allow platelet-specific expression may overcome these earlier issues and would create a mouse that expresses PAR1 and Par4 on platelets, but without further ectopic PAR1 expression. Here, we generate and characterise such a mouse. Despite correct targeting, this genetic approach failed to yield detectable expression or function of PAR1 on mouse platelets. When taken together with earlier studies using distinct approaches, this study indicates that forced expression of PAR1 in mouse platelets will be difficult to achieve and suggests that other options will be required for reliable preclinical screening of any future PAR antagonists.

Methods and Materials

Materials

Human α-thrombin and adenosine 5'-diphosphate sodium salt (ADP) were purchased from Sigma-Aldrich (St Louis, MO, USA). PAR1-activating peptide (PAR1-AP; TFLLR-NH_2) and PAR4-activating peptide (PAR4-AP; AYPGKF-NH_2) were synthesised at Monash Institute of Pharmaceutical Sciences (Melbourne, Australia) by Assoc Professor Philip Thompson. The mouse anti-P-selectin (FITC anti-mouse CD62P) and human anti-P-selectin (PE anti-human CD62P) antibodies were purchased from BD Biosciences (San Jose, CA, USA). PE anti-CD45.2,

PE anti-CD41a (human) antibody and PE anti-CD41 antibody (mouse) were purchased from Abcam (Melbourne, Australia). The PE-anti-GPIb-tail and non-immune rabbit IgG isotype were a generous gift from Assoc Professor Robert Andrews (Monash University, Melbourne, Australia). The anti-PAR1 antibody (ATAP2), anti-PAR3 antibody (8E8), HRP-anti-actin (I-19) and HRP- anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Generation of hPAR1-KI (*Pf4-Cre; Par3$^{LSL-PAR1}$*) mice

All mouse studies were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee (approval number E/1465/2014/M). *Pf4-Cre; Par3$^{LSL-PAR1}$* mice (hereafter referred to as hPAR1-KI mice) were generated by Ozgene (Perth, Australia). The targeting vector involved exon 2 of *Par3* (*F2rl2*) flanked with a lox-stop-lox and followed by exon 2 of *PAR1 (F2r)* (Fig 1A). Since exon 1 encodes the signal peptide and exon 2 encodes the entire mature receptor for both PAR1 and Par3 [19], Cre-mediated deletion is predicted to result in the replacement of Par3 with PAR1 and leaves the endogenous *Par3* promoter intact (Fig 1A). The BAC vector comprised unaltered exon 1 of *Par3*, then exon 2 of *Par3* followed by a stop codon and flanked by loxP sites, and followed by *PAR1*. The vector was injected into C57BL/6J blastocysts, chimeras were bred for germline transmission, and the targeted allele bred to homozygosity (*Par3$^{LSL-PAR1}$*). These mice were then bred with Pf4-Cre mice to induce deletion of *Par3* and permit the expression of *PAR1* specifically in platelets (*Pf4-Cre; Par3$^{LSL-PAR1}$*, hereafter referred to as hPAR1-KI mice). PCR genotyping of the targeted allele was performed using the following primers: common reverse (AGCTGAAAAATGGAGCGCTTG) with WT forward (TGGGTTCCTCATCCCGTTTG, predicted size 588bp) or mutant forward (TCCTTCACTTGTCTGGCCATC, predicted size 914bp) (Fig 1A). Deletion of *Par3* was confirmed by PCR using the following primers: forward (CAGTGTGTG GTTTTGTTTCACCT) and reverse (GCCAATCACTGCCGGAAAAG, predicted size 963bp) (Fig 1A).

Isolation of mouse and human platelets

All human studies were approved by the Monash University Human Research Ethics Committee (CF07/0141-2007/0025). Blood was collected after written informed consent was obtained from healthy adults (21–50 years old, of both sexes) who had not taken anti-platelet medications in the past 10 d. Mouse and human blood was drawn into syringes containing acid citrate dextrose (ACD; 1:7 v/v). Platelets were isolated by centrifugation as previously described [20].

Megakaryocyte DNA extraction

Bone marrow cells were aseptically flushed from the femora and tibiae of mice using a syringe and a 23-gauge needle containing Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% foetal bovine serum (FBS), penicillin, streptomycin and glutamine. Post-harvest, thrombopoietin (0.1 μg/mL) was added to the cells, which were allowed to culture for 4 d at 37°C, 5% CO_2. Cells were then collected and centrifuged at 50 g for 5 min and the pellet was re-suspended in 4 mL of PBS. The cells were layered over a two-step BSA density gradient and allowed to sediment for 45 min at room temperature. The megakaryocyte-rich population was collected and centrifuged at 50 g for 5 min, and the pellet was treated with DNA extraction buffer (Bioline) and digested at 75°C for 10 min.

Platelet aggregation

Platelet aggregation was measured by light transmission aggregometry in a 96-well plate format. Human and mouse washed platelets ($2x10^8$ platelets/mL) were added to wells of a clear, flat

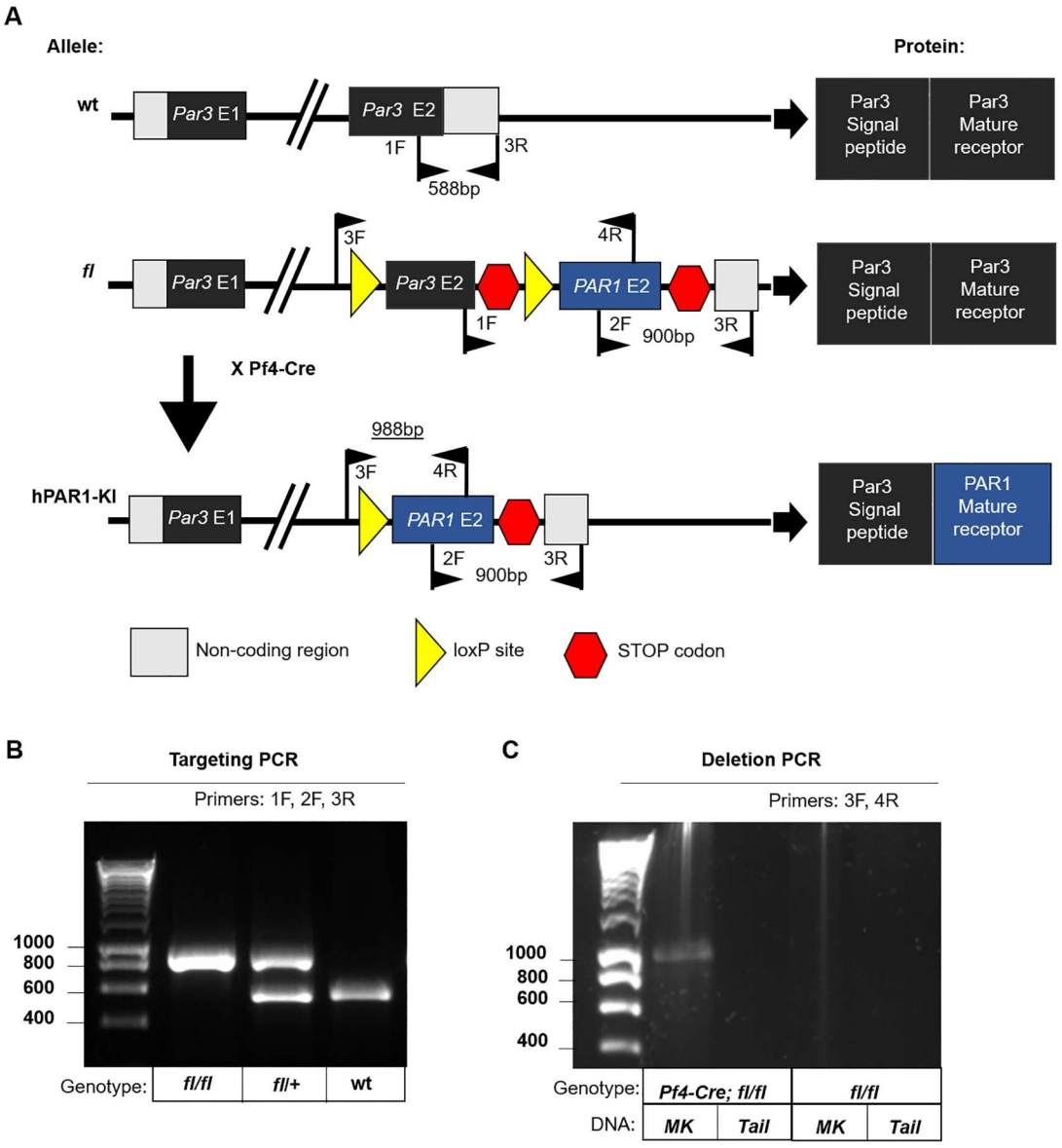


Fig 1. Generation of mice expressing PAR1 in place of Par3 in a platelet-specific manner. (A) Schematic of the strategy for platelet-specific expression of human PAR1 in place of mouse Par3. Top: wild-type *Par3* (wt) has two exons: exon 1 encodes the signal peptide and exon 2 encodes the complete mature receptor. Middle: In the targeted allele ($Par3^{LSL\text{-}PAR1}$; fl) an insert of exon 2 of human *PAR1* is placed downstream of exon 2 of *Par3*. *Par3* exon 2 is flanked by loxP sites, allowing Cre-mediated excision. Bottom: Cre-mediated deletion of *Par3* exon 2 allows expression of *PAR1* exon 2, resulting in a predicted chimeric protein consisting of the Par3 signal peptide fused to the mature PAR1 receptor (hPAR1-KI). Heterozygous targeted mice, $Par3^{LSL\text{-}PAR1/+}$ (fl/+); homozygous targeted mice, $Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$ (fl/fl); platelet-specific replacement of mPAR3 with hPAR1, Pf4-Cre; $Par3^{LSL\text{-}PAR1}$ (Pf4-Cre;fl/fl). **(B)** PCR genotyping of tail DNA using the primers indicated (black flags): wild type forward (1F), mutant forward (2F), common reverse (3R). **(C)** Cre-mediated deletion of *Par3* was confirmed by PCR genotyping of individual mice using DNA from both tail (not deleted) and megakaryocytes (MK; deleted), using a forward primer spanning the loxP site (3F) and *PAR1* reverse (4R). A PCR product of predicted size (988bp) was only generated using MK DNA from Pf4-Cre;fl/fl mice. Sequencing of this PCR product confirmed excision of floxed sequences (S3 Fig).

doi:10.1371/journal.pone.0165565.g001

bottom, 96-well tissue culture plate (Falcon). Platelets were stimulated with either thrombin (0.01–1 U/mL), PAR1-AP (10–100 μM), PAR4-AP (10–100 μM), or ADP (1–10 μM). The plate was analysed at 37°C in a FLUOstar OPTIMA plate reader (BMG Labtech) using a 595 nm excitation filter, for a period of 50 min (10 read cycles with 5 min double orbital shake period between each read). Ability to aggregate was calculated as $\frac{OD(No\ Agonist) - OD(Agonist)}{OD(No\ Agonist) - OD(blank)} \times 100$ at the time point where aggregation was at a maximum. Optical density was normalised against the blank (maximum) and unstimulated platelets (minimum) and expressed as % maximum.

P-selectin expression

Flow cytometry was used to detect the expression of P-selectin by activated platelets. Human and mouse platelets, prepared as outlined above, were incubated with an anti-P-selectin antibody and stimulated with thrombin (0.01–1 U/mL), PAR1-AP (10–100 μM), or PAR4-AP (30–300 μM) for 15 min at 37°C. Samples were then analysed using a FACSCalibur (Becton Dickinson) flow cytometer and FlowJo software.

Analysis of platelet PAR1 expression

Platelets isolated from either humans or mice ($5x10^7$ platelets/mL in Tyrode's buffer) were incubated with a PE-conjugated antibody against one of PAR1 (2.5 μg/10^6 cells; ATAP2), CD45 (platelet negative control antibody of matching isotype, 2.5 μg/10^6 cells), or CD41a (platelet positive control antibody of matching isotype, 2.5 μg/10^6 cells). In some experiments, isolated platelets were permeabilized with saponin (0.1% v/v) prior to incubation with antibodies. In these experiments, a PE-conjugated antibody against the cytoplasmic tail of GPIb was included to confirm effective platelet permeabilization [21]. In all cases, samples were analysed using a FACSCalibur flow cytometer and FlowJo Software. Positive expression was measured as a rightward shift in relative fluorescent intensity compared to the isotype control.

Western blot

Platelet proteins were analyzed in lysates of human or mouse isolated platelets ($1x10^9$ platelets/mL), prepared in Laemmli's buffer, denatured (95°C for 10 min), and run on 12% SDS-polyacrylamide gels at 170 V for 40 min. Proteins were transferred to PVDF membranes (Millipore, Lake Placid, NY, USA) at 250 mA for 2 h. Membranes were blocked with TBST (0.01 mM Tris, 50 mM NaCl, 0.01% v/v Tween-20) containing 5% skim milk powder for 30 min at room temperature, incubated with either an HRP-conjugated anti-actin antibody (1 μg/mL), anti-PAR1 antibody (1 μg/mL) or anti-PAR3 antibody (1 μg/mL) at 4°C overnight, followed by a HRP-conjugated anti-mouse IgG at room temperature for 2 h. Enhanced chemiluminescence (ECL) substrate (ThermoFisher Scientific, Waltham, MA, USA) was placed onto the membrane for 1 min before exposure (ChemiDoc Touch Imaging System, Bio-Rad, Hercules, CA, USA).

Results

Generation of hPAR1-KI (*Pf4-Cre; Par3$^{LSL\text{-}PAR1}$*) mice

We generated mice in which *PAR1* (*F2R*) was knocked into the *Par3* (*F2lr2*) locus and conditionally expressed in platelets upon Pf4-Cre-mediated excision. To this end, exon 2 of *PAR1* (encoding the entire mature receptor) was inserted downstream of exon 2 of *Par3* flanked by a lox-stop-lox (Fig 1A). This approach leaves the promoter and exon 1 of *Par3* (encoding the signal peptide) intact and results in replacement of *Par3* with *PAR1* after Cre-mediated excision (Fig 1A). This is predicted to yield the Par3 signal peptide linked to the complete mature

receptor of PAR1 (Fig 1A). The stability and surface expression of the predicted fusion protein was confirmed in HEK293T cells transiently transfected with vectors expressing the same sequence as that remaining after Cre-mediated excision of the targeted allele, with the predicted Par3/PAR1 fusion protein expressed at similar levels to native PAR1 (S1 Fig). We therefore went on to generate $Par3^{LSL\text{-}PAR1/+}$ mice and confirmed targeting by Southern blot (S2 Fig) and PCR genotyping (Fig 1B). Heterozygous mice were bred to homozygosity ($Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$) and crossed with Pf4-Cre mice [22], with experimental mice resulting from crosses of $Pf4\text{-}Cre;Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$ x $Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$ (Fig 1B). Cre positive offspring ($Pf4\text{-}Cre;Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$) are hereafter referred to as hPAR1-KI mice and their Cre negative littermates ($Par3^{LSL\text{-}PAR1/LSL\text{-}PAR1}$) as wild-type. Appropriate Pf4-Cre-mediated deletion in the platelet/megakaryocyte lineage was confirmed by PCR of DNA isolated from megakaryocytes versus tail biopsy (Fig 1C). We used a forward primer placed upstream of the loxP site and a reverse primer in each of *Par3* and *PAR1* as indicated (Fig 1A). As predicted, a band of expected size was only generated in megakaryocyte DNA from hPAR1-KI mice. Sequencing of this PCR product confirmed the predicted targeting and Cre-mediated deletion (S3 Fig).

Platelets from hPAR1-KI mice do not respond to PAR1-selective activation

Mice from these crosses were born at the expected rates, were normal in weight and appearance, and had normal whole blood counts (S1 Table). We assessed PAR1 function in platelets isolated from hPAR1-KI mice using two distinct markers of platelet activation: platelet aggregation and P-selectin expression. PAR1-selective activation by a PAR1-AP failed to aggregate platelets from hPAR1 mice at concentrations up to 100 μM (100 times the concentration required to cause platelet activation in human platelets [23]) (Fig 2A). This lack of response

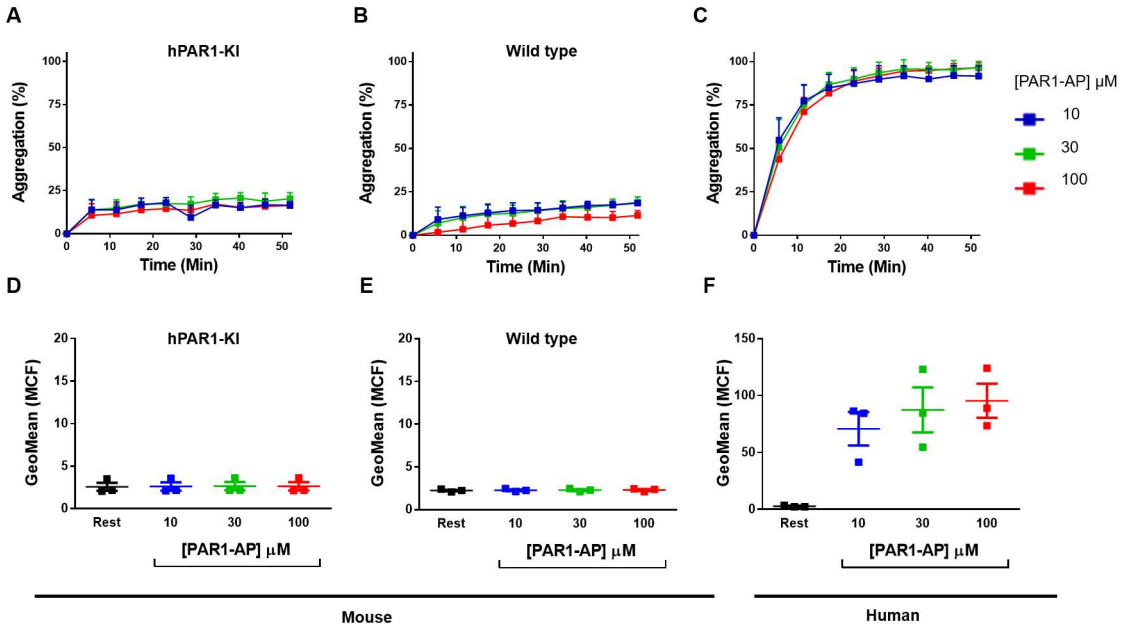


Fig 2. Platelets from hPAR1-KI mice do not respond to PAR1-selective activation. Platelets isolated from hPAR1-KI mice, wild type mice, or humans were stimulated with a PAR1-activating peptide (PAR1-AP; TFLLR) and examined for **(A-C)** platelet aggregation by light transmission aggregometry or **(D-F)** P-selectin expression by flow cytometry. Note that platelets from either hPAR1-KI or wild type mice failed to respond to PAR1-AP, even at concentrations that were supra-maximal in human platelets. Data are mean ± SEM of n = 3 experiments.

doi:10.1371/journal.pone.0165565.g002

was identical in platelets from wild type mice (Fig 2B). As expected, PAR1-AP induced a robust aggregation response in human platelets (Fig 2C). A similar pattern of responses was observed when P-selectin expression was used as the marker of platelet activation, with PAR1-AP unable to elicit any response in platelets isolated from either hPAR1-KI (Fig 2D) or wild type (Fig 2E) mice, but a robust response in human isolated platelets (Fig 2F). Together, these data indicate lack of functional PAR1 in platelets from hPAR1-KI mice.

Lack of PAR1 expression on platelets from hPAR1-KI mice

We next examined PAR1 expression in platelets from hPAR1-KI mice. We were unable to detect PAR1 on the surface of platelets from hPAR1-KI mice by flow cytometry (Fig 3A). As with the functional experiments, platelets from wild type mice and humans served as effective negative and positive controls for PAR1 surface expression by this method, respectively (Fig 3B and 3C). In all experiments, the platelet specific marker, CD41, and normal forward/side scatter properties were used to confirm the integrity of the platelets being assessed (Fig 3).

We also tested for PAR1 expression in permeabilized platelets. In these experiments, an antibody against the cytoplasmic tail of GPIbα [21] was used as a positive control for successful permeabilization. As predicted, this antibody only recognised an epitope on the internal surface of the platelet membrane, with no staining of intact platelets and strong staining in permeabilized platelets from both mice and humans (S4 Fig). However, we were unable to detect any PAR1 in permeabilized platelets from hPAR1-KI mice (Fig 4A). Again, platelet integrity was confirmed by CD41 expression in all cases, while permeabilization was confirmed by

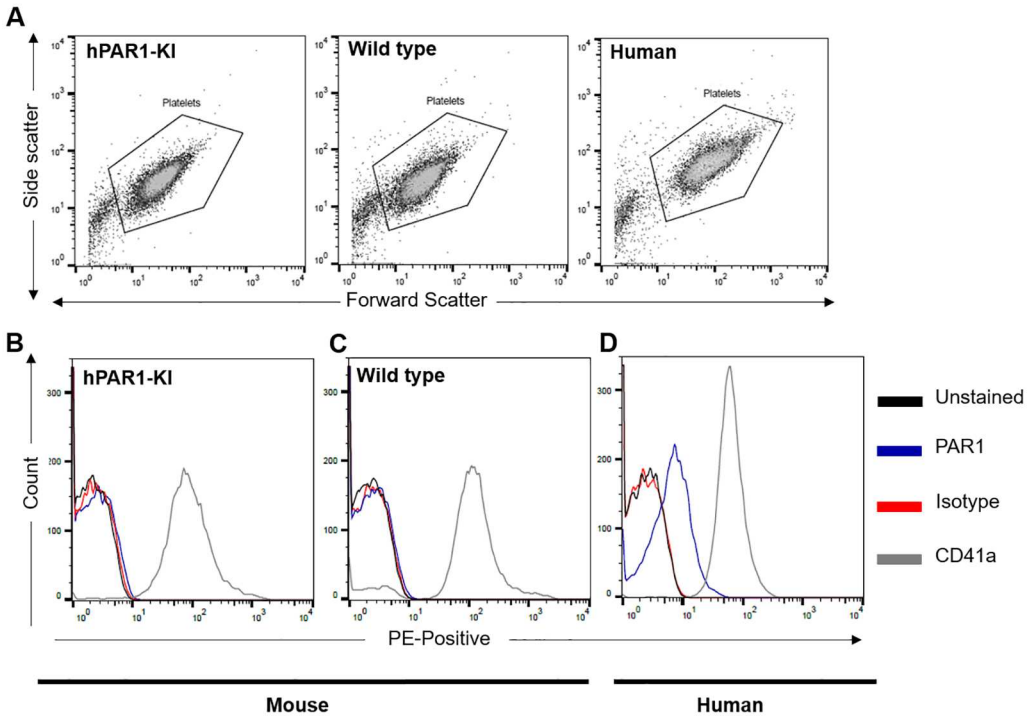


Fig 3. PAR1 is undetectable on the surface of platelets from hPAR1-KI mice. Platelets isolated from hPAR1-KI mice, wild type mice, or humans were either left unstained (black) or were incubated with a PE-conjugated antibody against one of PAR1 (blue) or CD41a (grey; positive platelet control), or an isotype control (red). **(A)** Platelets were gated by forward and side scatter as shown. **(B-D)** PAR1 was detected on human platelets but not platelets from either hPAR1-KI or wild type mice. Data shown are representative traces of n = 3 individual experiments.

doi:10.1371/journal.pone.0165565.g003

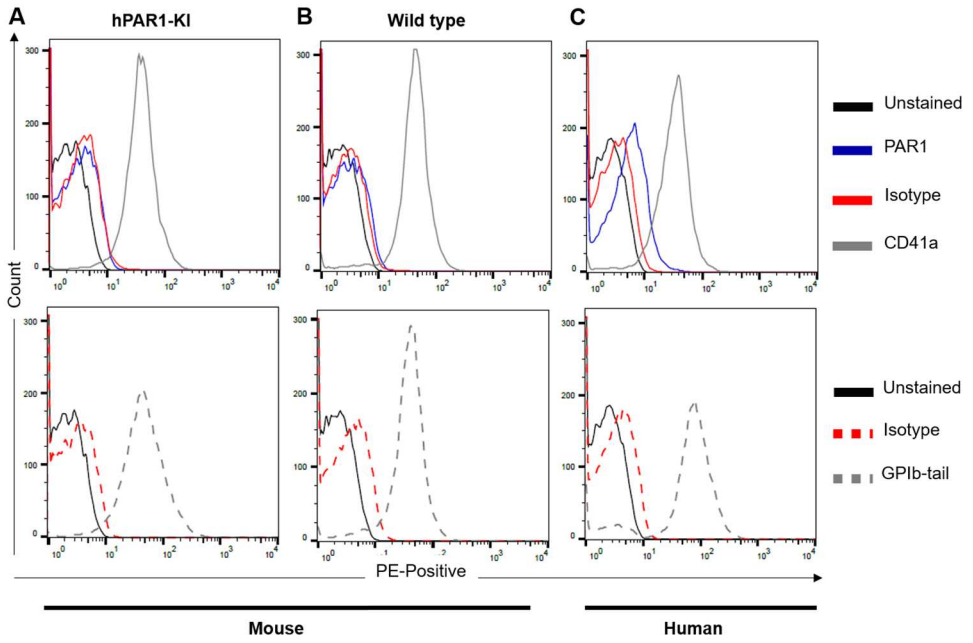


Fig 4. PAR1 is undetectable within platelets from hPAR1-KI mice. Platelets isolated from **(A)** hPAR1-KI mice, **(B)** wild type mice, or **(C)** humans were fixed and permeabilized prior to incubation with a PE-conjugated antibody against one of PAR1 (blue), CD41a (grey; positive control for platelets), isotype (red), or the cytoplasmic tail of GPIbα (grey dash; positive control for permeabilization). PAR1 was detected in human platelets but not in platelets from either hPAR1-KI or wild type mice. Data shown are representative traces of n = 3 individual experiments.

doi:10.1371/journal.pone.0165565.g004

detection with the anti-GPIbα-tail antibody (Fig 4). Platelets from wild type mice and humans again served as controls (Fig 4B and 4C), indicating the anti-PAR1 antibody still binds its epitope in permeabilized cells.

Finally, we also examined PAR1 expression by Western blot. Here, a band at the predicted size of approximately 75 kD was detected in lysates of human platelets, but not of wild-type or hPAR1-KI mouse platelets (Fig 5), mirroring the flow cytometry data. Together, these findings indicate PAR1 is not expressed at detectable levels in platelets from hPAR1-KI mice.

PAR3 expression and function is disrupted in platelets from hPAR1-KI mice

Our genetic strategy is predicted to disrupt Par3 expression in platelets. This was confirmed genetically by PCR genotyping and sequencing (Fig 1). Regardless, given the lack of PAR1 expression or function in platelets from hPAR1-KI mice, we therefore also probed for Par3 protein in these platelets. We never detected Par3 expression by Western blot in human platelets, but routinely observed a band of the previously reported size (approximately 50kDa) in wild type mouse platelets (Fig 5). This band was absent in platelet lysates from all hPAR1-KI mice examined (Fig 5), further confirming disruption of *Par3* in hPAR1-KI mice. To further examine this, we tested for the predicted functional effects of *Par3*-deficiency.

Platelets from *Par3*-/- mice exhibit diminished responsiveness to thrombin [24]. Here, we observed a similar effect in platelets from hPAR1-KI mice. We examined platelet aggregation and P-selectin expression in response to thrombin. Platelets from hPAR1-KI mice exhibited an approximately 3-fold decrease in sensitivity to thrombin in both assays when compared to platelets from wild type mice (Fig 6). Specifically, aggregation of hPAR1-KI platelets was only

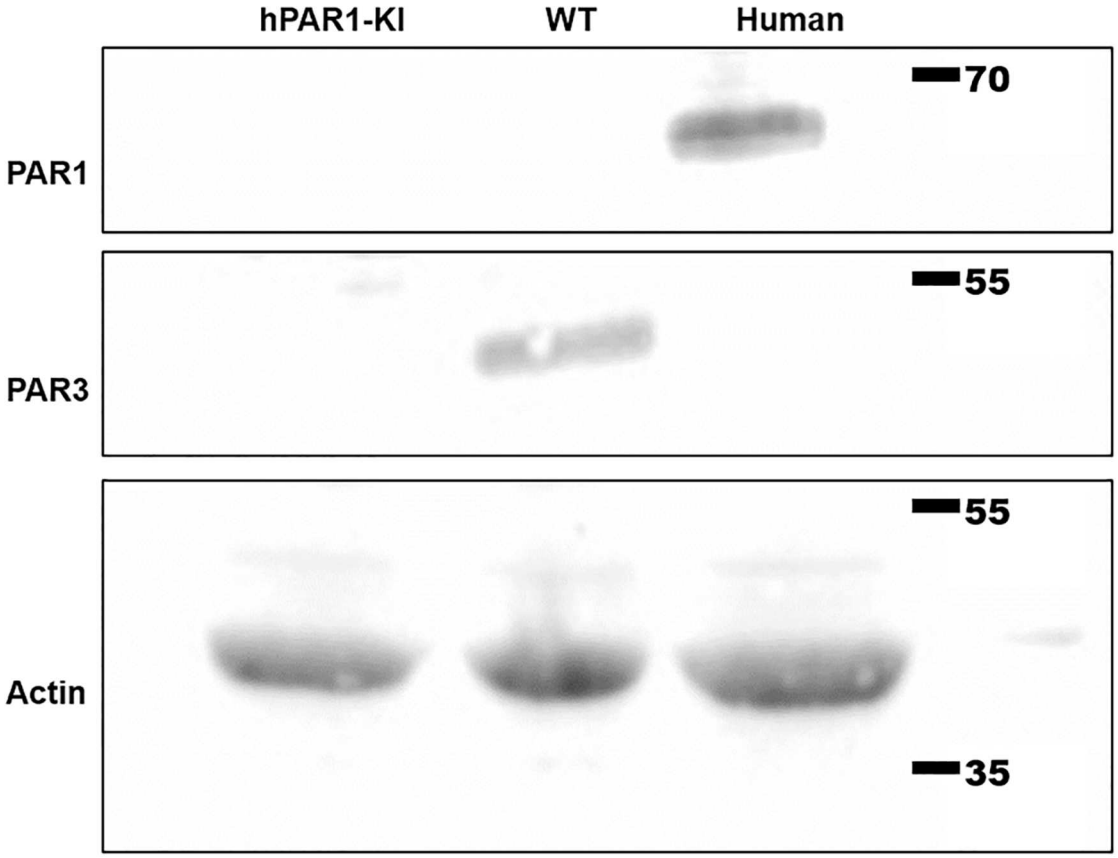


Fig 5. Protein for both PAR1 and Par3 is undetectable in platelets from hPAR1-KI mice. Whole cell lysates of platelets isolated from hPAR1-KI mice, wild type mice, or humans were probed for either PAR1 or Par3 protein by Western blot. PAR1 was readily detected in human platelets but not in platelets from wild type or hPAR1-KI mice. In the same samples, Par3 was detected in platelets from wild type mice but not in platelets from either hPAR1-KI mice or humans. Actin was used as a protein loading control for all lysates. Blots shown are representative of n = 3 experiments.

doi:10.1371/journal.pone.0165565.g005

achieved at thrombin concentrations of 0.3 U/mL and above (Fig 6A) compared with 0.1 U/mL and above for wild type platelets (Fig 6B). A similar pattern emerged when examining P-selection expression in response to thrombin: aggregation of hPAR1-KI platelets was only achieved at thrombin concentrations of 1 U/mL (Fig 6D) compared with 0.3 U/mL and above for wild type platelets (Fig 6E).

Importantly, hPAR1-KI platelets responded normally to other agonists, PAR4-AP and ADP (Fig 7). When comparing platelets from hPAR1-KI and wild type mice, near-identical concentration-responses were observed for ADP-induced aggregation (Fig 7A and 7B), PAR4-AP-induced aggregation (Fig 7D and 7E), and PAR4-AP-induced P-selectin expression (Fig 7G and 7H). These functional data are consistent with the phenotype observed in platelets from *Par3-/-* mice and, when combined with the failure to detect Par3 expression in hPAR1-KI platelets, suggest correct targeting and *Par3* deletion in hPAR1-KI mice.

Discussion

PARs are leading targets for new anti-platelet drugs, exemplified by the recent approval of the PAR1 antagonist, vorapaxar, and the early clinical development of the PAR4 antagonist, BMS-986120. Given the increased focus of examining PAR antagonists as anti-platelet agents, there

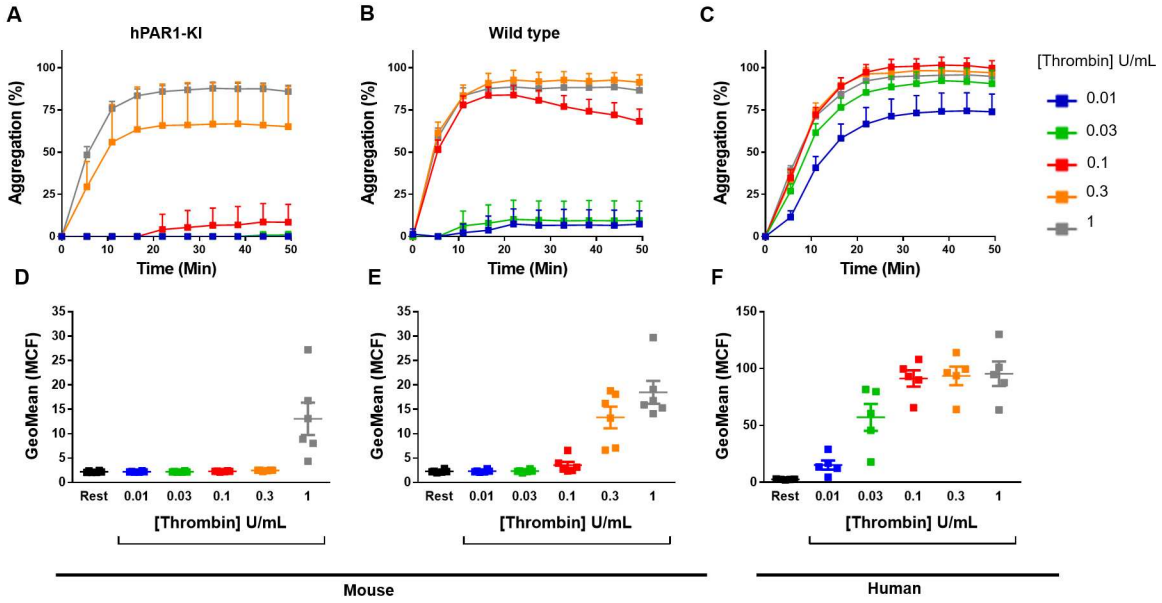


Fig 6. Platelets from hPAR1-KI mice exhibit diminished responses to thrombin. Platelets isolated from **(A,D)** hPAR1-KI mice, **(B,E)** wild type mice, or **(C,F)** humans were with thrombin and examined for **(A-C)** platelet aggregation by light transmission aggregometry or **(D-F)** P-selectin expression by flow cytometry. Note the approximate 3-fold decrease in sensitivity to thrombin-induced responses in platelets from hPAR1-KI mice versus wild-type mice in both assays. Data are mean ± SEM of n = 4–7 experiments.

doi:10.1371/journal.pone.0165565.g006

is much interest in developing an appropriate small animal model for *in vivo* testing of such drugs. This has proved to be a major limitation in the field, as the PAR1/PAR4 expression profile of human platelets is only shared amongst primates. Here, we attempted to create a genetically-modified mouse that mimics the PAR1/PAR4 expression profile of human platelets. To do this, we knocked the human *PAR1* gene in to the mouse *Par3* locus in a manner that provided for platelet-specific replacement of Par3 with PAR1. Correct targeting was confirmed genetically and was apparent functionally. Yet despite this, we were unable to detect expression or function of PAR1 in platelets from these mice, indicating the approach used here is insufficient to 'humanize' platelet PAR expression in mice by inducing PAR1 expression in place of Par3.

Forced PAR1 expression has been previously achieved using transgene-based approaches in other cell types. For example, mouse PAR1 (Par1) was specifically expressed in endothelial cells via the Tie2 promoter/enhancer [25] and in cardiomyocytes via the αMHC promoter [26]. In addition, PAR1 has been expressed in mouse mammary gland epithelium [27]. In contrast to these successes, a previous attempt failed to express either Par1 or PAR1 transgenes in mouse platelets via multiple platelet-specific promoters [18]. The reasons for the inability to express PAR1 in mouse platelets remain unknown. However, given the limitations and lack of control associated with transgene-based approaches, we used a knock-in approach in which *PAR1* was targeted to the *Par3* locus, essentially resulting in replacement of Par3 with PAR1, and with expression governed by the endogenous mouse *Par3* promoter.

Correct genetic targeting was confirmed by multiple methods, including Southern blot analysis of founders, PCR genotyping across the insert, and PCR analysis of correct Pf4-Cre-mediated excision in the DNA in isolated megakaryocytes. Correct targeting was also apparent by the absence of Par3 expression in platelets from hPAR1-KI mice. The disruption of Par3 expression was further supported by functional analyses of these platelets that revealed a

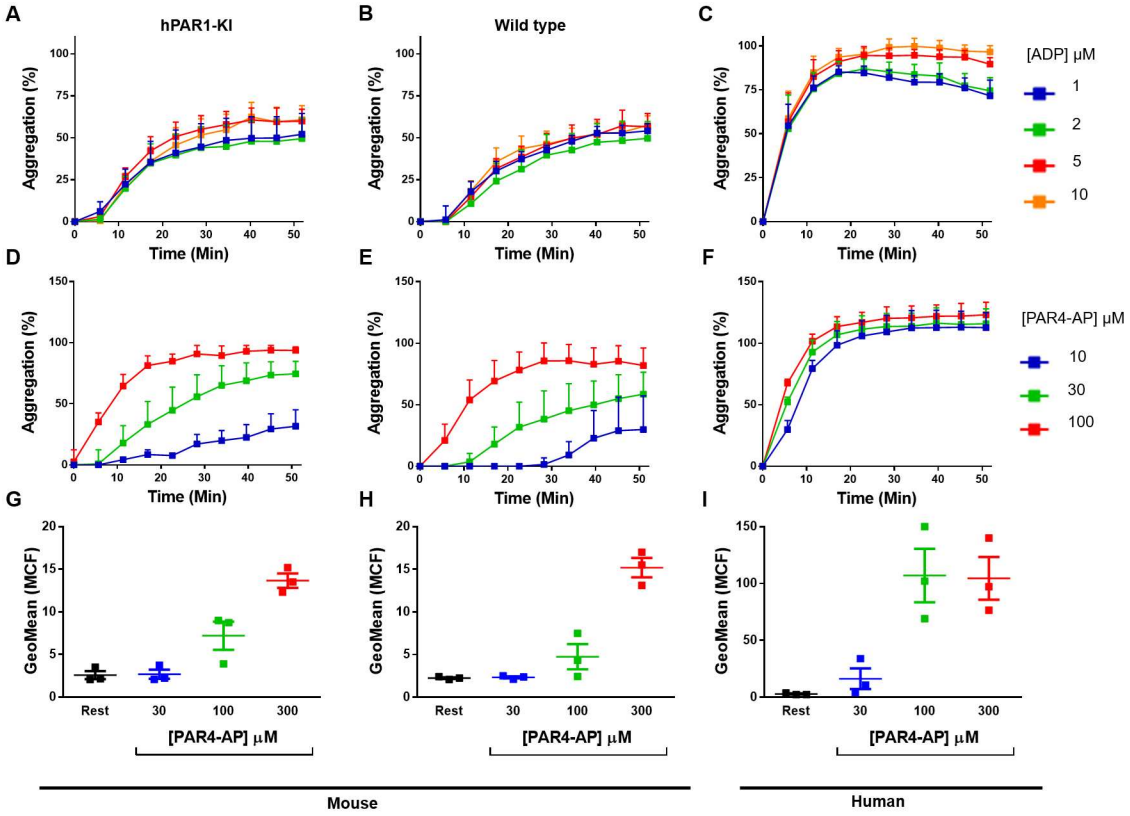


Fig 7. Platelets from hPAR1-KI mice respond normally to PAR1-independent platelet agonists. Platelets isolated from **(A,D,G)** hPAR1-KI mice, **(B,E,H)** wild type mice, or **(C,F,I)** humans were stimulated with either **(A-C)** ADP or **(D-I)** a PAR4-activating peptide (PAR4-AP) and assessed by **(A-F)** platelet aggregation or **(G-I)** P-selectin expression. Note the near-identical concentration-dependent responses in platelets from hPAR1-KI and wild type mice in all cases examined. Data are mean ± SEM of n = 3 experiments.

doi:10.1371/journal.pone.0165565.g007

phenotype remarkably similar to that observed in platelets from *Par3*$^{-/-}$ mice [24], with an approximately 3-fold decrease in sensitivity to thrombin-induced platelet activation. Together, this detailed analysis indicates correct targeting occurred in these mice and resulted in disruption of Par3 expression. However, no replacement PAR1 expression or function was detected.

The reason behind this lack of expression of PAR1 in mouse platelets is unknown. Our initial functional studies showed that hPAR1-KI platelets did not respond to a PAR1-specific agonist but responded normally to other agonists, suggesting that the receptor was either absent or unable to signal. We probed extensively for protein expression but were unable to detect PAR1 in platelets from hPAR1-KI mice via flow cytometry or Western blot. The lack of detection of PAR1 even in permeabilizsed platelets (by flow cytometry) or in whole cell platelet lysates (by Western blot) strongly suggest that the PAR1 protein is either not translated or is very rapidly degraded. The inability of mouse platelets to ectopically express PAR1 so far appears unique, but remains unexplained and could arise from a number of critical stages in the development of mature PAR1 protein. While possible that the Par3 signal peptide is insufficient to guide PAR1 to the cell surface [28], the highly conserved nature of signal peptides coupled with our observation of robust surface expression of the fusion protein in HEK293T cells suggest otherwise. Other potential explanations include as yet unidentified mechanisms that appear unique to the mouse platelet, such as poor or defective N-linked glycosylation of the receptor leading to receptor instability and signaling dysfunction [29, 30]. Since the primary aim of this study

was to generate a research tool for *in vivo* thrombosis studies, we did not explore the precise mechanism behind the lack of tolerance for PAR1 expression in mouse platelets. Whether or not replacement of the entire human *PAR1* gene in the mouse *Par3* locus will be sufficient to drive expression in mouse platelets remains unknown. Regardless, it appears that other approaches will need to be examined if a non-primate model is to be used for pharmacological screening of drugs targeting platelet PARs.

Supporting Information

S1 Fig. The receptor generated in hPAR1-KI mice can be expressed in HEK293T cells. The fusion protein predicted to be expressed by hPAR1-KI mice, consisting of the mPAR3 signal peptide attached to the mature hPAR1 receptor, can be expressed on the surface of transfected HEK293T cells. HEK293T cells were transfected with native hPAR1 (purple), the mPAR3/hPAR1 fusion (pink), or empty vector (yellow). Note the similar level of detectable expression of native hPAR1 and the mPAR3/hPAR1 fusion. Also shown is a positive control for surface expression (Thy-1) in cells transfected with the empty vector.
(TIFF)

S2 Fig. Southern blot analyses of targeted ES cells and founder mice. (A) ES cell clones were screened for targeting by Southern blot. Targeted clones are indicated by the expected additional band at 8.3kb (e.g. clones D1 and D4). **(B)** Founder mice screened by the same Southern blot approach as in (A), showing results for 13 mice from two litters. Seven of the 13 mice genotyped as heterozygous targeted, exhibiting both the wt (11.6 kb) and mutant band (8.3kb).
(TIFF)

S3 Fig. Sequence annotation for the hPAR1-KI allele. (A) Predicted genomic DNA sequence for the hPAR1-KI allele, showing *Par3* exon 1 highlighted in grey, the position of primers 3F and 4R in red, the loxP site in yellow, and *PAR1* exon 2 in blue. Red text indicates the sequence output of the PCR product from primers 3F and 4R, which was identical to the predicted genomic DNA sequence. **(B)** Annotated sequence of the predicted coding sequence and protein chimera, comprising the Par3 signal peptide (grey text) and PAR1 mature receptor (blue text). Red text indicates sequenced PCR product amplified from genomic DNA of hPAR1-KI mice, aligning to the predicted region of PAR1 coding sequence.
(TIFF)

S4 Fig. Confirmation of platelet permeabilization. Platelets isolated from a mouse (left) or human (right) were either left intact (top) or were permeabilized with saponin (0.1%; bottom) prior to incubation with an antibody against the intracellular C-terminal of GPIbα (grey) or isotype control (red). The GPIbα tail antibody produced a rightward shift over isotype only in permeabilized platelets of both species, confirming successful permeabilization. Data shown are representative traces of n = 3 individual experiments.
(TIFF)

S1 Table. Whole blood cell counts from wild type and hPAR1-KI mice.
(DOCX)

Acknowledgments

The authors wish to thank AMREP AS staff for animal care and members of the AMREP flow cytometry core facility for technical assistance and advice. The authors also thank Assoc Professors Philip Thompson, Elizabeth Gardiner and Robert Andrews for generously providing key reagents, and L. R. B. Cooper for valuable contributions. This work was supported by

grants to JRH from the National Health & Medical Research Council of Australia. JRH is a Future Fellow of the Australian Research Council.

Author Contributions

Conceptualization: SLF RAD JRH.

Formal analysis: SLF ACP MJM.

Funding acquisition: JRH.

Investigation: SLF ACP MJM RAD JRH.

Methodology: SLF ACP MJM RAD JRH.

Project administration: JRH.

Resources: RAD JRH.

Supervision: RAD JRH.

Validation: SLF ACP MJM.

Visualization: SLF.

Writing – original draft: SLF JRH.

Writing – review & editing: SLF ACP MJM RAD JRH.

References

1. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000; 407:258–64. doi: 10.1038/35025229 PMID: 11001069
2. French SL, Arthur JF, Tran HA, Hamilton JR. Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood reviews*. 2015; 29:179–89. doi: 10.1016/j.blre.2014.10.006 PMID: 25467961
3. Holinstat M, Bray PF. Protease receptor antagonism to target blood platelet therapies. *Clinical pharmacology and therapeutics*. 2016; 99:72–81. doi: 10.1002/cpt.282 PMID: 26501993
4. Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991; 64:1057–68. PMID: 1672265
5. Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, et al. A dual thrombin receptor system for platelet activation. *Nature*. 1998; 394:690–4. doi: 10.1038/29325 PMID: 9716134
6. Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *New England Journal of Medicine*. 2012; 366:1404–13. doi: 10.1056/NEJMoa1200933 PMID: 22443427
7. Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van de Werf F, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *New England Journal of Medicine*. 2012; 366:20–33. doi: 10.1056/NEJMoa1109719 PMID: 22077816
8. Ismat FA, Ma X, Wang Z, Frost CE, Ni YG, Yang J. Abstract TMP91: Phase I Assessment of the Safety, Tolerability, Pharmacokinetics and Pharmacodynamics of the Oral Protease-activated Receptor-4 Antagonist BMS-986120. *Stroke*. 2016; 47:ATMP91–ATMP.
9. Derian CK, Damiano BP, Addo MF, Darrow AL, D'Andrea MR, Nedelman M, et al. Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. *Journal of Pharmacology and Experimental Therapeutics*. 2003; 304:855–61. doi: 10.1124/jpet.102.042663 PMID: 12538843
10. Connolly AJ, Ishihara H, Kahn ML, Farese RV, Coughlin SR. Role of the thrombin receptor in development and evidence for a second receptor. *Nature*. 1996; 381:516–9. doi: 10.1038/381516a0 PMID: 8632823
11. Connolly T, Condra C, Feng D, Cook J, Stranieri M, Reilly C, et al. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thrombosis and Haemostasis*. 1994; 72:627–33. PMID: 7878643

12. Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature*. 2000; 404:609–13. doi: 10.1038/35007085 PMID: 10766244

13. Khan TA, Bianchi C, Voisine P, Sandmeyer J, Feng J, Sellke FW. Aprotinin inhibits protease-dependent platelet aggregation and thrombosis. *The Annals of thoracic surgery*. 2005; 79:1545–50. doi: 10.1016/j.athoracsur.2004.11.006 PMID: 15854931

14. Andrade-Gordon P, Derian CK, Maryanoff BE, Zhang H-C, Addo MF, Cheung W-M, et al. Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. *Journal of Pharmacology and Experimental Therapeutics*. 2001; 298:34–42. PMID: 11408522

15. Hamilton J, Cornelissen I, Coughlin S. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. *Journal of Thrombosis and Haemostasis*. 2004; 2:1429–35. doi: 10.1111/j.1538-7836.2004.00783.x PMID: 15304051

16. Lee H, Sturgeon S, Mountford J, Jackson S, Hamilton J. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. *British Journal of Pharmacology*. 2012; 166:2188–97. doi: 10.1111/j.1476-5381.2012.01944.x PMID: 22428607

17. Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:288–92. doi: 10.1073/pnas.0610188104 PMID: 17190826

18. Arachiche A, de la Fuente M, Nieman MT. Platelet Specific Promoters Are Insufficient to Express Protease Activated Receptor 1 (PAR1) Transgene in Mouse Platelets. *PloS one*. 2014; 9:e97724. doi: 10.1371/journal.pone.0097724 PMID: 24830314

19. Kahn ML, Hammes SR, Botka C, Coughlin SR. Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *Journal of Biological Chemistry*. 1998; 273:23290–6. PMID: 9722561

20. Mountford JK, Petitjean C, Putra HW, McCafferty JA, Setiabakti NM, Lee H, et al. The class II PI 3-kinase, PI3KC2alpha, links platelet internal membrane structure to shear-dependent adhesive function. *Nature Communications*. 2015; 6:6535. doi: 10.1038/ncomms7535 PMID: 25779105

21. Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Berndt MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. *Journal of Thrombosis and Haemostasis*. 2007; 5:1530–7. doi: 10.1111/j.1538-7836.2007.02590.x PMID: 17445093

22. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood*. 2007; 109:1503–6. doi: 10.1182/blood-2006-04-020362 PMID: 17032923

23. Lee H, Hamilton JR. The PAR1 antagonist, SCH79797, alters platelet morphology and function independently of PARs. *Thrombosis and Haemostasis*. 2013; 109:164. doi: 10.1160/TH12-06-0389 PMID: 23093354

24. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. *Blood*. 2002; 100:3240–4. doi: 10.1182/blood-2002-05-1470 PMID: 12384423

25. Griffin CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR. A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science*. 2001; 293:1666–70. doi: 10.1126/science.1061259 PMID: 11533492

26. Pawlinski R, Tencati M, Hampton CR, Shishido T, Bullard TA, Casey LM, et al. Protease-activated receptor-1 contributes to cardiac remodeling and hypertrophy. *Circulation*. 2007; 116:2298–306. doi: 10.1161/CIRCULATIONAHA.107.692764 PMID: 17967980

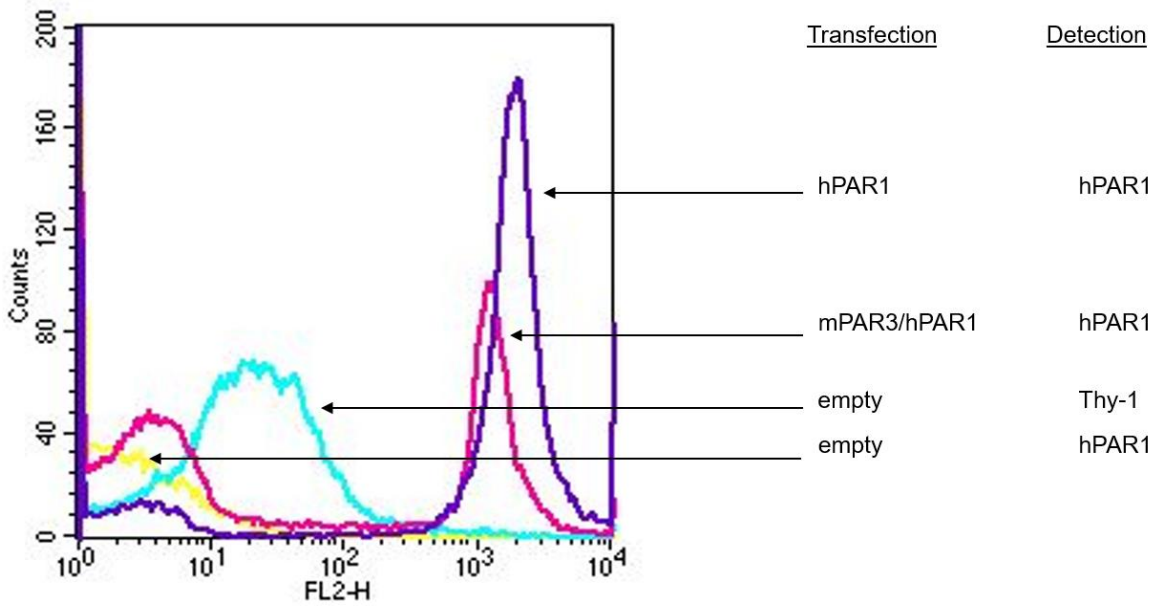
27. Yin YJ, Katz V, Salah Z, Maoz M, Cohen I, Uziely B, et al. Mammary gland tissue targeted overexpression of human protease-activated receptor 1 reveals a novel link to beta-catenin stabilization. *Cancer Research*. 2006; 66:5224–33. doi: 10.1158/0008-5472.CAN-05-4234 PMID: 16707447

28. Zampatis DE, Rutz C, Furkert J, Schmidt A, Wustenhagen D, Kubick S, et al. The protease-activated receptor 1 possesses a functional and cleavable signal peptide which is necessary for receptor expression. *FEBS letters*. 2012; 586:2351–9. doi: 10.1016/j.febslet.2012.05.042 PMID: 22659187

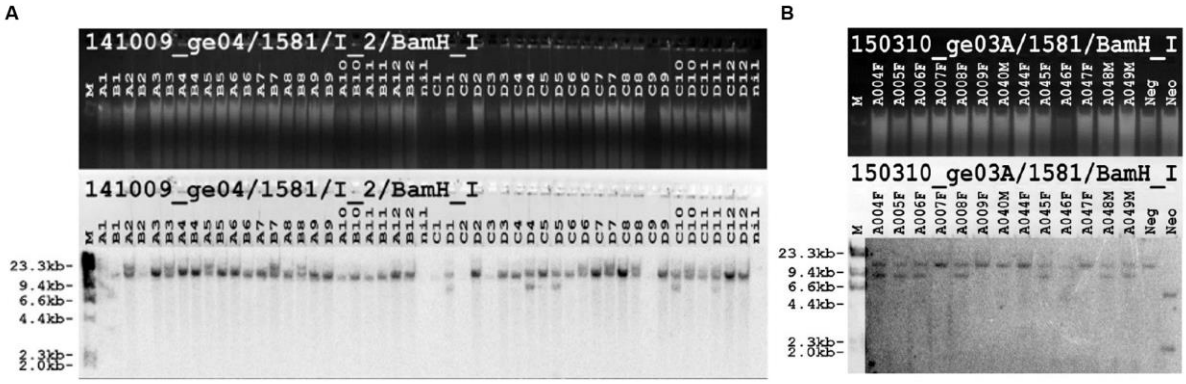
29. Soto AG, Trejo J. N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *The Journal of Biological Chemistry*. 2010; 285:18781–93. doi: 10.1074/jbc.M110.111088 PMID: 20368337

30. Tordai A, Brass LF, Gelfand EW. Tunicamycin inhibits the expression of functional thrombin receptors on human T-lymphoblastoid cells. *Biochemical and Biophysical Research Communications*. 1995; 206:857–62. doi: 10.1006/bbrc.1995.1122 PMID: 7832797

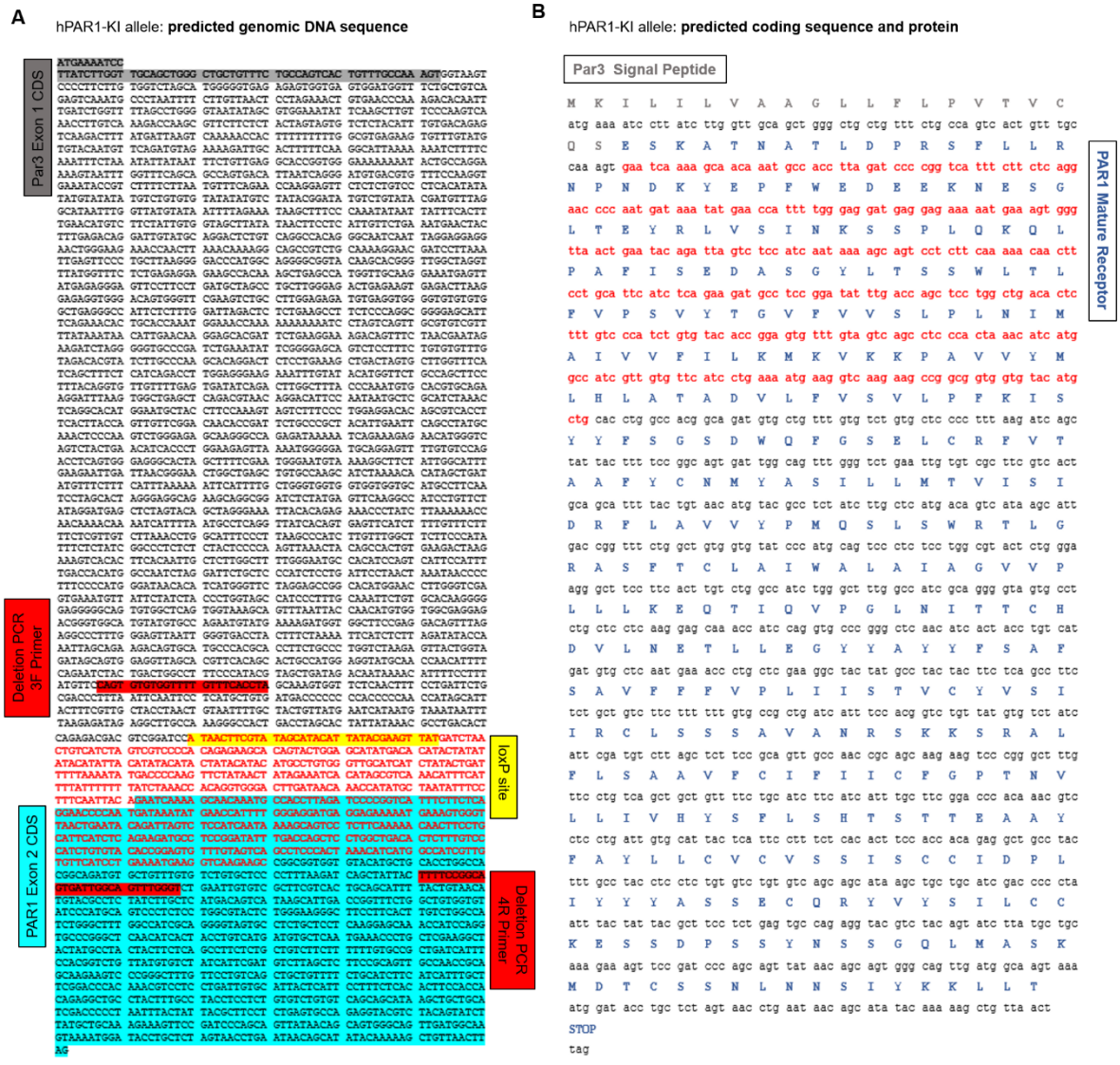
4.3 Supplementary Figures to French et al. "Humanizing the protease-activated receptor (PAR) expression profile in mouse platelets by knocking *PAR1* into the *Par3* locus reveals PAR1 expression is not tolerated in mouse platelets" (PLoS One, 2016)



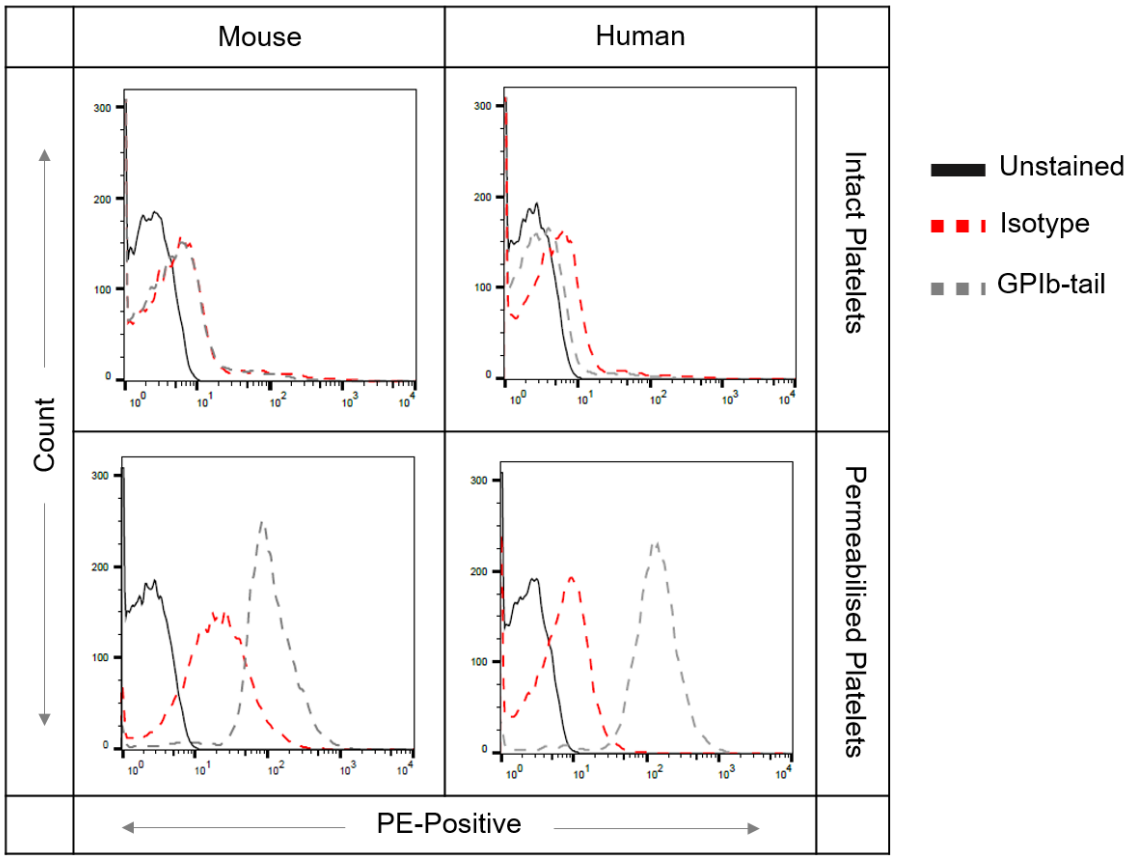
S1 Fig. The receptor generated in hPAR1-KI mice can be expressed in HEK293T cells. The fusion protein predicted to be expressed by hPAR1-KI mice, consisting of the mPAR3 signal peptide attached to the mature hPAR1 receptor, can be expressed on the surface of transfected HEK293T cells. HEK293T cells were transfected with native hPAR1 (purple), the mPAR3/hPAR1 fusion (pink), or empty vector (yellow). Note the similar level of detectable expression of native hPAR1 and the mPAR3/hPAR1 fusion. Also shown is a positive control for surface expression (Thy-1) in cells transfected with the empty vector.



S2 Fig. Southern blot analyses of targeted ES cells and founder mice. (A) ES cell clones were screened for targeting by Southern blot. Targeted clones are indicated by the expected additional band at 8.3kb (e.g. clones D1 and D4). **(B)** Founder mice screened by the same Southern blot approach as in (A), showing results for 13 mice from two litters. Seven of the 13 mice genotyped as heterozygous targeted, exhibiting both the wt (11.6 kb) and mutant band (8.3kb).



S3 Fig. Sequence annotation for the hPAR1-KI allele. (A) Predicted genomic DNA sequence for the hPAR1-KI allele, showing *Par3* exon 1 highlighted in grey, the position of primers 3F and 4R in red, the loxP site in yellow, and *PAR1* exon 2 in blue. Red text indicates the sequence output of the PCR product from primers 3F and 4R, which was identical to the predicted genomic DNA sequence. **(B)** Annotated sequence of the predicted coding sequence and protein chimera, comprising the Par3 signal peptide (grey text) and PAR1 mature receptor (blue text). Red text indicates sequenced PCR product amplified from genomic DNA of hPAR1-KI mice, aligning to the predicted region of PAR1 coding sequence.



S4 Fig. Confirmation of platelet permeabilization. Platelets isolated from a mouse (left) or human (right) were either left intact (top) or were permeabilized with saponin (0.1%; bottom) prior to incubation with an antibody against the intracellular C-terminal of GPIb (grey) or isotype control (red). The GPIb tail antibody produced a rightward shift over isotype only in permeabilized platelets of both species, confirming successful permeabilization. Data shown are representative traces of n=3 individual experiments.

S1 Table. Whole blood cell counts from wild type and hPAR1-KI mice.

	Wild type	hPAR1-KI
WBC (10^3/μL)	7.3 ± 3.2	7.1 ± 2.9
RBC (10^6/μL)	9.4 ± 1.2	9.6 ± 0.7
PLT (10^3/μL)	887 ± 50	888 ± 98

WBC = white blood cells, RBC = red blood cells, PLT = platelets.

Chapter 5 - Inhibition of protease-activated receptor 4 (PAR4) with function-blocking antibodies provides equivalent antithrombotic activity in the face of the hyper-reactive Thr120 PAR4 variant

5.1 Introduction

Despite the lack of an appropriate small animal model to test any novel PAR4 inhibitors *in vivo*, the studies of Chapter 2 coupled with other preclinical and clinical advances with PAR4 antagonists pointed to the overall feasibility of this approach. Specifically, Chapter 2 showed that selective inhibition of PAR4, but not of PAR1, impairs platelet procoagulant function, leading to marked reductions in thrombin generation and fibrin formation during human thrombus formation. This distinct antithrombotic mechanism of action suggests PAR4 inhibition is a viable alternative approach for novel therapy. Toward this goal, a series of small molecule PAR4 inhibitors have been developed, with one of these (BMS-986120) undergoing extensive preclinical testing in non-human primate models and another (BMS-986141) undergoing a phase 2 trial for prevention of transient ischemic attack (NCT02671461). When taken together, there is now substantial rationale for PAR4 antagonism as an antithrombotic approach.

However, as discussed in Chapter 1, a number of PAR4 polymorphisms have recently been described – at least one of which appears to be clinically relevant and may impact on the efficacy of PAR4 antagonists. The recently reported PAR4 variant (at rs773902, encoding a threonine or alanine at amino acid position 120; Thr^{120} or Ala^{120}), is expressed in 20 – 80% of individuals depending on the population. The Thr^{120} variant was shown to render the receptor more sensitive to agonists and less sensitive to antagonists – specifically, near-resistance to the small molecule orthosteric PAR4 antagonist, YD-3. These surprising findings suggest the effectiveness of current strategies to inhibit PAR4 may vary significantly between individuals

and indicate that a different approach to receptor inhibition may be required for indiscriminate PAR4 antagonism across the population.

To address this, the studies of Chapter 5 generated the first human monoclonal inhibitory antibodies against the PAR4 thrombin-cleavage site and examined their activity against the Ala^{120} and Thr^{120} PAR4 variants. This approach yielded a potent, specific, and highly effective inhibitory antibody that inhibits PAR4 and provides marked antithrombotic effects independently of PAR4 genotype. These findings reveal antibody-mediated inhibition of PAR4 cleavage and activation provides robust antithrombotic activity independently of PAR4 variant expression, and provide rationale for such an approach for improved antithrombotic therapy.

This chapter has been submitted for publication as:

French SL, Thalmann C, Sleeman MA, Hamilton JR. Inhibition of protease-activated receptor 4 (PAR4) with function-blocking antibodies provides equivalent antithrombotic activity in the face of the hyper-reactive Thr120 PAR4 variant.

Inhibition of protease-activated receptor 4 (PAR4) with function-blocking antibodies provides equivalent antithrombotic activity in the face of the hyper-reactive Thr120 PAR4 variant

Authors: Shauna L French[1], Claudia Thalmann[2,3], Mark W Sleeman[2,3], Justin R Hamilton[1]

Australian Centre for Blood Diseases[1], Department of Physiology[2], Biomedicine Discovery Institute[3], Monash University, Melbourne, Australia

Correspondence:

Dr Justin Hamilton

Australian Centre for Blood Diseases

Monash University

L2, AMREP Building, The Alfred

Commercial Rd

Melbourne, Victoria, 3004

AUSTRALIA

Tel - +61 3 9903 0125

Email – Justin.Hamilton@monash.edu

Keywords: protease-activated receptor, thrombin, platelets, thrombosis, antibody

Abstract

Thrombin activates human platelets via two protease-activated receptors (PARs), PAR1 and PAR4, and both are antithrombotic drug targets – a PAR1 inhibitor is approved for clinical use and a PAR4 inhibitor is in trial. However, a sequence variant in human PAR4 (rs773902, encoding Thr120 in place of Ala120), expressed in 20 – 80% of individuals depending on the population, renders the receptor more sensitive to agonists and less sensitive to antagonists. Here, we develop the first human monoclonal function-blocking antibody to human PAR4 (hPAR4) and show it provides equivalent efficacy against the Ala120 and Thr120 PAR4 variants. This candidate was generated from a panel of anti-hPAR4 antibodies and was screened for specific inhibitory activity against the Ala120 and Thr120 PAR4 variants in cultured cells, isolated platelets, and an *ex vivo* whole blood thrombosis assay. The antibody binds hPAR4 with high selectivity (16-fold over PAR1, PAR2 and PAR3) and affinity ($K_D \approx$ 0.4 nM) and is capable of near-complete inhibition of thrombin cleavage of either the Ala120 or Thr120 PAR4 variant transfected into HEK293T cells. Platelets from individuals expressing the Thr120 PAR4 variant exhibit increased thrombin-induced aggregation and phosphatidylserine exposure versus those with the Ala120 PAR4 variant, yet the hPAR4 antibody inhibited these responses equivalently ($IC_{50} \approx 5$ µg/ml). In support of these *in vitro* findings, the antibody significantly impairs platelet procoagulant activity in an *ex vivo* thrombosis assay, with equivalent inhibition of fibrin formation and overall thrombus size in blood from individuals expressing the Ala120 or Thr120 PAR4 variant. These findings reveal antibody-mediated inhibition of PAR4 cleavage and activation provides robust antithrombotic activity independently of PAR4 variant and provide rationale such an approach for antithrombotic therapy.

Introduction

Protease-activated receptors (PARs) are G protein-coupled receptors that are present on the surface of a range of cells and respond to a variety of proteases (for review, see (1)). Human platelets express two PARs, PAR1 and PAR4, and these receptors are primarily responsible for mediating the platelet-activating effects of the key coagulation protease, thrombin (2). Due to this central function in platelet biology, both platelet PARs have been the focus of antithrombotic drug development. The initial strategy was to inhibit PAR1 as it has an approximate 30-fold greater affinity for thrombin than PAR4. This approach led to the recent approval of the first PAR1 inhibitor, vorapaxar, for the prevention of myocardial infarction and peripheral arterial disease (3,4). However, the clinical utility of vorapaxar is limited by increased rates of major bleeding in patients on the drug (5). As a result, there is now emerging interest in targeting PAR4 as a safer antithrombotic approach.

There is substantial rationale for developing PAR4 inhibitors as antithrombotics (for review, see (6,7)). One key point of distinction between PAR1 and PAR4 is the different signaling kinetics of the two receptors and the impact this has on the regulation of platelet function. Specifically, PAR4 contains an anionic sequence downstream of the thrombin cleavage site which serves to prolong the thrombin-receptor interaction (8). One effect of the lower affinity but more prolonged interaction between thrombin and PAR4 versus PAR1 is that activation of PAR4 induces a more sustained, albeit weaker, intracellular signal than the robust and acute signal elicited downstream of PAR1 (9). This has been most obviously observed with the kinetics of PAR-induced calcium signaling. In the setting of platelet function, prolonged calcium signaling drives the procoagulant response. Indeed, selective inhibition of PAR4, but not of PAR1, specifically impairs platelet procoagulant function, leading to marked reductions in thrombin generation and fibrin formation during human thrombus formation (10). This distinct antithrombotic mechanism of action suggests PAR4 inhibition is a viable alternative

approach for novel therapy. Toward this goal, a series of small molecule PAR4 inhibitors have been developed, with one of these (BMS-986141) undergoing a phase 2 trial for prevention of transient ischemic attack (NCT02671461). Proof-of-concept studies were performed using the related compound, BMS-986120, which afforded impressive antithrombotic activity in cynomolgous monkeys with a safety profile that exceeded that of the $P2Y_{12}$ antagonist, clopidogrel (11). Together, these studies provide strong rationale for pursuing PAR4 antagonists as novel antithrombotics.

However, a number of PAR4 polymorphisms have recently been described – at least one of which appears to be clinically relevant and may impact on the efficacy of PAR4 antagonists. The recently reported sequence variant in human PAR4 (rs773902, encoding either a threonine or alanine at amino acid position 120; Thr120 or Ala120), is expressed in 20 – 80% of individuals depending on the population, and renders the receptor more sensitive to agonists and less sensitive to antagonists (12,13). Specifically, platelets from individuals expressing the Thr120 variant of PAR4 exhibit increased responsiveness to a PAR4-selective activating peptide and resistance to the small molecule orthosteric PAR4 antagonist, YD-3, when compared with individuals expressing the Ala120 variant (13). In both cases, individuals heterozygous for the SNP displayed an intermediate phenotype. These surprising findings suggest the effectiveness of current strategies to inhibit PAR4 may vary significantly between individuals and indicate that a different approach to receptor inhibition may be required for indiscriminate PAR4 antagonism across the population.

To address this, we utilized VelocImmune® HumAb mice to generate the first human monoclonal inhibitory antibodies against PAR4. We targeted the thrombin cleavage site of the receptor and examined the activity of antibodies against the Ala120 and Thr120 PAR4 variants in cultured cells, isolated platelets, and an *ex vivo* whole blood thrombosis assay. Although there have been a number of previous reports of function-blocking PAR4 antibodies (14-16),

including our own (10), effective inhibition of thrombin-induced receptor activation has been limited. However, our approach yielded a potent, specific, and highly effective inhibitory antibody that inhibits PAR4 and provides marked antithrombotic effects independently of hPAR4 genotype. These findings reveal antibody-mediated inhibition of hPAR4 cleavage and activation provides robust antithrombotic activity independently of hPAR4 variant expression, and provide rationale for such an approach for improved antithrombotic therapy.

Materials and Methods

Materials

The following reagents were stored according to manufacturer's instructions: Alexa Fluor 488-conjugated goat anti-mouse IgG (BD Biosciences, San Jose, CA, USA), mouse IgG_1 isotype (Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD41a (BD Biosciences), FITC-conjugated anti-FLAG antibody (clone M2, Sigma-Aldrich, St Louis, MO, USA), Dylight650-conjugated anti-fibrin antibody (clone 59D8 (17) generous gift from Dr Vivien Chen, University of New South Wales, Australia), anti-CD9-PE (BD Biosciences), Alexa Fluor 488-conjugated annexin-V (Sigma-Aldrich), hPAR4 immunising peptide (GGDDSTPSILPAPRGYPGQVC-KLH), hPAR4 naked peptide (GGDDSTPSILPAPRGYPG QVC), hPAR4 biotinylated peptide (GDDSTPSILPAPRGYPGQVC-GGGGSKB), hPAR3 biotinylated peptide (AKPTLPIKTFRGAPPNSF-GGGGSKB), hPAR2 biotinylated peptide (SCSGTIQGTNRSSKGRSL-GGGGSKB), hPAR1 biotinylated peptide (SKATNATLDPRS FLLRNP-GGGGSKB) (all from Auspep, Melbourne, Australia), PAR4-activating peptide (PAR4-AP; AYPGKF) and PAR1-activating peptide (PAR1-AP; TFLLR) were synthesized by Prof Philip Thompson (Monash University, Australia), vorapaxar (Axon Medchem, Reston VA, USA), human α-thrombin (Sigma-Aldrich), calcium ionophore A23187 (Sigma-Aldrich), bovine type 1 collagen (Sigma-Aldrich), and hirudin (lepirudin, Celgene, Summit, NJ, USA). For thrombin measurements in whole blood, a fluorescence resonance energy transfer (FRET)-based thrombin activity sensor (Thr-SP, CPC Scientific, Sunnyvale, CA, USA), was linked to the anti-CD41a antibody via CLICK chemistry as previously described (18).

Antibody production and purification

Immunizations were undertaken in VelocImmune® HumAb mice (generously provided by Regeneron Pharmaceuticals), which have been genetically modified by replacing the variable

regions of the mouse heavy and light chain Ig loci with the corresponding human sequence to produce antibodies that have human variable regions with the mouse constant regions (19,20). All mouse studies were approved by the Monash University Animal Ethics Committee. 14 week old HumAb mice were injected subcutaneously with a KLH-coupled peptide corresponding to a region spanning the thrombin cleavage site of PAR4 (GDDSTPSILPAPR/GYPGQVC-KLH, where / indicates the thrombin cleavage site; 25 µg in Freund's complete adjuvant) with 3 boosts given every 2 weeks for 6 weeks. After the final boost, primary splenocytes were isolated and fused with myeloma Sp2/0 cells, and plated onto 96 well plates to generate antibody-producing hybridomas. Supernatants from the resulting hybridomas were screened for high affinity specific hPAR4 antigen-positive lines using microarray (Arraviet Super Marathon, ArrayJet, UK) and standard ELISA. Cross reactivity to hPAR1, hPAR2 and hPAR3 was also measured. Specific hPAR4 clones were subcloned to monoclonality by limiting dilution. Specific hybridoma lines were expanded, adapted to serum free and suspension culture, and purified anti-hPAR4 antibody was isolated by standard affinity chromatography procedures using Protein A/G sepharose.

Surface plasmon resonance binding studies

The binding kinetics of purified antibody were determined via surface plasmon resonance (SPR; Bio-Rad ProteOn XPR36). Biotinylated hPAR1, hPAR2, hPAR3 or hPAR4 peptides (25 µg/mL) were captured on the ProteOn NLC biosensor chip consisting of NeutrAvidin surface bound to the alginate polymer. Varying antibody concentrations (6.25 – 100 nM) were flowed across the channels. The data were analyzed using Proteon Manager software with a Langmuir interaction model that assumes a 1:1 binding interaction between the peptide and antibody.

Human blood samples

All human studies were approved by the Monash University Human Research Ethics Committee. Blood was collected after informed consent from healthy adults (21 – 50 years old, of both sexes) who had not taken anti-platelet medications in the past 10 d. Blood was drawn from the antecubital vein using a 19 gauge butterfly needle into syringes containing either one-seventh acid citrate dextrose (ACD; 7:1 v/v, final concentration) for platelet isolation or one-tenth volume trisodium citrate (0.32% w/v, final concentration) for whole blood flow experiments and DNA extraction.

PAR4 genotyping

Genomic DNA was extracted from the buffy coat of blood samples collected in citrate, using the DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Samples were genotyped at rs773902 using the TaqMan SNP Genotyping Assay (Assay C-7493801-10, ThermoFisher Scientific).

Flow cytometry

Antibody binding to native PAR4 on the platelet surface was determined using flow cytometry. Platelets were isolated from human whole blood collected in ACD as previously described (10). Isolated platelets (1 x 10^6 cells) were then incubated with anti-PAR4 antibody (10 µg/mL), anti-human CD41a (10 µg/mL), or isotype control (mouse IgG_1, 10 µg/mL) for 30 min at 37ºC. Cells were then fixed with paraformaldehyde (2% final concentration) for 30 min at room temperature. The suspension was centrifuged at 1000 x *g* for 2 min to obtain the platelet pellet, which was then resuspended in modified Tyrode's buffer (12 mM $NaHCO_3$, 10 mM HEPES pH 7.4, 137 mM NaCl, 2.7 mM KCl, 5.5 mM D-glucose, 1.8 mM $CaCl_2$, 5% bovine serum albumin) containing 10 µg/mL Alexa488-conjugated anti-mouse IgG. After 30 min at 37ºC the samples were centrifuged again and the platelet pellet resuspended in modified

Tyrode's buffer and analyzed using a flow cytometer (FACSCalibur; BD Biosciences) and FlowJo software (v10).

PAR4 cleavage assay

FLAG-tagged Ala120-PAR4 or Thr120-PAR4 expression vectors (pBJ-FLAG-PAR4-120A-296F and pBJ-FLAG-PAR4-120T-296F, a generous gift from Prof. Paul Bray (University of Utah, Salt Lake City, Utah)) were transiently transfected into HEK293T cells using Lipofectamine 2000 (ThermoFisher Scientific), as per the manufacturer's instructions. Cells were harvested 48 h after transfection, washed twice with PBS, and resuspended to 1 x 10^6 cells/mL. Cells were then pre-treated with either anti-PAR4 antibody (1 – 100 μg/mL), or matched isotype control (mouse IgG_1; 100 μg/mL) for 15 min at 37ºC, before being stimulated with thrombin (2 U/mL) for 10 min. The reaction was stopped with hirudin (8 U/mL). Samples were then incubated with FITC-conjugated anti-FLAG antibody (10 μg/mL) for 1 h at room temperature and analyzed by flow cytometry (FACSCalibur) and FlowJo software (v10).

Platelet aggregation

Platelet aggregation was measured by light transmission aggregometry in a 96-well plate format as previously described (21). Human isolated platelets (2 x 10^8/mL) were stimulated with varying concentrations of thrombin (0.0178 – 1 U/mL), PAR4-AP (10 – 100 μM), or PAR1-AP (3 – 30 μM). The plate was analysed at 37ºC in a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) using a 595 nm excitation filter, for a period of 50 min (10 read cycles with 5 min double orbital shake period between each read). Aggregation was calculated as $\frac{OD(No\ Agonist)-OD(Agonist)}{OD(No\ Agonist)-OD(blank)} \times 100$ at the time point where aggregation was at a maximum. Optical density was normalised against the blank (maximum) and unstimulated platelets (minimum) and expressed as % maximum. For antagonist studies, platelets were pre-

treated for 15 min at 37ºC with anti-PAR4 antibody (1 – 100 μg/mL), vorapaxar (90 nM), a combination of both, or their vehicle controls (mouse IgG_1 and 1% DMSO, respectively).

Phosphatidylserine exposure

Phosphatidylserine (PS) exposure on the platelet surface was used as a measure of platelet procoagulant activity. Here, isolated platelets (1 x 10^6 cells) were stimulated with increasing concentrations of either PAR4-AP (0.1 – 3 mM), thrombin (0.1 – 3 U/mL), or calcium ionophore (20 μM) for 30 min at 37ºC. Alexa Fluor 488-conjugated annexin V (1:100 dilution) was added after agonist stimulation for PS detection. After the 30 min stimulation, samples were fixed with paraformaldehyde (2% final concentration) and resuspended in a modified Tyrode's buffer for flow cytometry analysis, as above (FACSCalibur).

Whole blood thrombosis assay

Human whole blood collected in citrate (3.2%) was pre-incubated for 15 min at 37ºC with PE-conjuated anti-CD9 antibody (4 μg/mL), Thr-SP (5 μM), and a Dylight650-conjugated anti-fibrin antibody (5 μg/mL), plus either the anti-PAR4 antibody (0.1 mg/mL) or hirudin (800 U/mL). Whole blood was re-calcified with 10 mM $CaCl_2$ (final concentration) to initiate coagulation, and drawn over glass microslides (1 x 0.1 mm internal diameter; Vitrotubes, Vitrocom, NJ, USA) coated with bovine type 1 collagen (250 μg/ml) using a Harvard pump (Instech Laboratories, PA, USA) at a fixed flow rate of 0.06 mL/min, resulting in a wall shear rate of 600 s^{-1}. Tri-colour confocal fluorescence images were recorded at 488, 561 and 647 nm excitation, collected through a 25x water immersion objective. Thrombi were formed over 3 min of blood flow after which calcium-free Tyrode's buffer was flowed over the thrombi. Confocal z-stacks encompassing the entire height of the thrombus field were continuously recorded (Z step 2 μm; Nikon A1r, with NIS software) over a period of 10 min. Offline analysis of thrombi parameters was performed using NIS software. Image series were initially

thresholded empirically, and then the same threshold applied to all subsequent experiments, since identical experimental and confocal settings were used throughout. Platelet thrombi were defined using anti-CD9-PE, and thrombin activity and fibrin volume were quantified using average fluorescence of the thrombus field. Data were normalised against the hirudin baseline, and expressed as a percentage of the control.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software (v6.0). Significance was defined at $p < 0.05$ as determined by either unpaired, two-tailed, Student's *t*-test or one-way ANOVA with Dunnet's post-hoc test for multiple comparisons, as indicated in the relevant figure legends.

Results

Development of a human monoclonal function-blocking antibody against PAR4

VelocImmune® HumAb mice were immunized with a KLH-linked peptide corresponding to a region of hPAR4 spanning the thrombin cleavage and activation site (GDDSTPSILPAPRGYPGQVC-KLH) (Figure 1A). Serum titres from routine bleeds were monitored and spleens from the highest producing mice were harvested. Splenocytes were then fused with Sp2/0 cells and resulting hybridomas (~ 5000) were screened for binding to the naked version of the immunizing peptide by microarray and confirmed secondarily by ELISA. ELISA-based antigen screening showed that clone mAb-5RC3 (mAb-RC3) bound naked hPAR4 peptide with a 16-fold selectivity over any of hPAR1, hPAR2, or hPAR3 peptides, and did not bind to mouse PAR4 (Figure 1B). SPR confirmed binding of clone mAb-RC3 to the target antigen and estimated it bound the immunizing peptide with a K_D of ~ 0.4 nM (Figure 1C). We also confirmed mAb-RC3 bound native PAR4 on human platelets. Here, mAb-RC3 (10 μg/mL) bound to a purified human platelet preparation, with a rightward shift in fluorescence intensity over that produced by a mouse IgG_1 isotype (Figure 1D).

mAb-RC3 inhibits thrombin cleavage of both Ala120 and Thr120 PAR4 variants

We next examined the function-blocking activity of the clone mAb-RC3 in a bioassay of thrombin-induced cleavage of PAR4. In HEK293Ts transfected with plasmids containing either Ala120-PAR4 or Thr120-PAR4 with an N-terminal FLAG-tag, thrombin caused a similar concentration-dependent loss of FLAG epitope, with a maximum cleavage of receptor of approximately 60% in both cases (Figure 2A). Under conditions of maximum PAR4 cleavage, mAb-RC3 inhibited receptor cleavage in a concentration-dependent manner (Figure 2B). This effect was not different between the Ala120 and Thr120 PAR4 variants (Figure 2B). Pre-treatment with the highest concentration of mAb-RC3 examined (100 μg/mL) almost

prevented thrombin cleavage of either PAR4 variant, with only ~ 5% residual loss of the FLAG epitope detected (Figure 2B).

mAb-RC3 inhibits the enhanced platelet aggregation elicited by the Thr120-PAR4 variant

To date, the heightened PAR4 response in platelets from individuals expressing the Thr120 PAR4 variant has only been demonstrated in response to the PAR4-specific agonist peptide, PAR4-AP, and only in a North American population (13). Therefore, we first confirmed this phenotype in our local (Australian) cohort. In line with previous observations (13), we observed a significant increase in platelet aggregation induced by PAR4-AP in individuals expressing the Thr120 variant versus those expressing the Ala120 variant (Figure 3A). Platelets from heterozygous individuals displayed a similar increase in responsiveness to PAR4-AP (Figure 3A). We next determined whether this hyper-reactivity in platelets from individuals with the Thr120 allele also occurred in response to the physiological PAR4 agonist, thrombin, since this has not been previously reported. Although thrombin–induced aggregation was increased in platelets from Thr120 individuals (homozygous or heterozygous) versus those from Ala120 individuals, this effect was less obvious than that observed in response to PAR4-AP (Figure 3B). To isolate the PAR4-dependant effects of thrombin in this assay, platelets were treated with the PAR1 antagonist, vorapaxar, at a concentration (90 nM) we have previously shown sufficiently blocks PAR1 activation under these conditions (21). Here, we observed a further dampening of the PAR4 genotype-dependent difference in platelet aggregation (Figure 3C). Regardless, mAb-RC3 caused a concentration-dependent inhibition of this thrombin-induced, PAR4-dependent, platelet aggregation. mAb-RC3 almost abolished aggregation at the highest concentrations examined (30 or 100 µg/mL), with indistinguishable effects across donors expressing the various PAR4 variants (Figure 3D) and with an estimated IC_{50} of 5.8 µg/mL (Figure 3E).

mAb-RC3 inhibits the enhanced platelet procoagulant response elicited by the Thr120-PAR4 variant

The generation of procoagulant platelets is important for thrombus growth and stability (22,23), and thrombin-induced procoagulant platelet production is mediated predominantly by PAR4 (10). Therefore, we next examined the sensitivity of thrombin-induced procoagulant platelet production (assessed by annexin V binding to surface-exposed PS) in donors expressing the Ala120 versus Thr120 PAR4 variant, and the ability of mAb-RC3 to inhibit this response. We observed a strikingly similar trend to the aggregation studies in Figure 3. First, PAR4-AP induced a greater increase in PS on platelets expressing the Thr120 PAR4 variant (heterozygous or homozygous) than on those expressing the Ala120 PAR4 variant (Figure 4A). Second, the same, less obvious, trend was observed in response to thrombin (Figure 4B). Third, mAb-RC3 concentration-dependently inhibited thrombin-induced PS exposure independently of PAR4 genotype, again nearly abolishing the response at the highest concentrations examined (100 µg/mL) in platelets expressing either of the PAR4 variants (Figure 4C).

mAb-RC3 inhibits human thrombus formation independently of PAR4 variant expression

Finally, the antithrombotic activity of mAb-RC3 was assessed in a human whole blood thrombosis assay in which coagulation remains intact (10). Specifically, we measured platelet deposition, thrombin generation, and fibrin deposition in real time in blood from individuals expressing the Ala120 or Thr120 PAR4 variant (Figure 5A). Perhaps surprisingly, there was no significant difference in the extent of any of platelet deposition, thrombin generation, or fibrin deposition between donors expressing the various PAR4 variants (Figure 5B,C). Importantly, and in support of the procoagulant platelet response experiments of Figure 4, pretreatment of blood with mAb-RC3 alone was sufficient to significantly reduce thrombin generation (~ 40%) and fibrin formation (~ 60%) in formed thrombi (Figure 5D,E). As in the

in vitro experiments, this reduction in thrombin and fibrin occurred independently of PAR4 genotype as it was evident in blood taken from individuals expressing the Ala120 or Thr120 PAR4 variant and in individuals who genotyped as heterozygous at the allele (Figure 5B,C). As predicted from the *in vitro* experiments in Figure 4, the effect of mAb-RC3 on fibrin formation (~ 60% reduction; Figure 6C) was more pronounced than that on platelet deposition (~ 30% reduction; Figure 6B). Together, the overall impact of mAb-RC3 pretreatment was a marked reduction in total thrombus volume (> 50%; Figure 6D). Again, the antithrombotic effect of mAb-RC3 was independent of PAR4 genotype, occurring in 12 of 14 individuals examined (Figure 6E). Indeed, mAb-RC3 only failed to provide an antithrombotic effect in the two individuals with the lowest baseline thrombotic response (Figure 6E). These studies indicate that PAR4 inhibition with the function-blocking monoclonal antibody developed here affords equally effective anti-platelet and antithrombotic effects in individuals regardless of their expression of the sequence variant of PAR4 at rs773902.

Discussion

We have generated the first human monoclonal function-blocking antibody against the platelet thrombin receptor, PAR4. We utilized VelocImmune® HumAb mice to generate human monoclonal antibodies directed against the thrombin cleavage site of PAR4 – a strategy similar to one we have used previously to generate a specific antagonist of thrombin-induced PAR4 activation (10). One clone (mAb-RC3) demonstrated specific and high-affinity binding to PAR4, and effective inhibition of thrombin-induced PAR4 cleavage and activation that markedly improves on previous antibody-based approaches (14-16). Importantly, mAb-RC3 inhibits the Ala120 PAR4 variant and the hyper-reactive Thr120 PAR4 variant equally well in isolated platelets and provides equivalent antithrombotic effects in blood from individuals regardless of their PAR4 genotype. These studies thereby provide a new agent for PAR4 inhibition that may have broad clinical utility.

There are now a number of PAR4 inhibitors (11,24,25), with one small molecule (BMS-986141) being evaluated in clinical trials (NCT02341638, NCT02671461). However, specificity and efficacy have proven especially challenging. Binding analyses performed here indicate mAb-RC3 binds PAR4 with high affinity (K_D ~ 0.5 nM) and with almost no cross reactivity to other PARs. Furthermore, mAb-RC3 appears significantly more effective than previous antibodies (10,14-16). In addition to the usual challenges of specificity and efficacy, the recent discovery of a clinically significant PAR4 variant (rs773902) has raised additional issues for PAR4 antagonism, with the Thr120-PAR4 variant exhibiting increased sensitivity to agonists and an insensitivity to at least one small molecule antagonist (13). The mAb-RC3 antibody developed here appears to completely overcome this, suggesting antibody-based inhibition of PAR4 may be required for effective and well-controlled antithrombotic activity across the population.

The Thr120-PAR4 variant has previously been associated with up-regulated platelet responses to a PAR4 activating peptide (13). Our findings extend on this to indicate the heightened response also occurs in response to PAR4's physiological agonist, thrombin, with platelets from individuals expressing the Thr120 PAR4 variant exhibiting increased thrombin-induced aggregation. The mechanism by which the amino acid change at position 120 (located in the second transmembrane domain) impacts PAR4 function remains unknown. Our data suggest it is not due to changes in receptor cleavage since the cleavage-site spanning mAb-RC3 was equally effective at inhibiting PAR4 cleavage and activation in Thr120 and Ala120 PAR4 variants. Whether or not interactions at the ligand binding domain within the second extracellular loop are involved remain untested, but are consistent with the findings from us and others (13) showing differences in responses to PAR4 activating peptides and small molecule antagonists that act at this site.

We have previously shown that PAR4 activation via thrombin drives procoagulant platelet activity (10). Here, we extended on these studies to provide the first evidence for a heightened production of procoagulant platelets in individuals expressing the Thr120-PAR4 variant. That this response was also inhibited by mAb-RC3 provides further rationale for its use as an antithrombotic approach. Although a similar trend of increased platelet procoagulant activity in response to PAR4-AP was observed with thrombin, consistent with our experiments examining platelet aggregation, the extent of any increase with thrombin was far less apparent than with PAR4-AP. This observation may explain the lack of correlation between PAR4 genotype and *ex vivo* thrombus formation observed here. We observed no differences in any of platelet deposition, thrombin generation, fibrin formation, or total thrombus volume between donors of different PAR4 genotypes. Whether the increased platelet sensitivity to thrombin is truly insufficient to cause effects in the more involved whole blood system will require more thorough examination – for example in a greater number of individuals and across a range of

thrombogenic conditions. However, regardless of the impact of the sequence variant of PAR4 at rs773902 on thrombus formation, mAb-RC3 alone was sufficient to provide a marked antithrombotic effect in almost all donors examined. As predicted by our in vitro studies, this effect was largely driven by a reduction in platelet procoagulant activity and subsequent fibrin formation, although some contribution by impaired platelet deposition was also apparent. The marked antithrombotic effect provided by mAb-RC3 in blood from donors of all PAR4 genotypes suggests directly targeting the thrombin cleavage site may be required for effective and consistent PAR4 inhibition across the population. Given the efficacy and relative safety of PAR4 inhibition in animal models (11), determining the antithrombotic effects of mAb-RC3 in human blood in combination with other anti-platelet agents that target distinct aspects of thrombus formation (e.g. aspirin, $P2Y_{12}$ receptor antagonists) will be of interest.

Conclusion

We have developed a human monoclonal PAR4 antibody that is a highly selective and effective inhibitor of PAR4 cleavage and activation. This function-blocking PAR4 antibody impairs thrombin-induced PAR4 cleavage, platelet activation, and pro-thrombotic activity equivalently in the face of expression of either the Ala120 or Thr120 PAR4 variant. These findings reveal a novel approach to PAR4 inhibition that overcomes the impaired sensitivity of existing approaches against the commonly expressed Thr120-PAR4 variant, and provide rationale for such an approach for improved antithrombotic therapy.

Acknowledgements

This work was supported by grants awarded to JRH from the NHMRC (#1047295) and the CASS Foundation (#SM16/7334) and funds to the Monash Antibody Technology Facility from Monash University (MWS). JRH is a Future Fellow of the Australian Research Council (#FT130100540). The authors wish to thank Regeneron Pharmaceuticals for provision of VelocImmune® HumAb mice, and Caroline Laverty and MATF staff for invaluable technical and project support.

References

1. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 2005;3:1800-14.

2. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000;407:258-64.

3. Tricoci P, Huang Z, Held C, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *N Engl J Med* 2012;366:20-33.

4. Morrow DA, Braunwald E, Bonaca MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med* 2012;366:1404-13.

5. French SL, Arthur JF, Tran HA, et al. Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Rev* 2015;29:179-89.

6. French SL, Hamilton JR. Protease-activated receptor 4: from structure to function and back again. *Br J Pharmacol* 2016;173:2952-65.

7. Hamilton JR, Trejo J. Challenges and Opportunities in Protease-Activated Receptor Drug Development. *Annu Rev Pharmacol Toxicol* 2017;57:349-73.

8. Jacques S, Kuliopulos A. Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem J* 2003;376:733-40.

9. Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* 2000;39:5458-67.

10. French SL, Arthur JF, Lee H, et al. Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood. *J Thromb Haemost* 2016;14:1642-54.

11. Wong PC, Seiffert D, Bird JE, et al. Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding. *Sci Transl Med* 2017;9: eaaf5294.

12. Edelstein LC, Simon LM, Montoya RT, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nat Med* 2013;19:1609-16.

13. Edelstein LC, Simon LM, Lindsay CR, et al. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood* 2014;124:3450-8.

14. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, et al. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Investig* 1999;103:879-87.

15. Mumaw MM, de la Fuente M, Noble DN, et al. Targeting the anionic region of human protease-activated receptor 4 inhibits platelet aggregation and thrombosis without interfering with hemostasis. *J Thromb Haemost* 2014;12:1331-41.

16. Mumaw MM, de la Fuente M, Arachiche A, et al. Development and characterization of monoclonal antibodies against Protease Activated Receptor 4 (PAR4). *Thromb Res* 2015;135:1165-71.

17. Ivanciu L, Toso R, Margaritis P, et al. A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia. *Nat Biotechnol* 2011;29:1028-33.

18. Welsh J, Colace T, Muthard R, et al. Platelet-targeting sensor reveals thrombin gradients within blood clots forming in microfluidic assays and in mouse. *J Thromb Haemost* 2012;10:2344-53.

19. Murphy AJ, Macdonald LE, Stevens S, et al. Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice. *Proc Natl Acad Sci U S A* 2014;111:5153-8.

20. Macdonald LE, Karow M, Stevens S, et al. Precise and in situ genetic humanization of 6 Mb of mouse immunoglobulin genes. *Proc Natl Acad Sci U S A* 2014;111:5147-52.

21. French SL, Paramitha AC, Moon MJ, et al. Humanizing the protease-activated receptor (PAR) expression profile in mouse platelets by knocking PAR1 into the Par3 locus reveals PAR1 expression is not tolerated in mouse platelets. *PLoS One* 2016;11:e0165565.

22. Munnix IC, Kuijpers MJ, Auger J, et al. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007;27:2484-90.

23. Hua VM, Abeynaike L, Glaros E, et al. Necrotic platelets provide a procoagulant surface during thrombosis. *Blood* 2015;126:2852-62.

24. Wu CC, Hwang TL, Liao CH, et al. Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. *Thromb Haemost* 2002;87:1026-33.

25. Young SE, Duvernay MT, Schulte ML, et al. Synthesis of indole derived protease-activated receptor 4 antagonists and characterization in human platelets. *PLoS One* 2013;8:e65528.

Figures

A

Protein	Sequence
hPAR4 native protein	GDDSTP SILPAP**RG**YP GQVC
hPAR4 immungoen	GDDSTP SILPAP**RG**YP GQVC-KLH

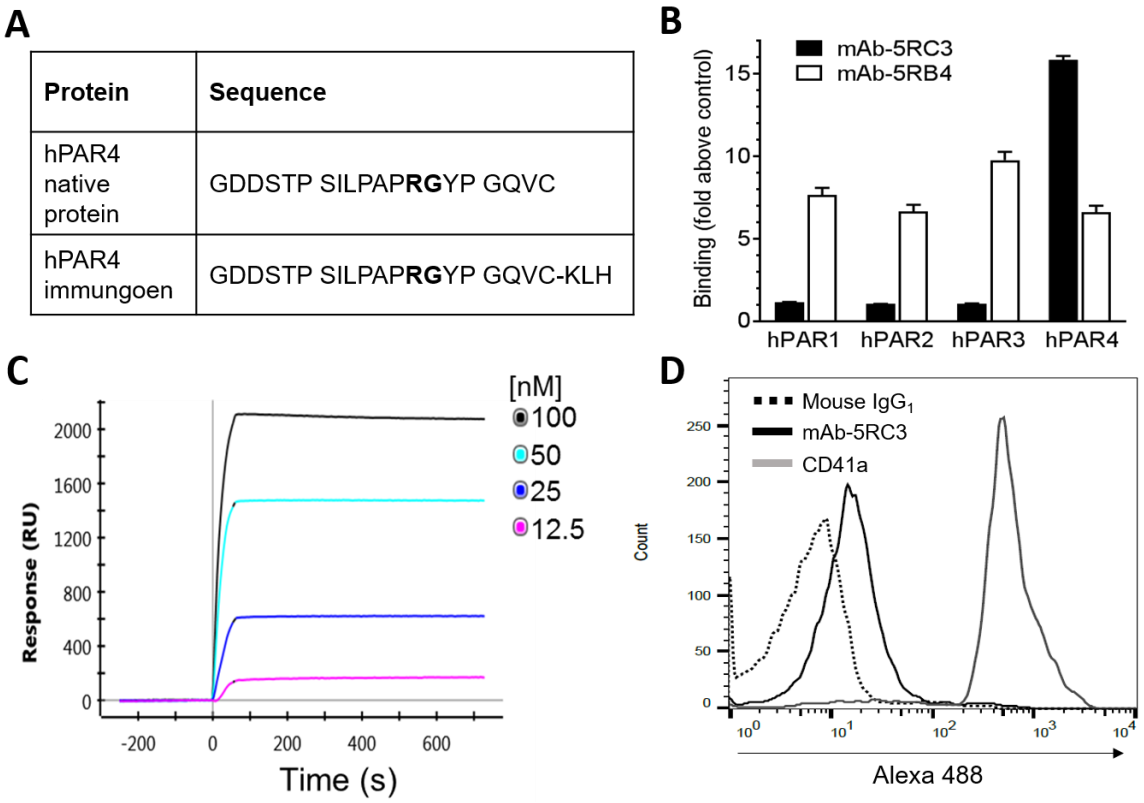


Figure 1. Development of a human monoclonal function-blocking antibody against human PAR4 (mAb-RC3). (A) HumAb mice were immunized with a KLH-coupled peptide corresponding to a region in the N-terminus of hPAR4 spanning the thrombin cleavage site (GDDSTPSILPAP**RG**YPGQVC-KLH, bold font indicated thrombin cleavage site). **(B)** Initial ELISA-based antigen screening showed mAb-5RC3 (mAb-RC3) bound native human PAR4 peptide (hPAR4) with a 16-fold selectivity over human PAR1, 2, or 3 peptides, while mAb-5RB4 bound all four peptides similarly. **(C)** Binding was confirmed by SPR analysis which showed that mAb-RC3 bound native PAR4 peptide with a K_D ~ 0.4 nM. **(D)** mAb-RC3 (10µg/mL) binding to human PAR4 on the platelet surface was confirmed by flow cytometry. Note a rightward shift in fluorescence intensity compared to the matched Isotype control (mouse IgG_1, dashed line). The platelet-specific anti-CD41a antibody (grey line) was used as a positive control. **(C-D)** Data are representative images of n=3 independent experiments.

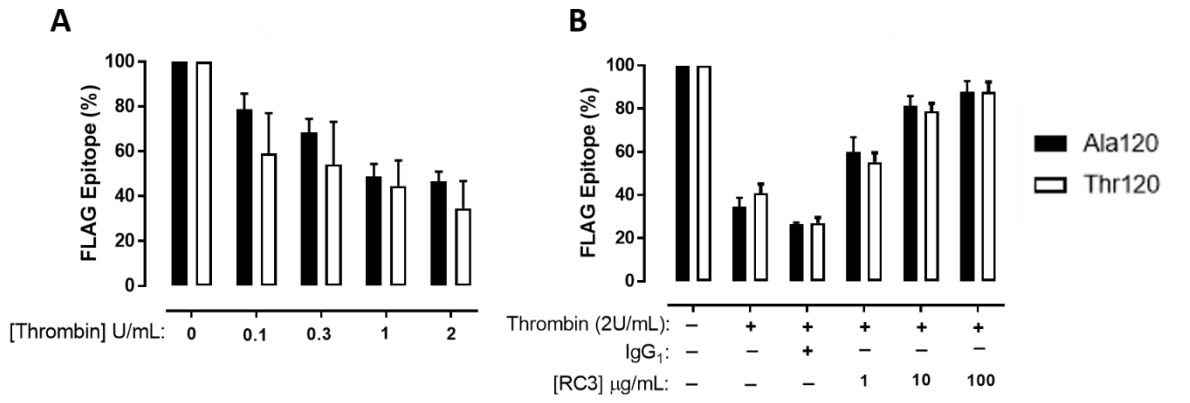


Figure 2. mAb-RC3 inhibits thrombin cleavage of both Ala120 and Thr120 PAR4 variants. Thrombin cleavage of PAR4 was assessed in HEK293T cells transiently transfected with either Ala120-PAR4 or Thr120-PAR4 variants with an N-terminal FLAG tag. **(A)** Cells were stimulated with increasing concentrations of thrombin (0.1 – 2 U/mL) and receptor cleavage measured as a loss of FLAG-epitope using a FITC-conjugated anti-FLAG antibody by flow cytometry. **(B)** Pre-incubation of transfected cells with mAb-RC3 (1-100 μg/mL) before thrombin stimulation (2 U/mL) provided equivalent and near-complete inhibition of thrombin cleavage of either PAR4 variant. Data is mean ± SEM of n=3 experiments.

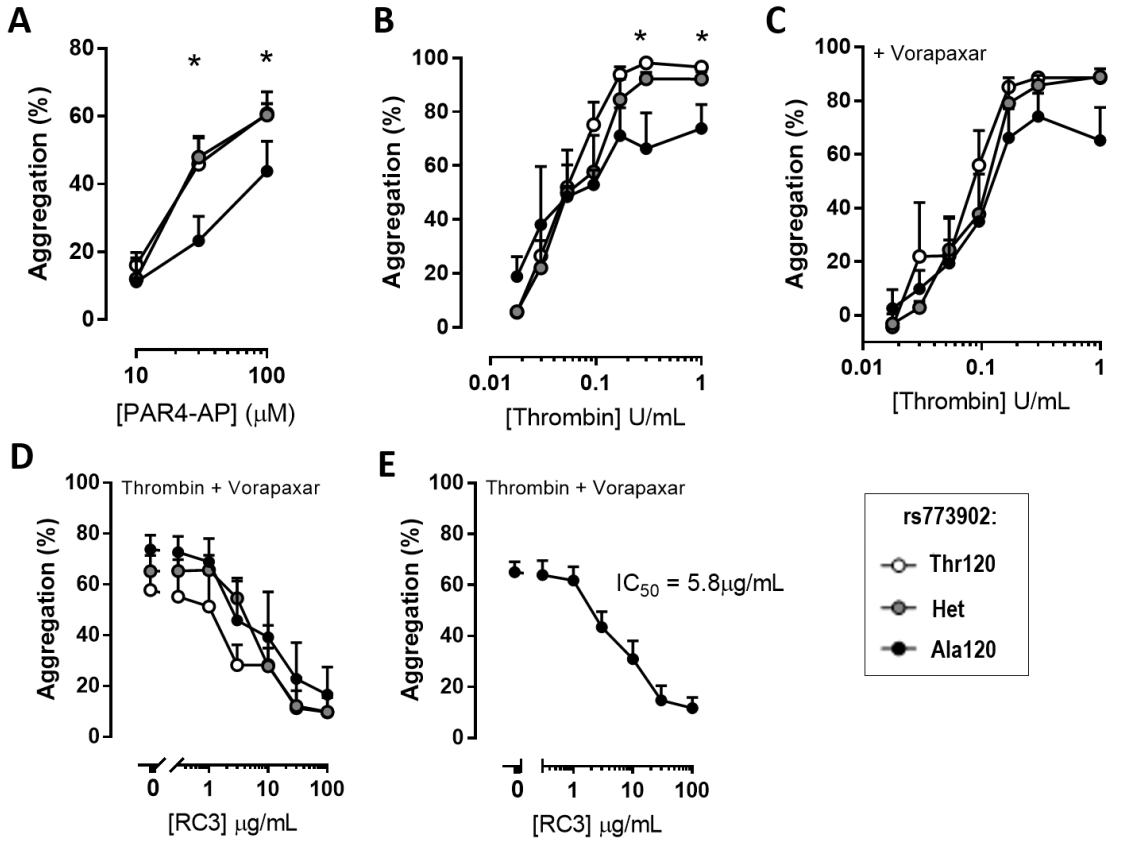


Figure 3. mAb-RC3 inhibits the enhanced platelet aggregation elicited by the Thr120-PAR4 variant. Aggregation of human isolated platelets from donors subsequently genotyped as homozygous at rs773902 for Thr120, Ala120, or as heterozygous. Shown are concentration-response curves to **(A)** PAR4-AP, **(B)** thrombin, and **(C)** thrombin in the presence of the PAR1 inhibitor vorapaxar (90 nM). Also shown are **(D)** concentration-inhibition curves to mAb-RC3 on responses to thrombin (0.1 U/mL) in the presence of vorapaxar (90 nM), indicating antibody-mediated PAR4 inhibition is equally effective across all genotypes, and **(E)** IC_{50} of mAb-RC3 derived from pooled data from **(D)**. All data are mean ± SEM from n=3-6 experiments per genotype. *, $P < 0.05$ (one-way ANOVA with Dunnet's test for multiple comparisons).

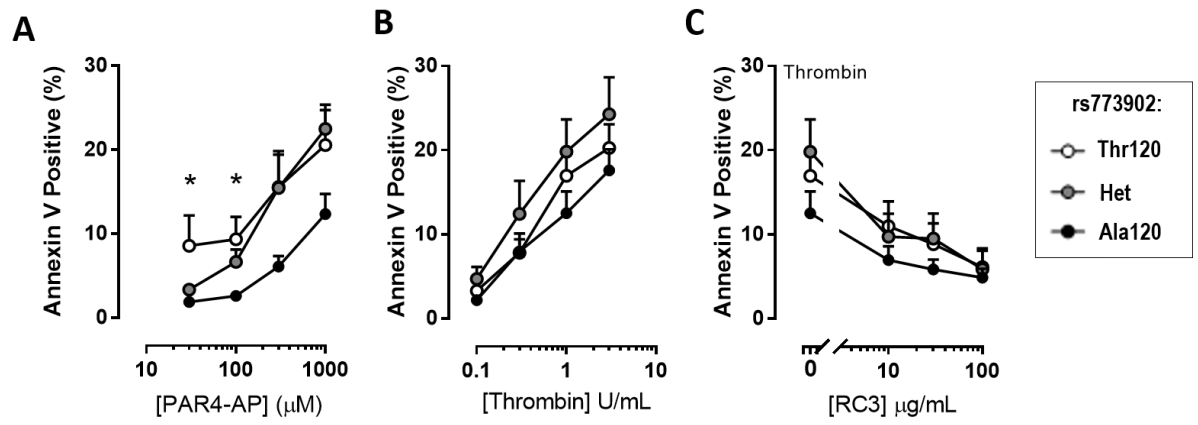


Figure 4. mAb-RC3 inhibits the enhanced platelet procoagulant response elicited by the Thr120-PAR4 variant. Production of procoagulant (annexin V-binding) platelets in human isolated platelet preparations from donors subsequently genotyped as homozygous at rs773902 for Thr120, Ala120, or as heterozygous. Shown are concentration-response curves to **(A)** PAR4-AP and **(B)** thrombin. Also shown **(C)** are concentration-inhibition curves to mAb-RC3 on responses to thrombin (1 U/mL), indicating antibody-mediated PAR4 inhibition is equally effective across all genotypes. All data are mean ± SEM from n=3-6 experiments per genotype. *, $P < 0.05$ (one-way ANOVA with Dunnet's test for multiple comparisons).

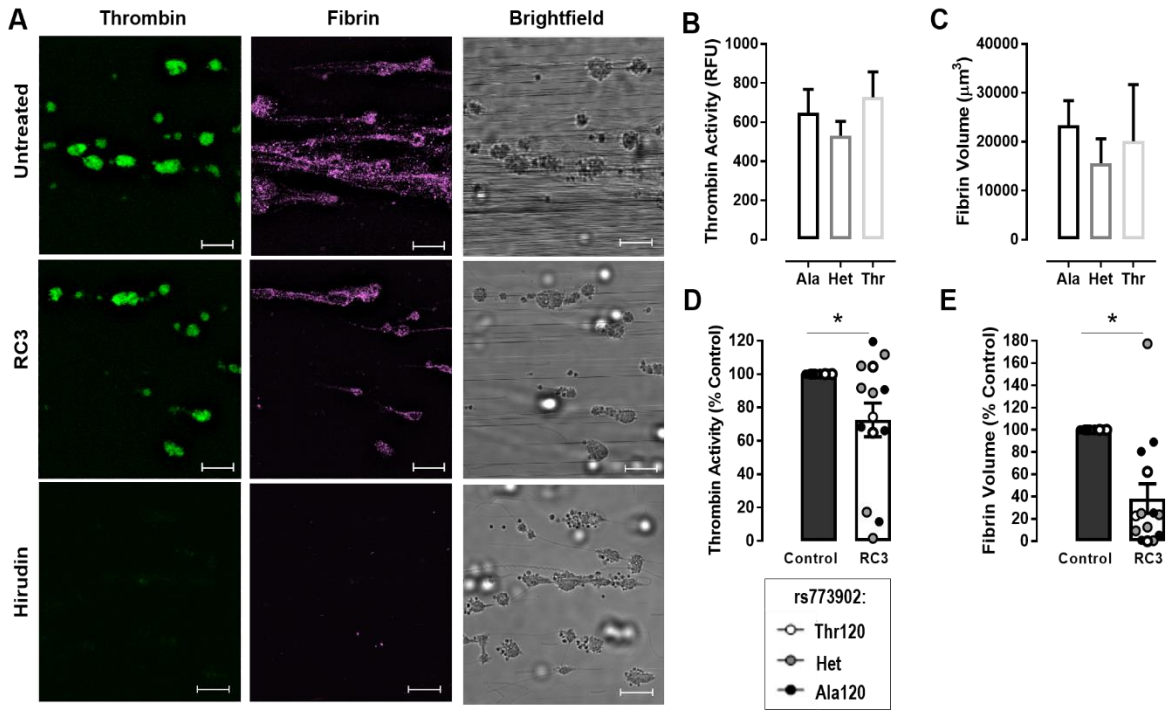


Figure 5. mAb-RC3 inhibits platelet procoagulant activity during *ex vivo* human whole blood flow. (A) Representative images and **(B-E)** quantitation of human thrombi formed after 3 minutes of a whole blood thrombosis assay using donors subsequently genotyped as homozygous at rs773902 as Thr120, Ala120, or as heterozygous. **(A)** Shown are thrombin (green, Thr-SP), fibrin (purple, anti-fibrin antibody) and brightfield image of thrombi. Note that the direct thrombin inhibitor, hirudin (800 U/mL), abolished thrombin activity and fibrin volume despite continued platelet deposition. Scale bar = 20μm. No differences in **(B)** thrombin or **(C)** fibrin volume were observed between thrombi formed in individuals expressing the indicated PAR4 genotypes. Pre-treatment with mAb-RC3 (RC3, 100 μg/mL) inhibited both **(D)** thrombin activity and **(E)** fibrin volume compared to untreated control. Data is mean ± SEM of N = 3-6 per genotype. *, $P < 0.05$ (unpaired Student's t test).

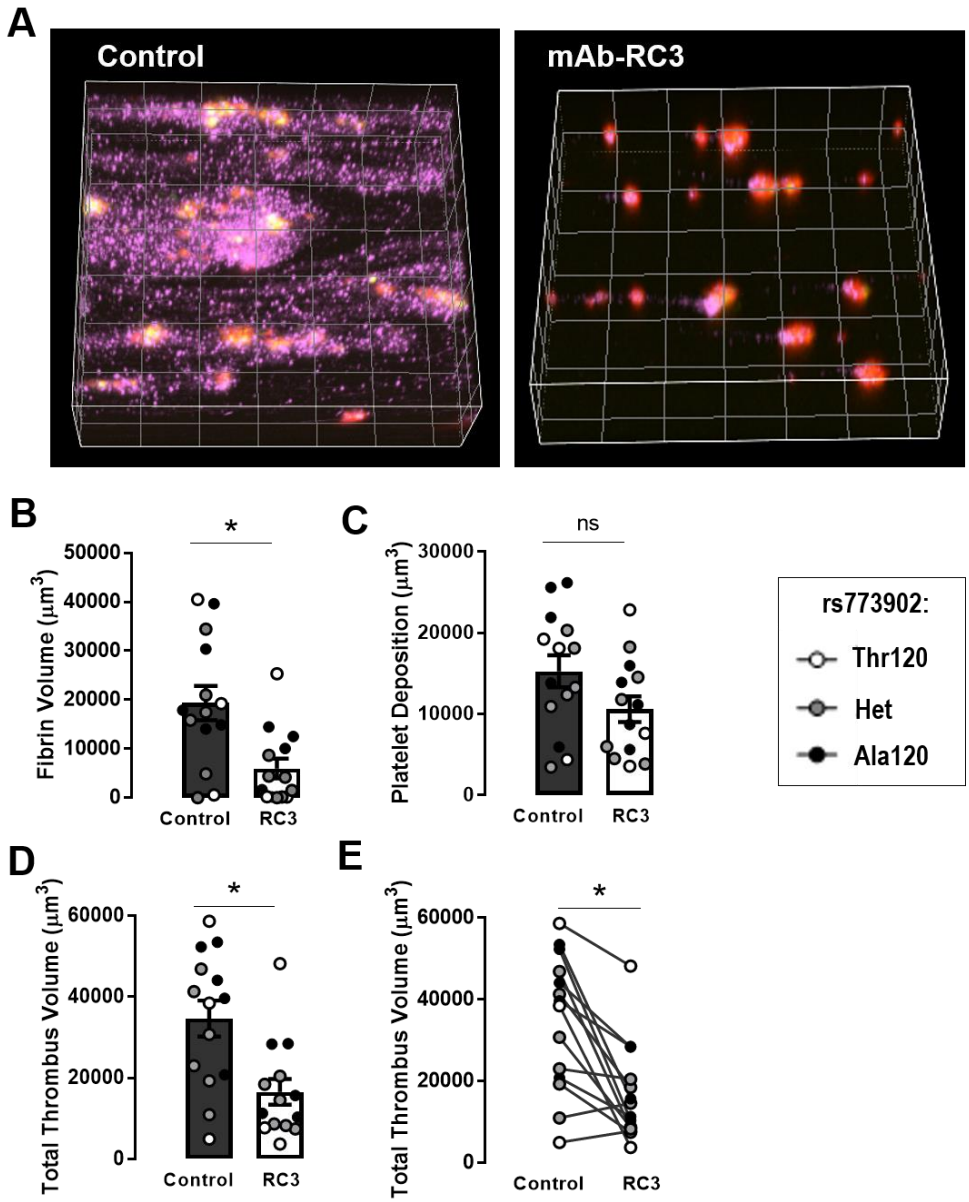


Figure 6. mAb-RC3 inhibits human thrombus formation independently of PAR4 variant expression. (A) Representative 3D reconstructions (each cubic edge = 20 µm) and **(B-E)** quantitation of human thrombi formed after 3 minutes of a whole blood thrombosis assay, showing platelets (red, anti-CD9), thrombin (green, Thr-SP), and fibrin (purple, anti-fibrin antibody). Note pre-treatment of blood with mAb-RC3 (RC3, 100µg/mL) significantly inhibited **(B)** fibrin volume but not **(C)** platelet deposition. This translated to **(D)** a significant reduction in total thrombus volume. Data is mean ± SEM of N = 14 (3-6 per genotype). A pairwise analysis is shown in **(E)**.*, $P < 0.05$ (unpaired Student's t-test). Ns = not significant.

Chapter 6 - General Discussion

6.1 Key findings of this thesis

The studies described in this thesis examined whether targeting the platelet thrombin receptor, PAR4, is a valid antithrombotic approach. Thrombin-induced platelet activation has been a long coveted antiplatelet strategy. However, the increases in bleeding associated with the clinical PAR1 antagonist, vorapaxar, suggested that safe and effective antithrombotic therapy would require a different approach to manipulating platelet-thrombin signalling. PAR4 is a lower affinity thrombin receptor, and has therefore drawn significant recent attention as a potential antithrombotic drug target that may have less impact on haemostasis. At the beginning of these studies, the function of PAR4 during platelet activation and thrombus formation in humans remained relatively unknown, in large part due to limitations in key reagents. In developing and utilising novel reagents to examine PAR4 function, the studies presented here have brought to light new roles of this receptor and the implications of its inhibition. The major findings of this thesis are:

1) PAR4 predominantly mediates thrombin-dependent platelet procoagulant activity – and subsequent thrombin and fibrin formation – largely independently of PAR1 (Chapter 2). This distinct antithrombotic mechanism suggests that PAR4 inhibition is a viable therapeutic strategy.
2) To assess PAR4 inhibition *in vivo*, two genetic mouse models were characterised: i) PAR4-/- mice, which have platelets that don't respond to thrombin (Chapter 3); and ii) a mouse genetically modified to mimic the human platelet-PAR expression profile (hPAR1-KI mice) (Chapter 4). Due to significant limitations that were revealed with both of these models, these studies suggest that alternative animal models will be required for meaningful preclinical evaluation of PAR4 antagonists.

3) A novel human monoclonal anti-PAR4 antibody was developed and characterised. This antibody provided marked antithrombotic effects, including equivalent effects against a common sequence variant in PAR4 that has been shown to be resistant to another PAR4 antagonist (Chapter 5). These studies indicate that blocking the thrombin-cleavage site of the receptor may be required for equivalent PAR4 inhibition across the population.

6.2 What is the role of PAR4 during thrombosis?

This thesis began by endeavouring to determine the specific functions of PAR4 on human platelets during thrombosis, in order to rationalise whether PAR4 inhibition was a valid antiplatelet strategy. At the time of these studies, suitably specific and effective inhibitors of PAR4 were not freely available. Therefore a function-blocking anti-PAR4 antibody was developed and used to probe for platelet activation events reliant on PAR4. The studies of Chapter 2 demonstrate that PAR4 alone, and not PAR1, appears to be more important in the regulation of platelet procoagulant activity – namely, thrombin-induced phosphatidylserine (PS) exposure, consequent platelet-mediated thrombin generation, and fibrin formation.

This role for PAR4 during thrombosis is consistent with what is known regarding receptor structure, activation kinetics, and downstream signalling profiles. It is well known that PAR4 activation generates a more prolonged intracellular Ca^{2+} signal than PAR1 activation[69], due to a longer interaction with thrombin[35] and slower desensitisation of the receptor[80]. It is also well known that a high and sustained rise in intracellular Ca^{2+} is required for PS externalisation, and that this surface binds tenase and prothrombinase complexes, leading to the local generation of thrombin and fibrin[102]. The studies in Chapter 2 build on this work by suggesting that PAR4 is predominantly responsible for mediating procoagulant effects of activated platelets, and demonstrating that PAR4 inhibition also directly impacts local thrombin and fibrin generation

under physiological flow conditions. Studies in Chapter 5 illustrate that PAR4 inhibition markedly reduces total thrombus volume, and that this reduction is predominantly due to impaired fibrin formation, rather than platelet deposition on collagen. Given that fibrin formation is an essential component of the stable clot, it follows that PAR4 inhibition could potentially impact overall thrombus stability, however further studies will be required to fully elucidate this. Regardless, these findings demonstrate that PAR4 acts at the interface of platelet aggregation and coagulation, and provides an antithrombotic effect in human blood via a distinct mechanism to other antiplatelet drug targets.

Whether PAR1 or PAR4 drives procoagulant responses has been controversial, with evidence of both receptors playing a role[75, 103-105]. However, this potentially relates to the lack of appropriate inhibitors available at the time of these earlier studies. The studies described in Chapter 2 and Chapter 5 of this thesis developed and characterised two effective and highly specific PAR4 inhibitors. Both function-blocking anti-PAR4 antibodies consistently inhibited thrombin-induced PS exposure, as well as whole blood thrombin and fibrin generation. Indeed, preclinical studies using the newest PAR4 antagonist, BMS-986120, support this notion[98]. BMS-986120 also demonstrated markedly more specificity for PAR4 over PAR1, as well as efficacy against thrombin-induced platelet activation[98]. When used *in vitro* it demonstrated Ca^{2+} signalling profiles similar to the ones generated here using an anti-PAR4 antibody[98]. The reagents generated throughout the course of this thesis are therefore of considerable research interest, as they may be used to uncover additional functions of PAR4 in a variety of conditions, including functions in cell types other than platelets.

6.3 What is the likely clinical utility of PAR4 antagonism?

PAR4 inhibition provides an antithrombotic effect via a mechanism distinct from all existing antiplatelet strategies. There are currently no antiplatelet drugs that directly target platelet

procoagulant activity, although the notion of specifically targeting this sub-population of activated platelets is gaining popularity[106]. The rationale for this is that targeting the procoagulant population preserves haemostatic functions of other platelets within the thrombus – namely those displaying the aggregating and contractile phenotype. Studies in Chapters 2 and 5 of this thesis show that specifically inhibiting PAR4 has no major impact on platelet deposition during human whole blood thrombosis, but does reduce fibrin deposition, which translates to a reduction in total thrombus volume. It is well documented that antiplatelet agents targeting platelet adhesion/aggregation pathways, and anticoagulant agents (global inhibition of thrombin) used in combination with the less efficacious antiplatelet drugs (aspirin/clopidogrel), significantly increase clinical bleeding events[107]. Therefore, as PAR4 antagonism acts at the interface of coagulation and platelet activation and leaves primary haemostatic functions intact (i.e. platelet adhesion and aggregation), it may lead to less clinical bleeding that is typically associated with other antiplatelet and anticoagulant drugs. Preclinical studies using the PAR4 antagonist, BMS-986120, strongly support this hypothesis. In non-human primate *in vivo* thrombosis models, PAR4 antagonism prevented thrombotic occlusion at doses that did not impact bleeding times – a therapeutic window not observed with clopidogrel[98]. However, it is of note that thrombin-induced platelet activation is less critical under conditions of high vascular shear[46], such as those known to occur in stenosed arteries. Therefore, it is unknown whether PAR4 antagonism alone will be sufficiently effective in reducing thrombotic events formed under these conditions, or whether additional antiplatelet interventions will be required. In this regard, studies examining the combined effects of PAR4 antagonism with other agents appear pressing.

Although these preclinical studies show a promising profile with an increased therapeutic window, the overall effect of PAR4 inhibition on haemostasis will require further elucidation. The studies in Chapter 3 of this thesis were the first to reveal a spontaneous bleeding phenotype

in mice genetically deficient in PAR4 – a phenotype which has been overlooked for nearly two decades. Although PAR4-/- mice represent a model of complete inhibition of thrombin-platelet signalling, whereas human platelets exposed to a PAR4 antagonist would presumably retain PAR1 signalling, it is not known if the bleeding phenotype is due to the complete absence of thrombin-platelet signalling, or the absence of the physiological function of PAR4 during thrombosis (i.e. procoagulant platelet generation). Chapter 4 of this thesis aimed to generate a mouse model with equivalent platelet-PAR expression to humans, in order to answer this question. However, the failure to achieve PAR1 expression in mouse platelets, in the context of previous failed attempts[108] and other notable differences between murine and human platelet biology, ultimately indicate that other animal models will be more appropriate to conduct meaningful studies. There is, however, some human genetic evidence to guide the appropriateness of PAR4 inhibition as an antithrombotic approach. Patients with Scott syndrome – a rare congenital disorder in which defective platelet PS exposure drastically diminishes their thrombin generation capacity – have a clinically significant bleeding phenotype[109]. Therefore, further studies will be required to determine whether specifically targeting platelet procoagulant activity *via* PAR4 antagonism is a viable option for safe and effective antithrombotic treatment.

The issue of population genetics is another clinical consideration for PAR4 antagonism. Recent studies have revealed a commonly expressed genetic variant of PAR4 (rs773902; encoding either Ala^{120} or Thr^{120}). The Thr^{120} PAR4 variant, expressed in 20 – 80% of people depending on the population, renders the receptor hyper-sensitive to agonists and hypo-sensitive to antagonists[100, 101]. Expression of the Thr^{120} variant is racially dimorphic, and present in high proportions in American blacks, sub-Saharan Africa, as well as in Melanesian and Papuan populations[101]. In the American cohort, the demographic in which the Thr^{120} PAR4 variant is dominant are known to have higher rates of cardiovascular disease, poorer outcomes,

and therefore more likely to be on antiplatelet drugs. On this point, the hyper-reactivity to PAR4 agonists observed in individuals with the Thr^{120} PAR variant persists in the face of current antiplatelet drugs, including vorapaxar[101]. Although the studies in Chapter 5 suggest that the Thr^{120} PAR4 variant alone is not sufficient to induce a heightened thrombotic phenotype in at least one *ex vivo* model, further studies with a greater number of donors and different thrombogenic conditions will be required to determine the extent to which PAR4 genotype has a direct impact on whole blood thrombosis. Regardless, the discovery of this commonly expressed PAR4 variant has led to PAR4 antagonism becoming highly desirable strategy for these at-risk populations.

6.4 What does the future hold for PAR4 antagonists?

The future of PAR4 antagonists is promising – there is a strong mechanistic rationale, preclinical studies have shown a desirable profile and potential to increase the therapeutic window, and the bio-pharmaceutical sector has an interest in continuing to develop improved compounds. The recently developed PAR4 antagonists – BMS-986120 and BMS-986141 – have undergone clinical trials. BMS-986120 was evaluated in a phase 1 dosing study, yet despite efficacy and a lack of adverse events no phase 2 studies of this compound were undertaken. Rather, BMS are investigating the related compound, BMS-986141, which underwent a phase 1 study (NCT02341638) and a subsequent phase 2 trial for the prevention of mini-stroke (NCT02671461). The trial (A Phase 2, Placebo Controlled, Randomized, Double-Blind, Parallel-Arm Study to Evaluate Efficacy and Safety of BMS- 986141 For the Prevention of Recurrent Brain Infarction in Subjects Receiving Acetylsalicylic Acid Following Acute Ischemic Stroke or Transient Ischemic Attack) had a primary efficacy endpoint of a composite of symptomatic ischemic stroke or unrecognized brain infarction, and a primary safety endpoint of a composite of adjudicated major bleeding and adjudicated clinically

relevant non-major bleeding during the treatment period. It was completed in April 2017 but has not yet been reported.

It is far too early to predict the likely clinical success and/or usefulness of PAR4 antagonists, and several key questions remain. Firstly, how well will PAR4 antagonism combine with current standard-of-care agents? This is a central point, since any trial will be conducted in the presence of standard-of-care, which frequently involves dual antiplatelet therapy. With the PAR1 antagonist vorapaxar, for example, the increased bleeding observed is believed to be due to poor compatibility with clopidogrel. Indeed, sub-study analyses show no additional bleeding in patients receiving aspirin plus vorapaxar versus those receiving aspirin alone[48, 50]. Here, it is interesting to note that BMS chose to investigate a patient group being treated with aspirin alone in its first phase 2 trial of its lead PAR4 antagonist. Also unknown are the specific indications most likely to be best served by a PAR4 antagonist. Again, sub-study analyses of the vorapaxar trials may provide pointers. These trials showed the most efficacy in reducing the rate of spontaneous myocardial infarction as well as in prevention of vascular complications associated with peripheral artery disease. This is perhaps unsurprising given the well-known role of thrombin generation in acute myocardial infarction, particularly in patients with a background of unstable angina and/or coronary artery disease[53]. Whether PAR4 antagonism will similarly demonstrate superior efficacy in these clinical situations where thrombin-induced platelet activation are implicated is an obvious place to start for future clinical trials.

Finally, the significant changes in PAR4 pharmacology associated with the Thr^{120} PAR4 variant – namely, the resistance to a small molecule PAR4 antagonist – have raised the question as to whether these compounds will also be less effective in some patients. It is also currently unknown how the change in amino acid from Ala to Thr at position 120 alters receptor pharmacology. The crystal structure of PAR4 has not yet been solved, although extrapolations from the crystal structure of PAR1 show critical hydrogen bonds at a similar region[110], which

could potentially effect the small molecule binding pocket and/or tethered ligand binding site. One major finding from Chapter 5 of this thesis is that antibody-based inhibition, via blocking the thrombin cleavage site, provides equivalent antithrombotic effects regardless of donor genotype. Further studies will be required to determine how the variant alters receptor biology, what the impact and significance of it will be clinically, and, what method of PAR4 antagonism will provide equivalent inhibition across the population.

6.5 Concluding Remarks

This thesis developed novel reagents to address key limitations in PAR4 research and used these to uncover previously unknown roles of PAR4 during thrombosis. In this way, these studies helped rationalise this receptor as a target for new antiplatelet drugs with a distinct mechanism of action. Although further insights are still to be gained regarding the utility of any PAR4 antagonism in clinical settings, the studies presented here have developed an antibody with therapeutic potential for the safe and effective prevention of arterial thrombosis that warrants investigation in further preclinical studies. These studies suggest targeting PAR4 represents a valid antithrombotic approach.

Appendix I

Blood Reviews 29 (2015) 179–189

Contents lists available at ScienceDirect

Blood Reviews

journal homepage: www.elsevier.com/locate/blre

REVIEW

Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent

Shauna L. French, Jane F. Arthur, Huyen A. Tran, Justin R. Hamilton *

Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria, Australia
Department of Clinical Haematology, Monash University, Melbourne, Victoria, Australia

ARTICLE INFO

Keywords:
Antiplatelet agents
Arterial thrombosis
Platelets
Protease-activated receptors
Thrombin

ABSTRACT

Twenty-three years after the discovery of the first thrombin receptor, now known as protease-activated receptor 1 (PAR1), the first drug targeting this receptor is available for human use. The PAR1 inhibitor, vorapaxar (Zontivity, MSD), was recently approved by the FDA for use in the USA for the prevention of thrombotic cardiovascular events in patients with a history of myocardial infarction or peripheral artery disease. In this review, we detail the rationale, development, as well as the clinical significance and considerations of vorapaxar, the original PAR antagonist and the latest anti-platelet agent in the pharmaco-armoury against arterial thrombosis.

1. Introduction

1.1. There is a significant clinical need for improved anti-thrombotic therapy

Cardiovascular disease, manifesting predominantly as ischaemic heart disease and ischaemic stroke, is by far the most common cause of death and disability in the world, accounting for approximately 30% of all deaths [1]. Strikingly, despite increased awareness and improved management, and in contrast to most other high-impact communicable and non-communicable diseases, the rates of cardiovascular disease rose over the past decade [1]. The increasing burden of conditions such as diabetes, obesity, and depression, as well as an overall ageing population, are likely to ensure that cardiovascular disease rates continue to rise into the foreseeable future, with each of these conditions having increased prevalence of cardiovascular-related morbidity and mortality.

Arterial thrombosis precipitates the most prevalent cardiovascular disease manifestations, most notably acute myocardial infarction (MI), ischaemic stroke, and peripheral artery disease (PAD). Current therapies for the prevention of arterial thrombosis are either anti-platelet agents, which prevent platelet activation, or anticoagulants, which primarily impair fibrin formation at therapeutic doses by inhibiting either the production or activity of thrombin. The efficacy of these agents is tempered by the attendant bleeding risk [2]. Given that activated platelets are the essential cellular component of arterial thrombi, anti-platelet drugs are the mainstay of current pharmacotherapy for arterial thrombosis, with the current recommendation for prevention of primary or secondary cardiovascular events in patients with acute coronary syndrome (ACS) being anti-platelet therapy alone [3]. Yet current standard-of-care treatments have limited efficacy and improved anti-platelet approaches are sought.

1.2. Current anti-platelet agents as anti-thrombotics

The processes controlling platelet function in the setting of arterial thrombosis have been extensively studied for the purpose of rationalising the development of improved anti-platelet therapies. Platelet behaviour during thrombus formation is generally agreed to involve, in order, cell adhesion, activation, and aggregation [4] (see Fig. 1) Understanding these processes has been highly informative in predicting the success of anti-platelet approaches.

i) *Adhesion*: In response to vascular damage, such as atherosclerotic plaque rupture in the case of ACS, platelets adhere to the damaged vessel wall through a complex between sub-endothelial proteins and cognate receptors on the platelet surface. Rapid initial adhesion is mediated by von Willebrand factor (vWF) binding to the glycoprotein (GP) Ib-IX-V receptor complex [5], although this interaction does not support stable adhesion and a second adhesive step between collagen and GPVI and/or the integrin $\alpha_2\beta_1$, as well as fibrinogen and the integrin $\alpha_{IIb}\beta_3$, is required for firm platelet adhesion to the vessel wall [4].
ii) *Activation*: following adhesion, the captured platelets are activated and undergo a series of morphological and functional changes

* Corresponding author at: Australian Centre for Blood Diseases, Monash University, L6, 89 Commercial Road, Melbourne, Victoria 3004, Australia. Tel.: +61 3 9903 0122; fax: +61 3 9903 0228.
E-mail address: Justin.Hamilton@monash.edu (J.R. Hamilton).

http://dx.doi.org/10.1016/j.blre.2014.10.006
0268-960X/

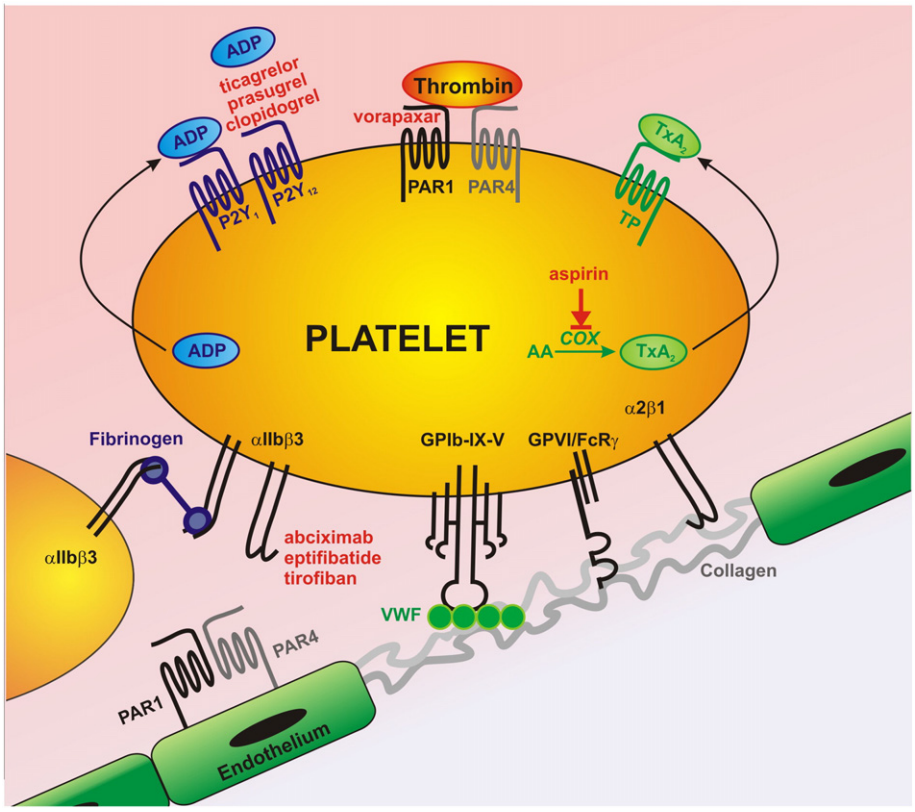


Fig. 1. Activation and adhesion receptors on the platelet. In response to vascular damage, platelets rapidly adhere to the vessel wall via the interaction of subendothelial von Willebrand factor (VWF, green spheres) with the platelet glycoprotein (GP) Ib-IX-V complex, and collagen (grey strands) with GPVI and integrin $\alpha_2\beta_1$. Adherent platelets become activated, and this activation is enhanced by signalling pathways initiated by: thromboxane receptors (TP) activated by thromboxane A2 (TxA_2) generated from arachidonic acid (AA) by cyclooxygenase (COX); $P2Y_1$ and $P2Y_{12}$ receptors activated by ADP; and PAR1 and PAR4 activated by thrombin. Platelet activation culminates in the activation of integrin $\alpha_{IIb}\beta_3$ which binds fibrinogen (blue dumbbell), VWF and fibronectin (not shown) to mediate platelet aggregation. Current anti-platelet agents (in red) include the $P2Y_{12}$ inhibitors clopidogrel, prasugrel and ticagrelor; the COX inhibitor, aspirin; $\alpha_{IIb}\beta_3$ inhibitors, abciximab, eptifibatide and tirofiban; and the most recent addition, vorapaxar, which inhibits PAR1.

including shape change, spreading, release of granular contents, and the local generation of thrombin at the platelet surface as an endpoint of coagulation [6]. This platelet activation is primarily driven by the triumvirate of thrombin, ADP, and thromboxane A_2 (TxA_2).

iii) *Aggregation*: regardless of the adhesion and activation mechanism, the final common result is the activation of the integrin $\alpha_{IIb}\beta_3$, which engages fibrinogen (also vWF and fibronectin) to mediate platelet aggregation and ultimately thrombus formation [6].

Based on the experiences with current and previously trialled anti-platelet drugs, the most useful therapeutic strategy is to interfere with the activation phase of the platelet response. Specifically, interfering with platelet activation may largely preserve the primary haemostatic functions of the platelet by sparing initial adhesion at sites of vascular injury. By way of example, antagonists of the major platelet integrin, $\alpha_{IIb}\beta_3$, such as tirofiban, eptifibatide, and abciximab, are by far the most potent platelet inhibitors as they inhibit platelet aggregation regardless of the activating pathway. However, these drugs all cause significant bleeding complications [7] due to their disruption of the haemostatic function of platelets also dependent on this pathway. Although $\alpha_{IIb}\beta_3$ inhibitors are currently indicated for acute use during percutaneous coronary intervention (PCI), the high bleeding risk and restrictive intravenous (i.v.) route of administration have limited their use in long-term preventative settings [8].

The current guidelines for treatment of ACS (comprising ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), and unstable angina) and associated diseases are dual therapy with aspirin (an inhibitor of thromboxane A_2 synthesis) and a $P2Y_{12}$ receptor antagonist, such as the thienopyridines, clopidogrel or prasugrel, or the cyclopentyl-triazolo-pyrimidine, ticagrelor [3] (American guidelines tend to favour the use of clopidogrel, whereas European guidelines favour either ticagrelor or prasugrel, with clopiodgrel as an alternative.). However, aspirin and clopidogrel prevent just 15 and 17% of lethal cardiovascular events respectively and are only marginally more effective in combination [2]. In addition, an increasing number of patients are being reported as resistant to these agents [9–11], while clopidogrel – a prodrug – is metabolised with significant variability within the population [12], providing yet another source of variable efficacy. Large scale clinical trials assessing the efficacy of aspirin and clopidogrel (together and separately) in reducing thrombotic risk in cardiovascular disease (CAPRIE [13] and CHARISMA [14]) have ultimately determined greater efficacy when used in combination, with an associated minor increase in risk of bleeding complications. Therefore, existing anti-platelet therapies have limitations in one or both of safety and efficacy, with no current agent (or combination of agents) affording sufficiently potent, safe, and orally active prevention of arterial thrombosis. As a result, improved anti-platelet approaches are required to meet the significant clinical need for the safe and effective prevention of arterial thrombosis.

2. Targeting protease-activated receptors as an anti-platelet approach

2.1. Rationale

2.1.1. Thrombin and thrombin receptors are important for platelet activation during thrombosis

The rationale for targeting thrombin-induced platelet activation as an anti-thrombotic approach has long held appeal. First, thrombin

functions at a time and place that is predicted to provide safe and effective anti-platelet activity. As outlined above, thrombin, in combination with ADP and TxA_2, mediates the platelet activation required for thrombus growth that follows initial cell adhesion. Second, thrombin is the most potent endogenous activator of platelets. This suggests that targeting thrombin-induced platelet activation may provide greater efficacy over existing mechanisms that block the platelet activating functions of TxA_2 (aspirin) and ADP (clopidogrel and co.). Third, existing drugs that block the production (e.g. rivaroxaban) or activity (e.g. dabigatran) of thrombin are effective as anti-thrombotics. However these agents provide global inhibition of thrombin's actions and, consequently, incur a significant bleeding risk — particularly at the high doses used for the prevention of platelet-rich arterial thrombi. Specifically targeting thrombin-induced platelet activation while leaving the other functions of thrombin intact may mitigate the bleeding risk and provide a more selective effect for arterial thrombosis prevention. In combination, these factors provided the impetus to develop inhibitors of platelet thrombin receptors.

Platelet responses to thrombin are mediated by G protein-coupled protease-activated receptors (PARs) [15,16]. PARs are expressed on the surface of numerous cell types. In the cardiovascular system this includes platelets [17–19], and also leukocytes [20], vascular endothelial and smooth muscle cells [21–23], cardiomyocytes [24,25], and cardiac fibroblasts [26]. Humans express four PARs, with PAR1, PAR3 and PAR4 being activated by thrombin [17–19], and PAR2 by trypsin, tryptase, coagulation factors VIIa and Xa, and membrane-bound serine proteases MTSP1 and TMPRSS2 [27–31]. Human platelets express two thrombin-sensitive PARs, PAR1 and PAR4, and activation of either receptor is capable of inducing platelet activation [17,18,32]. Thrombin-induced platelet activation initiates platelet shape change, promotes platelet aggregation, and provides the procoagulant surface that facilitates secondary coagulation reactions [15,32,33]. PAR1 is the 'high affinity' thrombin receptor on human platelets, responding more sensitively and rapidly to thrombin than PAR4 [32]. However PAR4 can also trigger full platelet activation with higher concentrations (10 to 30-fold) of thrombin [32,34]. Based on this difference in affinity the clinical strategy for PAR inhibition in humans has focussed on blocking PAR1 function.

The cleavage-based PAR activation mechanism is unique. PARs are all activated the same way, whereby the protease agonist cleaves the amino-terminus of the receptor to reveal a cryptic neo-amino terminal sequence known as the "tethered ligand" [17,35]. The newly exposed tethered ligand then activates the receptor by binding intramolecularly to the second extracellular loop [17,35]. This self-activation prompts the conformational change of the receptor that allows interactions with G proteins of the G_q, $G_{12/13}$ and $G_{i/z}$ families and consequent intracellular signalling events [36–38] (see Fig. 2). The interactions with $G_{12/13}$ drive Rho-dependent cytoskeletal responses involved in platelet shape change [38], whereas G_q-mediated signalling facilitates the processes important for platelet granule release, activation of cell surface integrins, and platelet aggregation [39]. $G_{i/z}$ proteins are involved in inhibition of adenylyl cyclase, thereby removing a brake on platelet activation, while the G_i protein family is the major source of $\beta\gamma$ subunits involved in modifying activities of enzymes such as PI3 kinase [37].

Although often described as a dual platelet thrombin receptor system, some distinctions between PAR1 and PAR4 have been shown, including independent intracellular signalling events and distinct activation kinetics. PAR1 is a "high affinity" thrombin receptor, as it contains a hirudin-like sequence ($K^{51}YEPF^{55}$) which binds exosite I of the protease and aligns the active site of thrombin with a specific cleavage site in the receptor [40,41]. PAR1 is activated by trace amounts of thrombin (sub-nM range) and is responsible for the initial and rapid rise in intracellular calcium induced in platelets by the coagulation protease [42]. However, the PAR1-induced signal is transient and requires additional support from other platelet agonists such as ADP [43]. In contrast to PAR1, the interaction between PAR4 and thrombin occurs primarily at the active site due to the absence of a hirudin-like thrombin binding domain in the amino-terminal of PAR4. Despite the lack of interaction between PAR4 and exosite I of thrombin, the receptor does bind at the active site with high affinity due to two optimally-positioned proline residues immediately amino-terminal to the thrombin cleavage site [44]. This interaction facilitates slowed dissociation of thrombin from the receptor and results in a slower but more sustained intracellular signalling profile [44]. As a result of these distinct receptor activation and signalling kinetics, PAR4, in contrast to PAR1, is capable of inducing irreversible platelet aggregation in the absence of additional agonist activation [43]. It has therefore been suggested that PAR1 and PAR4 complement each other during thrombin-induced platelet aggregation. However, the major clinical focus has been on inhibiting PAR1 for anti-thrombotic activity due to the significantly greater sensitivity of this receptor to thrombin. Initial pre-clinical studies toward this end were performed in animal studies.

2.1.2. Pre-clinical studies

As is the case in many other systems, mouse genetic models and other small animal *in vivo* thrombosis experiments provided pre-clinical proof-of-concept studies. However the interpretation of these studies has always been hampered by the differing platelet PAR profile in these traditional model systems. Of the most commonly used animal models, only non-human primates appear to have an identical platelet PAR profile to humans (PAR1 and PAR4). In contrast, platelets from mice [45], rats [46], and rabbits [47] express PAR3 and PAR4, while platelets from guinea-pigs express PAR1, PAR3 and PAR4 [48]. Despite these limitations, significant insights have been gained from mouse genetic experiments. Mouse platelets do not express PAR1, but instead have PAR3 and PAR4. In a further difference, although mouse PAR4 functions in an analogous manner to human PAR4, mouse PAR3 is incapable of mediating transmembrane signalling by itself, instead functioning as a cofactor that facilitates cleavage and activation of PAR4 at low thrombin concentrations [49,50]. Indeed, PAR3 appears to act as a high affinity receptor for thrombin as it also contains a hirudin-like sequence to facilitate thrombin binding, therefore acting to bind and localise thrombin to the platelet surface and promoting PAR4 cleavage and subsequent signalling. As this model predicts, platelets from PAR4−/− mice are unresponsive to thrombin [45,51] and provide a clean genetic model to examine the overall contribution of thrombin-induced platelet activation in (patho)physiology. In this regard, PAR4−/− mice are healthy and exhibit no evidence of spontaneous bleeding, but are protected against thrombosis in several distinct *in vivo* models [45,51–53]. Haemostasis is impaired upon challenge as evidenced by an increase in tail bleeding time [45,51]. Perhaps surprisingly, PAR3−/− mice are also protected against thrombosis in a series of *in vivo* models, albeit to a lesser extent than observed with PAR4-deficiency [49]. This is of interest given that PAR3−/− mice may be viewed as a model of PAR1 inhibition in humans, inasmuch as both sets of platelets rely on PAR4 for thrombin-induced platelet activation. Of note, prolonged tail bleeding times were also observed in PAR3−/− mice [49]. Despite the limitations of using mice as a model of human platelet PAR function, these early proof-of-concept studies provided insight into the relative importance of thrombin-induced platelet activation in the setting of *in vivo* thrombosis, and in large part drove the development of PAR1 antagonists as a novel anti-platelet approach.

The development of pharmacological PAR1 inhibitors proved a significant challenge — perhaps largely due to the unique nature of the PAR self-activation mechanism and the consequent issues regarding efficacy for any potential antagonists that must compete with an endogenous agonist with a substantial steric advantage. In addition, receptor cleavage is irreversible and stoichiometrically efficient: multiple receptors can be activated by a single protease molecule. Numerous strategies have been employed to overcome these issues, including peptidomimetics derived from the tethered ligand sequence [54,55], intracellular signalling-inhibiting 'pepducins' [56], as well as receptor function-blocking antibodies [57], all of which have contributed to the

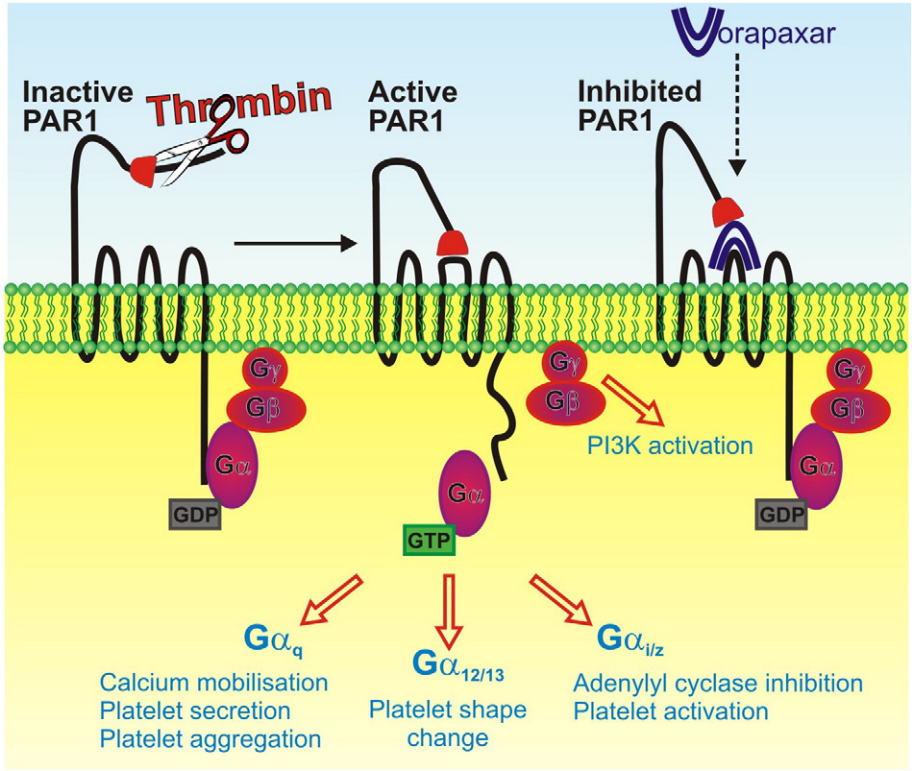


Fig. 2. Inhibition of PAR1 by vorapaxar. Thrombin cleaves the extracellular N-terminus of PAR1, exposing a tethered ligand (red cap) that binds to the second extracellular loop of the receptor and induces a conformational change that facilitates G protein interaction with the receptor and consequent intracellular signalling. PAR1 couples to G proteins of the G_q, $G_{12/13}$ and $G_{i/z}$ families to induce a multitude of intracellular signalling events in platelets. Vorapaxar prevents PAR1 activation by binding at or near the tethered ligand binding site, acting as a competitive antagonist of the endogenous activation mechanism of the receptor.

understanding of PAR biology and led to the eventual development of effective and specific small molecule antagonists.

The earliest PAR inhibitors were peptide-based agents and function-blocking antibodies and provided important tools for experiments investigating platelet PAR function in human cells. The peptide-based inhibitors were synthesised to mimic the endogenous tethered ligand sequence, but with modifications that prevented the receptor being activated once the antagonist had bound. The structure–activity insight gained from the earliest peptidomimetics and further optimisation of this class led to the development of RWJ-58259, which inhibited PAR1-mediated aggregation of human platelets and thrombus formation *in vivo* and *in vitro* in non-human primates [58]. The earliest function-blocking anti-PAR1 antibodies were rabbit polyclonal antibodies targeted against the thrombin cleavage site of the receptor. These agents also inhibited PAR1-dependent aggregation of human platelets [57]. In keeping with the anti-thrombotic activity observed with peptidomimetic antagonists of PAR1, function blocking anti-PAR1 antibodies were also effective anti-thrombotics in non-human primate models [57,58]. Despite this effectiveness, the use of peptide-based agents and therapeutic antibodies in these studies came with the usual pharmacodynamic and pharmacokinetic limitations of such approaches and resulted in investigations into designing selective and potent small molecule antagonists of PAR1. This effort ultimately yielded two agents that were examined in clinical trials: atopaxar (E5555) and vorapaxar (SCH530348).

2.2. Development

2.2.1. Atopaxar

Atopaxar is a small molecule PAR1 inhibitor. Developed by Eisai and formerly known as E5555, atopaxar is a bicyclic amidine derivative with a molecular weight of 609 Da that competitively binds at or near the tethered ligand binding site within the second extracellular loop of PAR1 [59]. In initial cell studies, atopaxar was shown to inhibit thrombin-induced aggregation of human platelets [60] and thrombin-induced intracellular calcium signalling in cultured smooth muscle cells [61]. It has an IC_{50} of 64 nM [60]. *In vivo*, atopaxar has been shown as an effective PAR1 antagonist in small animal models [60,61]. In rabbits, atopaxar reduced thrombin-dependent cerebral vasospasm – one of the major complications of subarachnoid haemorrhage – and was shown to dose-dependently decrease the basilar artery contractile response to thrombin as well as inhibit the upregulation of PAR1 expression in subarachnoid haemorrhage [61]. In a guinea pig model of photochemically-induced thrombosis, orally-administered atopaxar (30 mg/kg) prolonged the time to vessel occlusion by approximately 2-fold [60]. Given the promising efficacy of atopaxar in these small animal models, human trials were undertaken.

The safety of atopaxar was assessed in three phase 2 trials in patients with ACS (LANCELOT-ACS) and with chronic coronary artery disease (LANCELOT-CAD) [62–64]. These trials showed that overall clinically relevant bleeding increased numerically but not significantly, and that major bleeding rates increased in the ACS trial, but not in the CAD trial [62,63]. In addition, higher doses ($\geq$200 mg) resulted in an increase in liver enzymes and QT interval prolongation [62]. These effects were dose-dependent with significant liver function abnormalities observed in patients receiving the highest doses of atopaxar [62]. As a result, there are no phase 3 trials for atopaxar currently registered. For this reason, the remainder of this review will focus on the journey of vorapaxar through to FDA approval.

2.2.2. Vorapaxar

Vorapaxar is also a small molecule PAR1 inhibitor. Developed by Merck & Co (MSD; originally as Schering-Plough) and formerly known as SCH530348, vorapaxar is a synthetic tricyclic 3-phenylpyridine

analog of the naturally occurring alkaloid himbacine, and has a molecular weight of 591 Da. Similar to atopaxar, vorapaxar is a reversible, competitive antagonist of PAR1 that binds at or near the tethered ligand binding site within the second extracellular loop of PAR1, thus competing with the endogenous activation mechanism of the receptor [65]. Vorapaxar inhibits aggregation of human platelets induced by thrombin and a PAR1 agonist peptide (PAR1-AP) with an IC_{50} of 47 and 25 nM, respectively, and has a Ki of 8 nM [65]. Importantly, vorapaxar is administered as a bisulfate salt and has a high oral bioavailability (>90%) and is rapidly absorbed *via* the gastrointestinal tract, with a terminal plasma half-life of 126–269 h [66]. The drug is metabolised primarily by the liver *via* the CYP3A4 pathway and is eliminated almost exclusively as an amine metabolite [67]. As a result, drug interactions that have the potential to effect metabolism of vorapaxar include CYP3A4 inhibitors (e.g. ketoconazole) or inducers (e.g. rifampicin), which may increase or decrease the plasma concentration of vorapaxar respectively. In healthy subjects the drug was well tolerated and long-lasting: administration of a single loading dose (5 to 40 mg) inhibited PAR1-AP-induced platelet aggregation by >90% for more than 72 h [66]. Furthermore, administration of a daily dose of 1, 3, or 5 mg resulted in the same level of inhibition of the platelet aggregation through to day 7 of treatment [66]. To date, and in contrast to the phase 2 findings of atopaxar, no abnormalities in liver function or other adverse events have been associated with long term vorapaxar use.

Early human trials with PAR1 inhibitors showed trends toward clinical benefit in cardiovascular outcomes without an increase in bleeding risk. In a phase 2 trial, vorapaxar was assessed in 773 patients undergoing PCI and with a history of ACS and/or NSTEMI, most of whom were on dual antiplatelet therapy (aspirin plus clopidogrel) [66]. Patients received a loading dose of 10, 20, or 40 mg (or matching placebo) followed by a daily maintenance dose of 0.5, 1.0 or 2.5 mg. The primary endpoint was clinically significant major or minor bleeding, assessed by the Thrombolysis In Myocardial Infarction (TIMI) scale and was found to be not significantly different between the placebo and test groups, even at the highest dosing regimen [66]. Strikingly, although the study was not powered to determine efficacy, a trend toward a reduction in MI events was observed [66]. Given these promising findings, phase 3 trials of vorapaxar were developed using the highest dosing regimen from the phase 2 trials.

2.2.3. The Thrombin Receptor Antagonist for Clinical Event Reduction (TRACER) in acute coronary syndrome trial

Vorapaxar was assessed in two large scale phase 3 trials in patients with ACS (TRACER) and stable atherosclerosis (TRA 2°P-TIMI 50). The overall findings of these trials showed that vorapaxar, when combined with standard of care therapy, reduced the risk of cardiovascular events at the cost of increased bleeding [68,69].

TRACER was a randomised, double-blind, placebo-controlled multicentre trial that enrolled 12,944 patients with acute symptoms of coronary ischaemia plus either significant ST changes or elevations in cardiac necrosis markers (troponin or creatine kinase) [69]. Of the study group, 6473 patients received vorapaxar (40 mg single loading and 2.5 mg once daily maintenance) with 6471 receiving placebo. Ninety-six percent of all patients were receiving aspirin and 80% of those were also receiving a thienopyridine [69]. Assessments were performed during initial hospitalisation, with follow-up at 1, 4, 8, and 12 months post-hospitalisation, and every 6 months thereafter, with the aim of a total follow-up of 3 years. The primary end point for TRACER was a composite of cardiovascular death, MI, stroke, recurrent ischaemia, or urgent coronary revascularisation, with the secondary endpoint being a composite of death from cardiovascular causes, MI, or stroke. The key safety end points were a composite of moderate and severe bleeding according to the Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) classification as well as clinically significant bleeding according to the TIMI classification.

2.2.4. TRACER trial: efficacy

Ultimately, the primary endpoint of TRACER was not achieved: a non-significant reduction in the primary end point occurred in patients receiving vorapaxar (18.5%) versus placebo (19.9%) (hazard ratio (HR) 0.92; 95% confidence interval (CI) 0.85–1.01; P = 0.07) [69]. The key secondary end point occurred in 14.7% versus 16.4% of patients. Given that superior efficacy of vorapaxar in this setting was not observed, subsequent subgroup analyses were performed but were purely exploratory [69,70]. Analysis of individual end point components revealed that the greatest reduction was observed in the rate of MI (11.1% versus 12.5%; HR 0.88; 95% CI 0.79–0.88; P = 0.02). Interestingly, stroke rates between the two treatment groups were similar over 2 years: the vorapaxar group had lower rates of ischaemic stroke (1.1% versus 1.4%; HR 0.79; 95% CI 0.59–1.08; P = 0.14) but a higher rate of haemorrhagic stroke (0.3% versus 0.1%; HR 2.73; 95% CI 1.22–6.14; P = 0.02). Other efficacy endpoints are summarised in Table 1.

2.2.5. TRACER trial: safety

Patients receiving vorapaxar were more likely to experience bleeding events. Moderate or severe bleeding (assessed by GUSTO) occurred in 7.2% of patients receiving vorapaxar compared with 5.2% of those receiving placebo (HR 1.35; 95% CI 1.16–1.58; P < 0.001). Clinically significant (TIMI) bleeding occurred in 20.2% of patients in the vorapaxar group versus 14.6% in the placebo group (HR 1.43; 95% CI 1.31–1.57; P < 0.001). Most critically, however, intracranial haemorrhage increased more than 5-fold in the vorapaxar group (1.1% versus 0.2%; HR 3.39; 95% CI 1.78–6.45; P < 0.001) [69]. This effect was especially noted in patients with a prior history of stroke or transient ischaemic attack (TIA) and increased incrementally over time. Consequently, the planned 3-year follow-up of TRACER was terminated in January 2011 (five months early) in response to recommendations from the data and safety monitoring board.

2.2.6. TRACER trial: subgroup analyses

Exploratory subgroup analyses of the TRACER trial have yielded several interesting findings that may warrant further evaluation. Firstly, analysis of MI reduction in TRACER showed that vorapaxar treatment resulted in a 12% hazard reduction (HR 0.88; 95% CI 0.79–0.98; P = 0.021) of a first MI of any type [70]. Further, this reduction was largely accounted for by a specific decrease in the rate of type 1 (spontaneous) MI (5.9% vs 7.0%; HR 0.83; 95% CI 0.73–0.95; P = 0.007). This is noteworthy, as spontaneous MIs are the most commonly observed: 65% of MIs observed in TRACER were type 1 and were the most frequently observed coronary endpoint. These observations from the original trial data were supported by a recent meta-analysis of all placebo-controlled trials of either atopaxar or vorapaxar in CAD patients [71]. In addition to MI, vorapaxar reduced the primary endpoint in coronary artery bypass graft (CABG) patients with an estimated hazard reduction of 45% (43 patients in the vorapaxar treatment group versus 70 in placebo; 8.2% and 12.9%, respectively, HR 0.55; 95% CI 0.36–0.83; P = 0.005) [72]. There was a non-significant increase in both surgical and non-surgical bleeding in this cohort, and the overall results suggest that vorapaxar treatment may be a viable option in this setting. Investigations into the effect of standard-of-care therapy in addition to vorapaxar treatment indicated improved safety and efficacy in the vorapaxar groups who were not receiving high dose (>300 mg) aspirin and/or a thienopyridine [69,70].

2.2.7. TRACER trial: overall outcomes

Overall, TRACER concluded that vorapaxar in addition to standard anti-platelet therapy provided no significant net clinical benefit in patients with acute coronary syndromes. Furthermore, vorapaxar significantly increased the risk of major bleeding, most notably intracranial haemorrhage, in a subset of patients. These outcomes had a significant impact on the TRA 2°P-TIMI 50 trial. Firstly, the primary endpoint was revised to a composite of cardiovascular death, MI or stroke, with urgent

Table 1
Efficacy and safety endpoints of the TRACER and TRA 2°P-TIMI 50 trials.

	TRACER[a]				TRA 2°P-TIMI 50[b]			
	Vorapaxar Number of events (percent)	Placebo Number of events (percent)	Hazard ratio (95% CI)	P value	Vorapaxar Number of events (percent)	Placebo Number of events (percent)	Hazard ratio (95% CI)	P value
	n = 6473	n = 6471			n = 13,225	n = 13,224		
Primary endpoint	1031 (18.5)	1102 (19.9)	0.92 (0.85–1.01)	0.07	1028 (9.3)	1176 (10.5)	0.87 (0.80–0.94)	<0.001 (NNT = 83)
Secondary endpoint	822 (14.7)	910 (16.4)	0.89 (0.81–0.98)	0.02	1259 (11.2)	1417 (12.4)	0.88 (0.82–0.95)	0.001 (NNT = 83)
Cardiovascular death or myocardial infarction	755 (13.5)	843 (14.9)	0.90 (0.81–0.99)	0.03	789 (7.3)	913 (8.2)	0.86 (0.78–0.94)	0.002 (NNT = 111)
Cardiovascular death	208 (3.8)	207 (3.8)	1.00 (0.83–1.22)	0.96	285 (2.7)	319 (3.0)	0.89 (0.76–1.04)	0.15
Myocardial infarction	621 (11.1)	698 (12.5)	0.88 (0.79–0.98)	0.02 (NNT = 84)	564 (5.2)	673 (6.1)	0.83 (0.74–0.93)	0.001 (NNT = 122)
Stroke								
Any	96 (1.9)	103 (2.1)	0.93 (0.70–1.23)	0.61	315 (2.8)	324 (2.8)	0.97 (0.83–1.14)	0.73
Ischemic	74 (1.1)[c]	93 (1.4)	0.79 (0.59–1.08)	0.14	250 (2.2)	294 (2.6)	0.85 (0.72–1.01)	0.06
Haemorrhagic	22 (0.3)[c]	8 (0.1)	2.73 (1.22–6.14)	0.02	Data not reported			
Urgent coronary revascularisation	203 (3.8)	189 (3.5)	1.07 (0.88–1.31)	0.49	279 (2.5)	316 (2.6)	0.88 (0.75–1.03)	0.11
Recurrent ischemia with hospitalisation	79 (1.6)	69 (1.5)	1.14 (0.83–1.58)	0.42	Data not reported			
Stent thrombosis definite or probable	61/3549 (1.7)[c]	54/3526 (1.5)[c]	1.12 (0.78–1.62)	0.54	Data not reported			
Death vs any cause	334 (6.5)	318 (6.1)	1.05 (0.90–1.23)	0.52	540 (5.0)	565 (5.3)	0.95 (0.85–1.07)	0.41
Safety Endpoints	n = 6446	n = 6441			n = 13,186	n = 13,166		
GUSTO								
Moderate or severe bleeding	391 (7.2)	290 (5.2)	1.35 (1.61–1.58)	<0.001	438 (4.2)	267 (2.5)	1.66 (1.43–1.93)	<0.001
Severe bleeding	144 (2.9)	87 (1.6)	1.66 (1.27–2.16)	<0.001	Data not reported			
TIMI								
Clinically significant	1065 (20.2)	755 (14.6)	1.43 (1.31–1.57)	<0.001	1759 (15.8)	1241 (11.1)	1.46 (1.36–1.57)	<0.001
Major bleeding	208 (4.0)	136 (2.5)	1.53 (1.24–1.90)	<0.001	Data not reported			
Non-CABG major bleeding	131 (2.7)	71 (1.3)	1.85 (1.39–2.47)	<0.001	287 (2.8)	198 (1.8)	1.46 (1.22–1.75)	<0.001
CABG major bleeding	62/639 (9.7)	49/671 (7.3)	1.34 (0.92–1.95)	0.13	11/175 (7.6)	10/210 (6.1)	1.13 (0.48–2.66)	0.79
Bleeding requiring medical attention	784 (15.2)	564 (11.2)	1.41 (1.26–1.57)	<0.001	Data not reported			
Fatal bleeding	15 (0.4)	8 (0.2)	1.89 (0.80–4.45)	0.15	29 (0.3)	20 (0.2)	1.46 (0.82–2.58)	0.19
Intracranial haemorrhage	40 (1.1)	12 (0.2)	3.39 (1.78–6.45)	<0.001	102 (1.0)	53 (0.5)	1.94 (1.39–2.70)	<0.001

CI = confidence interval; NTT = number to treat; TIMI = Thrombolysis In Myocardial Infarction classification; GUSTO = Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries classification; CABG = coronary artery bypass graft.

[a] Primary endpoint is a composite of *cardiovascular death*, myocardial infarction, stroke, or urgent coronary revascularisation. Secondary end point is a composite of *cardiovascular death*, myocardial infarction or stroke. Percentages are cumulative Kaplan–Meier event rates at 2 years.

[b] Primary endpoint is a composite of *death from cardiovascular causes*, myocardial infarction or stroke. Secondary endpoint is a composite of death *from cardiovascular causes*, myocardial infarction, stroke or urgent coronary revascularisation. Percentages are cumulative Kaplan–Meier event rates at 3 years.

[c] Percentages are calculated from raw data, Kaplan–Meier event rates not included in data set.

coronary revascularisation becoming part of the secondary endpoint. Most importantly, patients enrolled in the TRA 2°P-TIMI 50 trial who had a history of TIA and/or stroke were removed from the trial in January 2011 (2 years post enrolment) after the increases in risk of intracranial bleeding were brought to light. These modifications permitted the completion of this trial.

2.2.8. Thrombin-Receptor Antagonist for Secondary Prevention of Atherothrombotic Ischaemic Events (TRA 2°P-TIMI 50) Trial

As with TRACER, TRAP 2°P-TIMI 50 was a randomised, double-blind, placebo-controlled, multicentre trial. It was a larger trial in which 26,449 patients were enrolled, 13,224 of whom received vorapaxar (2.5 mg daily, no loading dose) in addition to standard-of-care therapy [68]. Initial recruitment involved patients with stable atherosclerosis and a history of MI or ischaemic stroke (between 2 weeks and 12 months before enrolment) and/or PAD (either after revascularisation or with an ankle-brachial index of <0.85) [68]. As noted above, study treatment in patients who had a prior stroke was discontinued in January 2011, 2 years after the commencement of the trial. In the MI cohort, 98% were receiving aspirin and 78% were also receiving a thienopyridine; in the PAD patients, 88% were receiving aspirin and only 37% a thienopyridine. The primary end point was a composite of cardiovascular death, MI, or stroke, with the key secondary endpoint a composite of cardiovascular death, MI, stroke, or urgent coronary revascularisation. As with TRACER, the key safety endpoints were moderate or severe bleeding (GUSTO) and clinically significant bleeding (TIMI). The trial covered 3 years, with follow-up visits at 1, 4, 8, and 12 months, and then every 6 months thereafter until trial completion.

2.2.9. TRA 2°P-TIMI 50 trial: efficacy

The TRAP 2°P-TIMI 50 trial achieved its primary endpoint. At 3 years, the primary end point had occurred in 9.3% of patients receiving vorapaxar compared with 10.5% in the placebo group (Kaplan–Meier estimates at 3 years; HR 0.87; 95% CI 0.80–0.94; P < 0.001) [68]. The major secondary end point was also reduced (11.2% versus 12.4%; HR 0.88; 95% CI 0.83–0.95; P = 0.001). Again, of the individual components of these end points, MI was most significantly reduced (5.2% versus 6.1%; HR 0.83; 95% CI 0.74–0.93; P = 0.001). Despite these findings there was no significant difference in the rates of death from any cause (5.0% versus 5.3%; HR 0.95; 95% CI 0.85–1.07; P = 0.41) [68]. A summary of these endpoints is provided in Table 1.

2.2.10. TRA 2°P-TIMI 50 trial: safety

As observed in TRACER, bleeding complications were increased in patients receiving vorapaxar: moderate–severe bleeding (GUSTO) was increased (4.2% versus 2.5%; HR 1.66; 95% CI 1.43–1.99; P < 0.001), as was clinically significant bleeding and non-CABG-related major bleeding scores (TIMI) (15.8% versus 11.1% (HR 1.46; 95% CI 1.36–1.57) and 2.8 versus 1.8% (HR 1.46; 95% CI 1.22–1.75), respectively, P < 0.001 for both) [68]. In contrast, there was no significant difference in the rates of CABG-related major bleeding — a lack of effect also observed in the TRACER trial. Intracranial haemorrhage was increased two-fold in the vorapaxar group (1.0% versus 0.5%, HR 1.94; 95% CI 1.39–2.70;

P < 0.001) but, most critically, fatal bleeding was rare and was not different between the treatment groups (0.3% versus 0.2%, HR 1.46; 95% CI 0.82–2.58; P = 0.19) [68]. Bleeding was increased in women, those with a body weight <60 kg, and patients in the North American trials; however these groups did not test positive for significant interaction [68]. In contrast to TRACER, additional $P2Y_{12}$ therapy did not increase the bleeding risk of vorapaxar, yet concomitant aspirin treatment did. The aspirin-naive patient subgroup did not show any increase in clinically relevant bleeding [68,73].

2.2.11. TRA 2°P-TIMI 50 trial: subgroup analyses

TRA 2°P-TIMI 50 was designed to allow analyses of the large, predefined subgroups comprising the three distinct atherosclerotic patient cohorts: those with prior MI, ischaemic stroke, or PAD. In support of the TRACER findings, particular benefit was observed in the 17,779 patients with prior MI (8.1% versus 9.7%; HR 0.80; 95% CI 0.72–0.89, P < 0.001) [74]. Importantly, patients in this subgroup did not have increased rates of intracranial haemorrhage (0.6% versus 0.4%; P = 0.076) [74]. Interestingly, of the 3787 PAD patients, vorapaxar treatment did not reduce the risk of primary or secondary endpoint [75], but did produce some benefit in reducing the risk of peripheral vascular end points, notably limb ischaemia (2.3% versus 3.9%; HR 0.58; 95% CI 0.39–0.86; P = 0.006) and peripheral revascularisation (18.4% versus 22.2%; HR 0.84; 95% CI 0.73–0.97; P = 0.017) [75]. In concordance with the TRACER trial results, the ischaemic stroke subgroup of TRA 2°P-TIMI recorded no significant efficacy with vorapaxar treatment but an increase in bleeding was observed [76].

As a result of these trials, in May 2014 the FDA approved vorapaxar for the prevention of thrombotic cardiovascular events in patients with a history of MI or PAD. The drug will be sold under the trade name Zontivity. Given the clinical findings from TRACER and TRA 2°P-TIMI, Zontivity will come with a boxed warning contraindicating use in patients with a history of stroke or TIA.

2.3. Significance & considerations

2.3.1. When will PAR1 antagonists be of most benefit?

Understanding the relative contribution of thrombin-mediated platelet activation to thrombus formation in particular pathologies will likely be of significant use in predicting the clinical success of PAR1 inhibitors. Overall, in patients with no history of stroke or TIA, and with a body weight above 60 kg, the data from TRA 2°P-TIMI 50 translated into 6 fewer cardiovascular deaths at the cost of two intracranial haemorrhages for every 1000 patients treated with vorapaxar [73] (see Table 2). However, detailed analysis of the clinical data with vorapaxar use showed increased benefit of PAR1 inhibition in distinct clinical cohorts. PAR1 inhibition demonstrated a consistent reduction in the rate of type 1 (spontaneous) MI in vorapaxar-treated patients across the phase 2 and phase 3 studies [70]. This is perhaps unsurprising given the well-known role of thrombin generation in acute MI, particularly in patients with a background of unstable angina and/or coronary artery disease [77–79]. That these patients have a noted increase in plasma GPV levels (a marker of thrombin-induced platelet activation) weeks after an acute MI event [78] presumably reflects long-term thrombin-induced platelet activation in this setting. On this point, it is intriguing to note that plasma GPV levels in STEMI patients correlate with the likelihood of occlusion of infarcted arteries and of early recanalisation of these vessels [79].

In addition to acute MI, vorapaxar was shown to reduce the complications associated with PAD, notably limb ischaemia and the requirement for peripheral vascularisation [75]. Such efficacy of PAR1 inhibition in peripheral arteries might also have been predicted from the much previous work investigating the role of thrombin in distinct pathologies. Numerous studies have shown thrombin inhibitors to be more effective at the lower shear rates experienced in peripheral arteries [80–82]. This phenomenon was previously thought due to decreased fibrin production and a consequent impairment of thrombus stability [82], however more recent work has shown that thrombin-dependent platelet activation (via PARs) is also more prevalent at lower blood shear rates [83], presumably due to limited assembly of blood borne coagulation factors on the surface of activated platelets at higher blood flow/shear rates [84].

Table 2
Suggested clinical place of vorapaxar in cardiovascular disease.

Indicated	Contraindicated
Patients with	History of:
1) Coronary artery disease 2) Peripheral arterial disease 3) In combination with existing antiplatelet drugs	1) Stroke 2) TIA 3) Intracranial haemorrhage
	Active pathological bleeding
	Underlying bleeding risk:
	1) Decreased renal and/or hepatic function 2) Currently taking anticoagulants 3) Under 60 kg and/or over 75 years 4) History of bleeding disorders
	Pregnant or breastfeeding women

TIA = transient ischaemic attack;

With the ever-present push toward personalised medicine, identifying the relative role of thrombin-induced platelet activation in patients may be of interest. Clearly, the one-size-fits-all approach to anti-platelet therapy provides a wide spectrum of responses to standard-of-care therapy. On this point, one recent study used thromboelastography and ADP-induced platelet aggregation to predict ischaemic events in aspirin- plus clopidogrel-treated patients 6 months after PCI. After stratification by platelet-fibrin clot strength measured by thromboelastography, this study found that 58% of patients in the highest quartile for ADP-induced aggregation (high residual platelet reactivity) had an ischaemic event by 2 months versus only 2% of patients in the lowest quartile [85,86]. In this regard, patients identified as having high residual platelet reactivity following standard-of-care treatment might receive the greatest benefit from additional administration of the new PAR1 antagonist.

2.3.2. How safe is PAR1 antagonism?

In addition to the specific pathologies likely to benefit from PAR1 inhibition (or not), understanding the interaction of vorapaxar with other current anti-platelet drugs is clearly of importance and requires further investigation given the discrepancies in the existing trial data. The TRACER trial showed that bleeding rates (GUSTO) increased in patients receiving vorapaxar in combination with a thienopyridine compared to those not (HR 1.45; 95% CI 1.23–1.71 (no thienopyridine) versus HR 0.95; 95% CI 0.65–1.40 (with thienopyridine); P = 0.04 for interaction) [69]. In contrast, no increase in bleeding was observed in patients co-administered vorapaxar and a thienopyridine in the TRA 2°P-TIMI 50 trial [68]. A further difference between the trial results concerned concomitant aspirin usage, with patients receiving high dose aspirin (>300 mg daily at both baseline and time of discharge) recording a consistent, albeit non-statistically significant, trend toward higher bleeding and ischaemic outcomes in TRACER [87] but not in TRA 2°P-TIMI 50 [68]. The TRA 2°P-TIMI 50 trial did however report an increase in bleeding in North American patients, and it is worth noting that this patient group was considerably more likely to be receiving high dose aspirin than patients from other countries [68,87]. Regardless of these differences between the trials, bleeding in patients receiving multiple anti-platelet agents is likely to be a major consideration. It remains unknown whether the bleeding complications observed in these trials were due to PAR1 inhibition per se, the concurrent use of three anti-platelet drugs, or to a particular drug combination. On this point, it is worth noting that recent animal studies suggest that strategies that block platelet PAR function

Table 3
Comparison of efficacy and safety of antithrombotic agents in patients with acute coronary syndromes.

	Vorapaxar		Clopidogrel	Prasugrel	Ticagrelor	Rivaroxaban
Trial details	TRA 2°P-TIMI-50[a] Vorapaxar + aspirin / clopidogrel vs placebo; 2.5 mg daily/3 years; % = Kaplan–Meier at 3 years	TRACER[b] Vorapaxar + aspirin /clopidogrel vs placebo; 40 mg loading dose; 2.5 mg daily/2 years; % = Kaplan–Meier at 2 years	CHARMISA[c] Clopidogrel (75 mg daily) + aspirin vs aspirin alone; % = Kaplan–Meier at 27.6 months	TRITON-TIMI-38[d] Prasugrel + aspirin vs clopidogrel; 60 mg loading dose; 10 mg daily/15 months; % = Kaplan–Meier at 15 months	PLATO[e] Ticagrelor + aspirin vs clopidogrel; 180 mg loading dose, 90 mg twice daily/ 12 months; % = Kaplan–Meier at 12 months	ATLAS-ACS 2-TIMI-51[f] Rivaroxaban + aspirin / clopidogrel vs placebo; 2.5 mg twice daily/31 months; % = Kaplan–Meier at 24 months.
Patient characteristics	Number – 26,449 History of MI, ischemic stroke or PAD	Number – 12,944 Evidence of ACS without ST elevation	Number – 15,603 Documented coronary disease, cerebrovascular disease, PAD or multiple atherothrombotic risk factors; over 45 years of age	Number – 13,608 Moderate to high-risk ACS with scheduled PCI	Number – 18,624 Hospitalisation for ACS, with or without ST-elevation	Number – 15,526 Recent ACS (hospitalisation within 7 days)
	Vorapaxar (%) vs placebo (%); HR (95% CI)	Vorapaxar (%) vs placebo (%); HR (95% CI)	Clopidogrel + aspirin (%) vs aspirin (%); HR (95% CI)	Prasugrel (%) vs clopidogrel (%); HR (95% CI)	Ticagrelor (%) vs clopidogrel (%); HR (95% CI)	Rivaroxaban (%) vs placebo (%); HR (95% CI)
Composite of death from cardiovascular causes, myocardial infarction or stroke	9.3% vs 10.5% HR 0.87 (0.80–0.94) P < 0.001	14.7% vs 16.4% HR 0.89 (0.81–0.98) P = 0.02	6.8% vs 7.3% HR 0.93 (0.83–1.05) P = 0.22	9.9% vs 12.1% HR 0.81 (0.73–0.90) P < 0.001 (nonfatal MI and stroke)	9.8% vs 11.7%[g] HR 0.84 (0.77–0.92) P < 0.001	9.1% vs 10.7% HR 0.84 (0.72–0.97) P = 0.02
Myocardial infarction	5.2% vs 6.1% HR 0.83 (0.74–0.93) P = 0.001	11.1% vs 12.5% HR 0.88 (0.79–0.98) P = 0.02	1.9% vs 2.0% (nonfatal) HR 0.94 (0.75–1.18) P = 0.59	7.3% vs 9.5% (nonfatal) HR 0.76 (0.67–085) P = <0.001	5.8% vs 6.9% HR 0.84 (0.75–0.95) P = 0.005	6.1% vs 6.6% HR 0.90 (0.75–1.09) P = 0.27
Stroke (any)	2.8% vs 2.8% HR 0.97 (0.83–1.14) P = 0.73	1.9% vs 2.1% HR 0.93 (0.70–1.23) P = 0.61	1.9% vs 2.4% (nonfatal) HR 0.79 (0.64–0.98) P = 0.03	1.0% vs 1.0% (nonfatal) HR 1.02 (0.71–1.45) P = 0.93	1.5% vs 1.3% HR 1.17 (0.91–1.52) P = 0.22	1.4% vs 1.2% HR 1.13 (0.74–1.73) P = 0.56
Death from any cause	5.0% vs 5.3% HR 0.95 (0.85–1.07) P = 0.41	6.5% vs 6.1% HR 1.05 (0.90–1.23) P = 0.52	4.8% vs 4.8% HR 0.99 (0.86–1.14) P = 0.90	3.0% vs 3.2% HR 0.95 (0.78–1.16) P = 0.64	4.5% vs 5.9% HR 0.78 (0.69–0.91) P < 0.001	2.9% vs 4.5% HR 0.68 (0.53–0.87) P = 0.002
Key bleeding end points (TIMI except where indicated)	Non-CABG related major bleeding 2.8% vs 1.8% HR 1.46 (1.22–1.75) P < 0.001 Clinically significant 15.8% vs 11.1% HR 1.46 (1.36–1.57) P < 0.001	Non-CABG related major bleeding 2.7% vs 1.3% HR 1.85 (1.39–2.47) P < 0.001 Clinically significant 20.2% vs 14.6% HR 1.43 (1.31–1.57) P < 0.001	GUSTO Severe bleeding 1.7% vs 1.3% HR 1.25 (0.97–1.61) P = 0.09	Non-CABG related major bleeding 2.4% vs 1.8% HR 1.32 (1.03–1.68) P = 0.03	Non-CABG related major bleeding 2.8% vs 2.2% HR 1.25 (1.03–1.53) P = 0.03 Major bleeding 7.9% vs 7.7% HR 1.03 (0.93–1.15) P = 0.57	Non-CABG related bleeding 1.8% vs 0.6% HR 3.46 (2.08–5.77) P < 0.0001 TIMI bleeding requiring medical attention 12.9% vs 7.5% HR 1.79 (1.55–2.07) P <0.001
Fatal bleeding	0.3% vs 0.2% HR 1.46 (0.82–2.58) P = 0.19	0.4% vs 0.2% HR 1.89 (0.80–4.45) P = 0.15	0.3% vs 0.2% HR 1.53 (0.83–2.82) P = 0.17	0.4% vs 0.1% HR 4.19 (1.58–11.11) P = 0.002	0.3% vs 0.3% HR 0.87 (0.48–1.59) P = 0.66	0.1% vs 0.2% HR 0.67 (0.24–1.89) P = 0.45
Intracranial bleeding	1.0% vs 0.5% HR 1.94 (1.39–2.70) P < 0.001	1.1% vs 0.2% HR 3.39 (1.78–6.45) P < 0.001	0.3% vs 0.3% HR 0.96 (0.56–1.65) P = 0.89	0.3% vs 0.3% HR 1.12 (0.58–2.15) P = 0.74	0.3% vs 0.2% HR 1.87 (0.98–3.58) P = 0.06	0.4% vs 0.2% HR 2.83 (1.02–7.86) P = 0.04

HR = hazard ratio; CI = confidence interval; MI = myocardial infarction; PAD = peripheral artery disease; ACS = acute coronary syndromes; PCI = percutaneous coronary infusion; TIMI = Thrombolysis In Myocardial Infarction classification; GUSTO = Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries classification; CABG = coronary artery bypass graft.

[a] TRA 2°P-TIMI-50 (Thrombin-Receptor Antagonist in the Secondary Prevention of Atherothrombotic Events), Steering Committee, 2012. ClinicalTrials.gov number, NCT00526474.

[b] TRACER (Thrombin Receptor Antagonist Vorapaxar in Acute Coronary Syndromes) Steering Committee, 2012. ClinicalTrials.gov number, NCT00527943.

[c] CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidence) Steering Committee, 2006. ClinicalTrials.gov number, NCT00050817.

[d] TRITON TIMI-38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel) Steering Committee, 2007. ClinicalTrials.gov number, NCT00097591.

[e] PLATO (The Study of Platelet Inhibition and Patient Outcomes) Steering Committee, 2009. ClinicalTrials.gov number, NCT00391872.

[f] ATLAS-TIMI-51 (Anti-Xa Therapy to Lower Cardiovascular Events in Addition to Standard Therapy in Subjects with Acute Coronary Syndrome) Steering Committee, 2012. ClinicalTrials.gov number, NCT00809965. Data are presented as modified intention to treat mITT analysis with corresponding p values.

[g] PLATO's primary endpoint was defined as a composite of death from vascular causes, myocardial infarction or stroke. Death from vascular causes was defined as death from cardiovascular causes or cerebrovascular causes and any death with or without another known cause.

are more likely to provide safe and effective anti-thrombotic activity when used in combination with aspirin than with clopidogrel [52,88]. Whether or not vorapaxar is likely to be useful in patients classified as "clopidogrel non-responders" remains to be determined but is likely to be of value. Of note, such "clopidogrel non-responders" have been shown to exhibit increased platelet aggregation in response to PAR1 activation [89], further emphasising the potential for vorapaxar in this patient group.

In addition to the moderate increase in bleeding rates across the entire patient cohort, the phase 3 trials of vorapaxar revealed important and significant safety concerns for specific demographics (see Table 2). The FDA approval of vorapaxar came with explicit contraindications for patients with a history of stroke, TIA, intracranial haemorrhage, or active pathological bleeding. Vorapaxar use in geriatric patients or those with severe hepatic impairment was also not recommended due to the inherent increase in bleeding risk in these demographics. Safety and efficacy of vorapaxar in paediatrics or in pregnant or nursing women have not yet been established, but given the controversies surrounding anti-thrombotic management of these populations [90,91] further studies into the safety and efficacy of vorapaxar in these patient groups may be warranted.

Finally, despite a lack of overt off-target effects of vorapaxar throughout these clinical trials, the widespread expression pattern of PAR1 in the cardiovascular system (and beyond) suggests that the long-term effects of systemic PAR1 inhibition will require careful consideration.

2.3.3. How does vorapaxar compare with other antithrombotic agents?

Several large-scale clinical trials have been conducted to determine the optimal anti-thrombotic management of ACS patients, including studies on the anti-platelet drugs aspirin, clopidogrel [13,14], prasugrel [92], and ticagrelor [93], as well as anticoagulants such as the direct Xa inhibitor, rivaroxaban [94]. A comparison of these trial results is shown in Table 3. While clearly inappropriate to directly compare the safety and efficacy of these agents across distinct trials with their disparate clinical groups, it is of interest to observe that each drug shows benefit over standard of care therapy in the reduction of at least one clinical end point. With the recent addition of vorapaxar to the anti-thrombotic phamaco-armoury, an updated review of the trial data may be warranted.

2.3.4. What does the future hold for PAR inhibitors?

The use of vorapaxar beyond the well-defined settings and treatment regimens indicated by TRA 2°P-TIMI 50 awaits further investigation, and approval outside of the United States lies ahead. Given that vorapaxar is orally available, reversible, and sufficiently potent, there are no "second generation" PAR1 antagonists on the horizon as yet. For the time being, it is very much a case of "wait and see" for this first PAR1 inhibitor to be approved. What does the longer term future hold for platelet PAR inhibitors?

With the significant clinical effort behind the development of PAR1 inhibitors as anti-thrombotics, it is surprising that relatively little is known regarding the contribution of PAR4 to human platelet function during arterial thrombosis. Whether or not PAR4 represents a useful anti-platelet target is unknown, although some evidence suggests that PAR4 has distinct functions to PAR1 on human platelets. If true, PAR4 inhibitors may provide a useful adjunct and/or stand-alone therapy for the prevention of arterial thrombosis. For example, previous studies have shown that PAR1 and PAR4 differentially release distinct alpha-granule populations [95] and mediate diverse contributions to the procoagulant function of platelets [96]. Recent work has focussed on developing suitable tools to allow investigations of platelet PAR4 function, in particular PAR4-specific antagonists and mouse models in which the platelet PAR profile is 'humanised' to express PAR1 and PAR4 (rather than PAR3 and PAR4). To this end, several PAR4 antagonists have been developed, including pepducins [97], function-blocking antibodies [98] and the small molecule antagonist, YD-3 [99]. Initial proof-of-concept studies have shown that PAR4 inhibition provides anti-thrombotic activity, albeit in caveated mouse models [53,98,100] and *in vitro* studies [96,101]. Unfortunately, recent efforts to develop a 'humanised' platelet PAR mouse have been less productive, with an unsuccessful attempt to express PAR1 as a transgene in PAR3-deficient mice [102]. Further insights into platelet PAR biology utilizing these new tools will improve our understanding of the relative roles of platelet thrombin receptors in (patho)physiology, potentially providing additional rationalisation of these receptors as therapeutic targets.

3. Conclusion

The recent approval by the USA's Food and Drug Administration of the first PAR1 antagonist as a novel anti-platelet agent represents a potentially important breakthrough in the treatment of thrombotic cardiovascular events and marks the long path from receptor discovery to clinical drug use. While the journey from the initial discovery of this platelet thrombin receptor in the early 1990s to the approval for use of the first receptor antagonist in 2014 represents a significant achievement and advance in our knowledge of this important platelet activation mechanism, it is not unreasonable to think that significant further insights are yet to be gained.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

J.R.H. is a Future Fellow of the Australian Research Council and is supported by grants from the National Health and Medical Research Council of Australia and the CASS Foundation. The authors thank D.K. Lynch for important contributions.

References

[1] Alwan A. Global status report on noncommunicable diseases 2010. World Health Organization; 2011.
[2] Trialists' Collaboration A. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 2002;324(7329):71–86.
[3] Hamm CW, Bassand J-P, Agewall S, Bax J, Boersma E, Bueno H, et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. The Task Force for the Management of Acute Coronary Syndromes (ACS) in Patients Presenting Without Persistent ST-segment Elevation of the European Society of Cardiology (ESC). Eur Heart J 2011;32(23):2999–3054.
[4] Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate–receptor interactions in platelet thrombus formation under flow. Cell 1998;94(5):657–66.
[5] Vicente V, Kostel P, Ruggeri Z. Isolation and functional characterization of the von Willebrand factor-binding domain located between residues His1-Arg293 of the alpha-chain of glycoprotein Ib. J Biol Chem 1988;263(34):18473–9.
[6] White JG. Platelet structure. In: Michelson AD, editor. Platelets. Third ed. London: Academic Press; 2013. p. 117–45.
[7] Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists: what are the relevant issues concerning their pharmacology and clinical use? Circulation 1999;100(4):437–44.
[8] Kastrati A, Mehilli J, Neumann F-J, Dotzer F, ten Berg J, Bollwein H, et al. Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT 2 randomized trial. JAMA 2006;295(13):1531–8.
[9] Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting response variability, drug resistance, and the effect of pretreatment platelet reactivity. Circulation 2003;107(23):2908–13.
[10] Goodman T, Ferro A, Sharma P. Pharmacogenetics of aspirin resistance: a comprehensive systematic review. Br J Clin Pharmacol 2008;66(2):222–32.
[11] Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. J Am Coll Cardiol 2003;41(6):961–5.
[12] Järemo P, Lindahl T, Fransson SG, Richter A. Individual variations of platelet inhibition after loading doses of clopidogrel. J Intern Med 2002;252(3):233–8.
[13] Committee CS. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 1996;348(9038):1329–39.

[14] Bhatt DL, Fox KA, Hacke W, Berger PB, Black HR, Boden WE, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. N Engl J Med 2006;354(16):1706–17.
[15] Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000; 407(6801):258–64.
[16] Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, et al. A dual thrombin receptor system for platelet activation. Nature 1998;394(6694):690–4.
[17] Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991;64(6):1057–68.
[18] Xu W-f, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, et al. Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci 1998; 95(12):6642–6.
[19] Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, et al. Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 1997;386: 502–6.
[20] Howells GL, Macey MG, Chinni C, Hou L, Fox MT, Harriott P, et al. Proteinase-activated receptor-2: expression by human neutrophils. J Cell Sci 1997; 110(7):881–7.
[21] Bono F, Lamarche I, Herbert J. Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). Biochem Biophys Res Commun 1997;241(3):762–4.
[22] Molino M, Woolkalis MJ, Reavey-Cantwell J, Praticó D, Andrade-Gordon P, Barnathan ES, et al. Endothelial cell thrombin receptors and PAR-2 — two protease-activated receptors located in a single cellular environment. J Biol Chem 1997;272(17):11133–41.
[23] Hamilton JR, Chow JM, Cocks TM. Protease-activated receptor-2 turnover stimulated independently of receptor activation in porcine coronary endothelial cells. Br J Pharmacol 1999;127(3):617–22.
[24] Glembotski CC, Irons C, Krown K, Murray S, Sprenkle A, Sei C. Myocardial alpha-thrombin receptor activation induces hypertrophy and increases atrial natriuretic factor gene expression. J Biol Chem 1993;268(27):20646–52.
[25] Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, et al. Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. Circ Res 2000;86(10):1054–61.
[26] Sabri A, Short J, Guo J, Steinberg SF. Protease-activated receptor-1 mediated DNA synthesis in cardiac fibroblast is via epidermal growth factor receptor transactivation distinct PAR-1 signaling pathways in cardiac fibroblasts and cardiomyocytes. Circ Res 2002;91(6):532–9.
[27] Nystedt S, Emilsson K, Larsson AK, Strömbeck B, Sundelin J. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. Eur J Biochem 1995;232(1):84–9.
[28] Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, et al. Interactions of mast cell tryptase with thrombin receptors and PAR-2. J Biol Chem 1997; 272(7):4043–9.
[29] Camerer E, Huang W, Coughlin SR. Tissue factor-and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci 2000;97(10): 5255–60.
[30] Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J Biol Chem 2000;275(34):26333–42.
[31] Wilson S, Greer B, Hooper J, Zijlstra A, Walker B, Quigley J, et al. The membrane-anchored serine protease, TMPRSS2, activates PAR-2 in prostate cancer cells. Biochem J 2005;388:967–72.
[32] Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 1999;103(6):879–87.
[33] Sims P, Wiedmer T, Esmon CT, Weiss H, Shattil S. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J Biol Chem 1989;264(29):17049–57.
[34] Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. Biochemistry 2000;39(18):5458–67.
[35] Scarborough R, Naughton M, Teng W, Hung D, Rose J, Vu T, et al. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. J Biol Chem 1992; 267(19):13146–9.
[36] Hung D, Wong Y, Vu T-K, Coughlin S. The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. J Biol Chem 1992;267(29):20831–4.
[37] Offermanns S. Activation of platelet function through G protein-coupled receptors. Circ Res 2006;99(12):1293–304.
[38] Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. J Cell Biol 1999;144(4):745–54.
[39] Offermanns S, Toombs CF, Hu Y-H, Simon MI. Defective platelet activation in Gαq-deficient mice. Nature 1997;389(6647):183–6.
[40] Liu L-W, Vu T, Esmon C, Coughlin S. The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. J Biol Chem 1991; 266(26):16977–80.
[41] Nieman MT, Schmaier AH. Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. Biochemistry 2007;46(29):8603–10.
[42] Jacques SL, LeMasurier M, Sheridan PJ, Seeley SK, Kuliopulos A. Substrate-assisted catalysis of the PAR1 thrombin receptor — enhancement of macromolecular association and cleavage. J Biol Chem 2000;275(52):40671–8.
[43] Covic L, Singh C, Smith H, Kuliopulos A. Role of the PAR4 thrombin receptor in stabilizing platelet–platelet aggregates as revealed by a patient with Hermansky–Pudlak syndrome. Thromb Haemost 2002;87(4):722–7.
[44] Jacques S, Kuliopulos A. Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. Biochem J 2003; 376:733–40.
[45] Sambrano GR, Weiss EJ, Zheng Y-W, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 2001;413(6851):74–8.
[46] Hollenberg MD, Saifeddine M, Al-Ani B. Proteinase-activated receptor-2 in rat aorta: structural requirements for agonist activity of receptor-activating peptides. Mol Pharmacol 1996;49:229–33.
[47] Khan TA, Bianchi C, Voisine P, Sandmeyer J, Feng J, Sellke FW. Aprotinin inhibits protease-dependent platelet aggregation and thrombosis. Ann Thorac Surg 2005; 79(5):1545–50.
[48] Andrade-Gordon P, Maryanoff BE, Derian CK, Zhang HC, Addo MF, Darrow AL, et al. Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. Proc Natl Acad Sci U S A 1999;96(22):12257–62.
[49] Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. Blood 2002;100(9):3240–4.
[50] Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. Nature 2000;404(6778):609–13.
[51] Hamilton J, Cornelissen I, Coughlin S. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. J Thromb Haemost 2004;2(8):1429–35.
[52] Lee H, Sturgeon S, Mountford J, Jackson S, Hamilton J. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. Br J Pharmacol 2012;166(7):2188–97.
[53] Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. Proc Natl Acad Sci U S A 2007;104(1):288–92.
[54] Andrade-Gordon P, Derian CK, Maryanoff BE, Zhang HC, Addo MF, Cheung W, et al. Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J Pharmacol Exp Ther 2001;298(1):34–42.
[55] Hollenberg MD, Saifeddine M, Sandhu S, Houle S, Vergnolle N. Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists in vivo. Br J Pharmacol 2004; 143(4):443–54.
[56] Kuliopulos A, Covic L. Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. Life Sci 2003;74(2):255–62.
[57] Cook JJ, Sitko GR, Bednar B, Condra C, Mellott MJ, Feng D-M, et al. An antibody against the exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the African green monkey. Circulation 1995;91(12):2961–71.
[58] Derian CK, Damiano BP, Addo MF, Darrow AL, D'Andrea MR, Nedelman M, et al. Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in non-human primates. J Pharmacol Exp Ther 2003;304(2):855–61.
[59] Matsuoka T, Kogushi M, Kawata T, Kimura A, Chiba K-i, Musha T, et al. 1102-48 Inhibitory effect of E5555, an orally active thrombin receptor antagonist, on intimal hyperplasia following balloon injury. J Am Coll Cardiol 2004;43(5):A68.
[60] Kogushi M, Matsuoka T, Kawata T, Kuramochi H, Kawaguchi S, Murakami K, et al. The novel and orally active thrombin receptor antagonist E5555 (Atopaxar) inhibits arterial thrombosis without affecting bleeding time in guinea pigs. Eur J Pharmacol 2011;657(1):131–7.
[61] Kai Y, Hirano K, Maeda Y, Nishimura J, Sasaki T, Kanaide H. Prevention of the hypercontractile response to thrombin by proteinase-activated receptor-1 antagonist in subarachnoid hemorrhage. Stroke 2007;38(12):3259–65.
[62] Wiviott SD, Flather MD, O'Donoghue ML, Goto S, Fitzgerald DJ, Cura F, et al. Randomized trial of atopaxar in the treatment of patients with coronary artery disease: the Lessons From Antagonizing the Cellular Effect of Thrombin-Coronary Artery Disease Trial. Circulation 2011;123(17):1854–63.
[63] O'Donoghue ML, Bhatt DL, Wiviott SD, Goodman SG, Fitzgerald DJ, Angiolillo DJ, et al. Safety and tolerability of atopaxar in the treatment of patients with acute coronary syndromes: the Lessons From Antagonizing the Cellular Effects of Thrombin-Acute Coronary Syndromes Trial. Circulation 2011;123(17):1843–53.
[64] Goto S, Ogawa H, Takeuchi M, Flather MD, Bhatt DL. Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. Eur Heart J 2010;31(21):2601–13.
[65] Kosoglou T, Reyderman L, Tiessen RG, van Vliet AA, Fales RR, Keller R, et al. Pharmacodynamics and pharmacokinetics of the novel PAR-1 antagonist vorapaxar (formerly SCH 530348) in healthy subjects. Eur J Clin Pharmacol 2012;68(3):249–58.
[66] Becker RC, Moliterno DJ, Jennings LK, Pieper KS, Pei J, Niederman A, et al. Safety and tolerability of SCH 530348 in patients undergoing non-urgent percutaneous coronary intervention: a randomised, double-blind, placebo-controlled phase II study. Lancet 2009;373(9667):919–28.
[67] Ghosal A, Lu X, Penner N, Gao L, Ramanathan R, Chowdhury SK, et al. Identification of human liver cytochrome P450 enzymes involved in the metabolism of SCH 530348 (Vorapaxar), a potent oral thrombin protease-activated receptor 1 antagonist. Drug Metab Dispos 2011;39(1):30–8.
[68] Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. N Engl J Med 2012;366(15):1404–13.
[69] Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van de Werf F, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. N Engl J Med 2012;366(1):20–33.

[70] Leonardi S, Tricoci P, White HD, Armstrong PW, Huang Z, Wallentin L, et al. Effect of vorapaxar on myocardial infarction in the thrombin receptor antagonist for clinical event reduction in acute coronary syndrome (TRA·CER) trial. Eur Heart J 2013;34:1723–31.
[71] Capodanno D, Bhatt D, Goto S, O'Donoghue M, Moliterno D, Tamburino C, et al. Safety and efficacy of protease-activated receptor-1 antagonists in patients with coronary artery disease: a meta-analysis of randomized clinical trials. J Thromb Haemost 2012;10(10):2006–15.
[72] Whellan DJ, Tricoci P, Chen E, Huang Z, Leibowitz D, Vranckx P, et al. Vorapaxar in acute coronary syndrome patients undergoing coronary artery bypass graft surgery: subgroup analysis from the TRACER trial (Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome). J Am Coll Cardiol 2014;63(11):1048–57.
[73] Duerschmied D, Bode C. Vorapaxar expands antiplatelet options. Which patients may benefit from thrombin receptor antagonism? Hamostaseologie 2011;32(3):221–7.
[74] Scirica BM, Bonaca MP, Braunwald E, De Ferrari GM, Isaza D, Lewis BS, et al. Vorapaxar for secondary prevention of thrombotic events for patients with previous myocardial infarction: a prespecified subgroup analysis of the TRA 2 P-TIMI 50 trial. Lancet 2012;380(9850):1317–24.
[75] Bonaca MP, Scirica BM, Creager MA, Olin J, Bounameaux H, Dellborg M, et al. Vorapaxar in patients with peripheral artery disease results from TRA2° P-TIMI 50. Circulation 2013;127(14):1522–9.
[76] Morrow DA, Alberts MJ, Mohr JP, Ameriso SF, Bonaca MP, Goto S, et al. Efficacy and safety of vorapaxar in patients with prior ischemic stroke. Stroke 2013;44(3):691–8.
[77] Merlini PA, Bauer KA, Oltrona L, Ardissino D, Cattaneo M, Belli C, et al. Persistent activation of coagulation mechanism in unstable angina and myocardial infarction. Circulation 1994;90(1):61–8.
[78] Szczeklik A, Dropinski J, Radwan J, Krzanowski M. Persistent generation of thrombin after acute myocardial infarction. Arterioscler Thromb Vasc Biol 1992;12(5):548–53.
[79] Huisse M-G, Lanoy E, Tcheche D, Feldman LJ, Bezeaud A, Anglès-Cano E, et al. Prothrombotic markers and early spontaneous recanalization in ST-segment elevation myocardial infarction. Thromb Haemost 2007;98(2):420.
[80] Inauen W, Baumgartner HR, Bombeli T, Haeberli A, Straub PW. Dose- and shear rate-dependent effects of heparin on thrombogenesis induced by rabbit aorta subendothelium exposed to flowing human blood. Arteriosclerosis 1990;10(4):607–15.
[81] Kirchhofer D, Sakariassen KS, Clozel M, Tschopp TB, Hadvary P, Nemerson Y, et al. Relationship between tissue factor expression and deposition of fibrin, platelets, and leukocytes on cultured endothelial cells under venous blood flow conditions. Blood 1993;81(8):2050–8.
[82] Fressinaud E, Sakariassen KS, Rothschild C, Baumgartner HR, Meyer D. Shear rate-dependent impairment of thrombus growth on collagen in nonanticoagulated blood from patients with von Willebrand disease and hemophilia A. Blood 1992;80(4):988–94.
[83] Lee H, Sturgeon SA, Jackson SP, Hamilton JR. The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. Thromb Haemost 2012;107(2):328.
[84] Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, et al. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. PLoS One 2011;5(4):e10415.
[85] Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. J Am Coll Cardiol 2007;50(19):1822–34.
[86] Gurbel PA, Bliden KP, Guyer K, Cho PW, Zaman KA, Kreutz RP, et al. Platelet reactivity in patients and recurrent events post-stenting: results of the PREPARE POST-STENTING study. J Am Coll Cardiol 2005;46(10):1820–6.
[87] Mahaffey KW, Huang Z, Wallentin L, Storey RF, Jennings LK, Tricoci P, et al. Association of aspirin dose and vorapaxar safety and efficacy in patients with non-ST-segment elevation acute coronary syndrome (from the TRACER Trial). Am J Cardiol 2014;113(6):936–44.
[88] Cornelissen I, Palmer D, David T, Wilsbacher L, Concengco C, Conley P, et al. Roles and interactions among protease-activated receptors and P2ry12 in hemostasis and thrombosis. Proc Natl Acad Sci 2010;107(43):18605–10.
[89] Kreutz RP, Breall JA, Kreutz Y, Owens J, Lu D, Bolad I, et al. Protease activated receptor-1 (PAR-1) mediated platelet aggregation is dependant on clopidogrel response. Thromb Res 2012;130(2):198–202.
[90] Askie LM, Duley L, Henderson-Smart DJ, Stewart LA. Antiplatelet agents for prevention of pre-eclampsia: a meta-analysis of individual patient data. Lancet 2007;369(9575):1791–8.
[91] Bates SM, Greer IA, Hirsh J, Ginsberg JS. Use of antithrombotic agents during pregnancy: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004;126(Suppl. 3):627S–44S.
[92] Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007;357(20):2001–15.
[93] Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2009;361(11):1045–57.
[94] Mega JL, Braunwald E, Wiviott SD, Bassand J-P, Bhatt DL, Bode C, et al. Rivaroxaban in patients with a recent acute coronary syndrome. N Engl J Med 2012;366(1):9–19.
[95] Italiano Jr JE, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 2008;111(3):1227–33.
[96] Duvernay M, Young S, Gailani D, Schoenecker J, Hamm HE. Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets. Mol Pharmacol 2013;83(4):781–92.
[97] Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. Nat Med 2002;8:1161–5.
[98] Mumaw M, la Fuente M, Noble D, Nieman M. Targeting the anionic region of human protease activated receptor 4 (PAR4) inhibits platelet aggregation and thrombosis without interfering with hemostasis. J Thromb Haemost 2014;12:1–11.
[99] Wu CC, Huang SW, Hwang TL, Kuo SC, Lee FY, Teng CM. YD-3, a novel inhibitor of protease-induced platelet activation. Br J Pharmacol 2000;130(6):1289–96.
[100] Lee H, Hamilton JR. Physiology, pharmacology, and therapeutic potential of protease-activated receptors in vascular disease. Pharmacol Ther 2012;134(2):246–59.
[101] Judge HM, Jennings LK, Moliterno DJ, Hord E, Ecob R, Tricoci P, et al. PAR1 antagonists inhibit thrombin-induced platelet activation whilst leaving the PAR4-mediated response intact. Platelets 2014;0:1–7.
[102] Arachiche A, de la Fuente M, Nieman MT. Platelet specific promoters are insufficient to express protease activated receptor 1 (PAR1) transgene in mouse platelets. PLoS One 2014;9(5):e97724.

Appendix II

British Journal of Pharmacology

British Journal of Pharmacology (2016) **173** 2952–2965 2952

Themed Section: Molecular Pharmacology of G Protein-Coupled Receptors

REVIEW ARTICLE

Protease-activated receptor 4: from structure to function and back again

Correspondence Justin R. Hamilton, Australian Centre for Blood Diseases, Monash University, Level 2 AMREP Building, The Alfred, Commercial Road, Melbourne, VIC 3004, Australia. E-mail: justin.hamilton@monash.edu

Received 2 August 2015; **Revised** 22 January 2016; **Accepted** 29 January 2016

Shauna L French and Justin R Hamilton

Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

Protease-activated receptors are a family of four GPCRs (PAR1–PAR4) with a number of unique attributes. Nearly two and a half decades after the discovery of the first PAR, an antagonist targeting this receptor has been approved for human use. The first-in-class PAR1 antagonist, vorapaxar, was approved for use in the USA in 2014 for the prevention of thrombotic cardiovascular events in patients with a history of myocardial infarction or with peripheral arterial disease. These recent developments indicate the clinical potential of manipulating PAR function. While much work has been aimed at uncovering the function of PAR1 and, to a lesser extent, PAR2, comparatively little is known regarding the pharmacology and physiology of PAR3 and PAR4. Recent studies have begun to develop the pharmacological and genetic tools required to study PAR4 function in detail, and there is now emerging evidence for the function of PAR4 in disease settings. In this review, we detail the discovery, structure, pharmacology, physiological significance and therapeutic potential of PAR4.

LINKED ARTICLES

This article is part of a themed section on Molecular Pharmacology of G Protein-Coupled Receptors. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v173.20/issuetoc

Abbreviations

DRG, dorsal root ganglion; IP_3, inositol trisphosphate; MLC, myosin light chain; PAR, proteinase-activated receptor; TBD, thrombin binding domain

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
α_{2A}-Adrenoceptor	Calpain
B_2 receptor	Cathepsin G
$P2Y_{12}$ receptor	MAPK
PAR1	PKC
PAR2	PLA_2
PAR3	PLCβ
PAR4	Rho-kinase
Ligand-gated ion channels[b]	
P2X1 receptors	

LIGANDS
ADP
Bradykinin
Thrombin
Trypsin
TXA2, thromboxane A2
Vorapaxar

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c).

DOI:10.1111/bph.13455

Introduction

Protease-activated receptors (PARs) have been studied in some detail since the discovery of the prototype receptor, PAR1, in 1991. Humans express four PARs, designated PAR1 to PAR4 (please refer to the Concise Guide to Pharmacology; Alexander *et al.*, 2015a,b,c), that are broadly expressed and have diverse functions across multiple physiological systems. PARs form a small GPCR family characterized by their unique self-activation mechanism following cleavage by specific serine proteases – typically key effector proteases of coagulation and inflammatory pathways. As a result, extensive research has been conducted into the roles of PARs in thrombosis and other cardiovascular diseases, as well as a range of inflammatory conditions. To date, the research spotlight has been firmly focussed on PAR1 and PAR2, with PAR3 and PAR4 largely waiting in the wings.

PAR1 was discovered while attempting to elucidate the mechanism by which thrombin activates platelets and other cells (Vu *et al.*, 1991). PAR1 is a high-affinity thrombin receptor (Vu *et al.*, 1991). The other receptors of this family were subsequently cloned: PAR3 and PAR4 are also activated by thrombin (Ishihara *et al.*, 1997; Xu *et al.*, 1998; Kahn *et al.*, 1998b), with PAR2 thrombin-insensitive and primarily activated by trypsin and trypsin-like proteases (Nystedt *et al.*, 1995). The structure and function of PAR1 and PAR2 are well characterized, and have aided the clinical development of inhibitors of these receptors. The first PAR1 antagonist, vorapaxar, (trade name Zontivity), was approved in late 2014 by the FDA for use in the USA as an anti-platelet agent in patients with cardiovascular disease (Tricoci *et al.*, 2012; Morrow *et al.*, 2012; French *et al.*, 2015). In addition, PAR2 antagonists have undergone pre-clinical studies for the treatment of inflammation in a variety of settings, including arthritis (Kelso *et al.*, 2006; Lohman *et al.*, 2012). Despite these significant advances, there has been comparatively little progress in understanding the functions of PAR4 in health and disease and in targeting this receptor for therapeutic gain. Recent setbacks in the clinical development of PAR1 and PAR2 inhibitors, coupled with an emerging body of evidence suggesting potential utility of targeting PAR4 in disease settings, has re-ignited interest in the physiology and pharmacology of PAR4. This review outlines our current understanding of the physiology, pharmacology, clinical significance and therapeutic potential of PAR4, provided by the recent scientific advances in this area.

Receptor structure

PAR4 was first cloned in 1998 (Xu *et al.*, 1998; Kahn *et al.*, 1998b) and is the most recently cloned member of the PAR family. It is a 300 amino acid seven transmembrane-spanning domain GPCR (Figure 1) that retains most of the core features of the other PARs despite its genetic differences from the other members of the family (Xu *et al.*, 1998; Kahn *et al.*, 1998b). The human PAR4 gene is remarkably smaller than those of the other PARs, and it resides at a distinct location on chromosome 19p12, with the genes for PARs 1–3 located in tandem on chromosome 5q13 (Xu *et al.*, 1998; Kahn *et al.*, 1998a). This genetic divergence of PAR4 from the other PARs is thought to have arisen from a remote gene duplication and subsequent translocation event which gave rise to ancestral PAR4 and PAR1/PAR2/PAR3 genes (Kahn *et al.*, 1998a). More recently, two gene duplication events have occurred to separate PAR1 and PAR2 from PAR3; and then PAR1 from PAR2 (Kahn *et al.*, 1998a). These genetic differences may underlie some of the key differences in receptor structure and function within the PAR family.

The N-terminus of PAR4 contains a hydrophobic signal peptide sequence, with a signal peptidase cleavage site present at Ser^{17}/Gly^{18}. The extracellular amino-terminus also contains a serine protease cleavage site at Arg^{47}/Gly^{48} that is essential for receptor activation. Mutation of the serine protease cleavage site (Arg^{47} ➔ Ala) renders the receptor completely unresponsive to proteolytic activation. Site-specific receptor cleavage unmasks a cryptic tethered ligand sequence (GYPGQV) (Xu *et al.*, 1998), which then binds intramolecularly to a defined region in the second extracellular loop of the receptor (Figure 1), resulting in conformational change of the receptor and subsequent coupling to intracellular effectors, in common with other GPCR family members. While this proteolytic activation mechanism is common amongst PARs, there are several notable differences in the structure of PAR4 and the other receptors of the family. First, both the extracellular amino-terminus and intracellular carboxy terminus have little sequence similarity to the corresponding regions of other PARs (Xu *et al.*, 1998). Second, the tethered ligand binding site of PAR4 contains only three core amino acids (CHD; Figure 1) of the consensus sequence conserved in PARs 1–3 (ITTCHDV) (Xu *et al.*, 1998). PAR4 also lacks the high-affinity thrombin binding domain that is present in the other two thrombin receptors, PAR1 and PAR3 (Vu *et al.*, 1991; Mathews *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998b).

Receptor activation and membrane trafficking

The serine proteases capable of cleaving PAR4 are generally key regulators of coagulation (e.g. thrombin and coagulation factor Xa) and/or inflammation (e.g. trypsin and cathepsin G released from neutrophils, and bacterial proteases such as gingipains) (Xu *et al.*, 1998; Kahn *et al.*, 1998b; Sambrano *et al.*, 2001; Cottrell *et al.*, 2004). Additional serine proteases that are specific for arginine/lysine cleavage include coagulation factors VIIa, IXa, Xa, XIa, urokinase and plasmin; however, none of these have significant activity on PAR4, with the exception of factor Xa, which showed small effects at non-physiological concentrations (Xu *et al.*, 1998). Initial studies indicated that PAR4 is activated by thrombin and trypsin at similar concentrations, with an EC_{50} of ~5 nM each (Xu *et al.*, 1998) – significantly higher than the EC_{50} for thrombin at either PAR1 or PAR3 (~0.2 nM). The comparatively lower affinity of PAR4 for thrombin is a reflection of differences in the macromolecular association between the receptor and enzyme. PAR1 is a high-affinity thrombin receptor due to the presence of a hirudin-like thrombin binding domain (TBD) in its N-terminal exodomain (Mathews *et al.*, 1994). The TBD sequence, $K^{51}YEPF^{55}$, interacts with exosite I of thrombin and upon binding causes significant allosteric

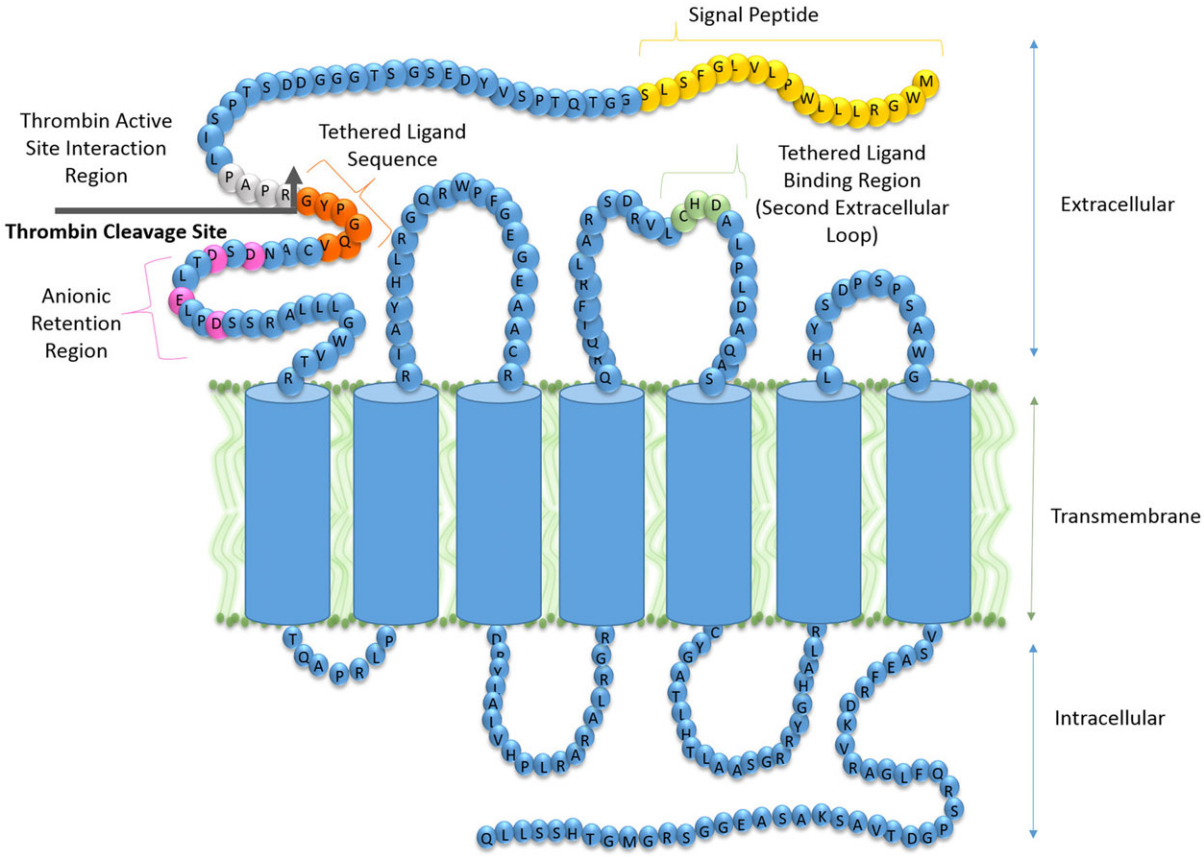


Figure 1

Protease-activated receptor 4: structure. The proposed structure of the 300 amino acid GPCR, human PAR4. Site-specific proteolytic cleavage of the receptor amino-terminus (thrombin cleavage site, Arg^{47}/Gly^{48}; black arrow) reveals a neo-amino-terminus (tethered ligand sequence; G^{48}YPGQV; orange) which binds intramolecularly to the second extracellular loop of the receptor (tethered ligand binding region; green). PAR4 contains two regions for enhanced thrombin interaction – the anionic retention region (pink) which interacts with thrombin's exosite; and the PAPR sequence (grey) which binds at the active site of thrombin with high affinity.

effects essential for rapid association of thrombin (Jacques *et al.*, 2000). PAR4 does not contain this TBD (Xu *et al.*, 1998). In fact, the evidence indicates that PAR4 has only limited interaction with exosite I of thrombin, as γ-thrombin (which lacks a functional exosite I) activates PAR4 as effectively as α-thrombin (Xu *et al.*, 1998), and mutations in the exosite I of thrombin have significantly less effect on cleavage of PAR4 than of PAR1 (Ayala *et al.*, 2001). Instead, PAR4 primarily interacts with the active site of thrombin via two optimally positioned proline residues (P^{44} and P^{46}) in the receptor (Jacques and Kuliopulos, 2003) just upstream of the thrombin cleavage site at Arg^{47}/Gly^{48}. A possible role for Leu^{43} in the thrombin–receptor interaction has also been identified and believed to be in facilitating high-affinity binding of the PAR4 amino-terminal to thrombin's active site (Jacques and Kuliopulos, 2003). PAR4 also contains an anionic cluster, Asp^{57}…Asp^{59}…Glu^{62}…Asp^{65}, just C-terminal of the thrombin cleavage site that is thought to interact with cationic residues that border exosite I of thrombin (Ayala *et al.*, 2001) and slow the dissociation rate of thrombin from PAR4 (Jacques and Kuliopulos, 2003). This is reflected in more sustained intracellular signals elicited by PAR4 activation compared with the more transient signals in response to PAR1 activation (Covic *et al.*, 2002c). These differences in receptor structure and activation mechanisms between PAR1 and PAR4 indicate that these two receptors have the potential to initiate distinct intracellular signalling kinetics and/or events.

Receptor trafficking mechanisms are another key regulator of receptor signalling. This is of particular importance for PARs due to the irreversible, cleavage-based, endogenous activation of these receptors, with continued responsiveness to agonists requiring new receptors to be trafficked to the cell surface. While much is known regarding the receptor trafficking and response regulation of PAR1 and PAR2 (for a detailed review, see Soh *et al.*, 2010), little is known about such mechanisms for PAR4. Defining these pathways is of clear interest. On this point, one apparently important distinction between PAR4 and PARs 1 and 2 relates to receptor desensitization. After receptor cleavage and activation, PARs are rapidly internalized on a phosphorylation signal in regions of the C-terminus of the receptor (Shapiro *et al.*, 1996). However, PAR4 has a shorter C-terminus than either of PAR1 or PAR2 and does not have many of the phosphorylation sites shown to be necessary for desensitization of these receptors (Shapiro

et al., 2000). As a result, agonist-triggered phosphorylation and consequent receptor internalization is significantly slower for PAR4 than for PAR1 or PAR2 (Shapiro *et al.*, 2000), providing a further mechanism for the comparatively prolonged intracellular signalling downstream of PAR4 activation.

Following internalization, PARs are sorted for either degradation (lysosomes) or recycling (endosomes). While activated receptors are predominately packaged into lysosomes for complete signal termination, unactivated receptors can cycle tonically between endosomes and the cell surface to ensure ongoing sensitivity to agonists in the absence of *de novo* receptor synthesis (Hoxie *et al.*, 1993; Shapiro *et al.*, 1996). An additional membrane trafficking pathway for PAR4 is provided by efficient transport from the endoplasmic reticulum (ER), via the Golgi for post-translational modifications such as glycosylation. PAR4 was recently shown to contain an arginine-based ER retention motif within the second intracellular loop that may regulate cell surface expression of the receptor (Cunningham *et al.*, 2012). Intriguingly, co-expression of PAR2 appeared to interfere with this ER retention signal and enhance PAR4 expression on the surface of a human keratinocyte cell line (Cunningham *et al.*, 2012), providing a potential mechanism for cell type and/or context-dependent discrepancies in the regulation of PAR4 surface expression.

Intracellular signalling

Cleavage and activation of PARs prompts a conformational change in the receptor that allows G protein coupling and initiates multiple intracellular signalling events. G_q, G_i and $G_{12/13}$ have all been demonstrated to mediate signals in response to activation of PARs, with PAR4 specifically being shown to couple to both G_q and $G_{12/13}$ family proteins (Coughlin, 2000; Faruqi *et al.*, 2000), but not G_i/G_o (Voss *et al.*, 2007) (Figure 2).

PAR4 coupling to $G_{12/13}$ initiates binding of RhoGEFs (guanine-nucleotide exchange factors that activate Rho) to the α-subunit. Consequent Rho activation induces a series of Rho-dependent cytoskeletal responses and PLC activation (Figure 2). For example, in platelets, activation of Rho-kinase-dependent cytoskeletal responses via $G_{12/13}$-mediated PAR4 signalling triggers platelet shape change (Offermanns *et al.*, 1994; Coughlin, 2000; Dorsam *et al.*, 2002) – one of the key initial events in platelet aggregation during thrombosis. In endothelial cells, $G_{12/13}$-mediated PAR4 signalling via Rho is manifested in cytoskeletal responses that drive increases in vascular and colonic paracellular permeability (Dabek *et al.*, 2009; Dabek *et al.*, 2011). Evidence also exists for PAR4 activation eliciting a prolonged signal via $G_{12/13}$, which is thought to involve the regulation of MLC phosphorylation and RhoA (Dabek *et al.*, 2011; Duvernay *et al.*, 2013).

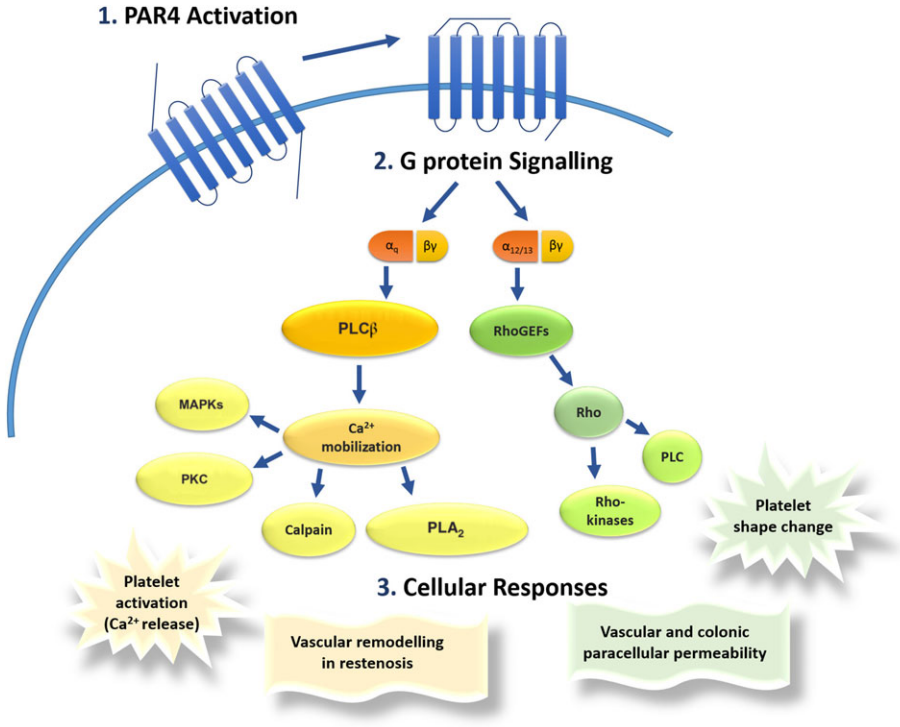


Figure 2

Protease-activated receptor 4: signalling. Following cleavage and activation of the receptor, PAR4 signals via coupling to Gq and/or G12/G13 family members. The best characterized signalling downstream of G_q is via PLCβ-mediated phosphoinositide hydrolysis and resultant intracellular calcium mobilization. This promotes the activity of several calcium-regulated kinases and phosphatases (MAPKs, PKCs, PLA_2 and calpain) which underlie PAR4-induced cellular responses such as platelet activation and vascular remodelling. The best characterized signalling downstream of PAR4- $G_{12/13}$ coupling is via RhoGEF and consequent Rho activation, which underlie PAR4-induced cellular responses such as cytoskeletal responses in platelets and vascular smooth muscle and endothelial cells.

PAR4 coupling to G_q causes intracellular calcium mobilization via activation of PLCβ and consequent phosphoinositide hydrolysis (Figure 2). PAR4-induced calcium mobilization promotes the activity of several calcium-regulated kinases and phosphatases (e.g. MAPKs, PKCs, PLA_2 and calpain) (Coughlin, 2000), with wide-ranging effects. For example, PAR4 activation mediates several key platelet responses that are induced by G_q -dependent calcium mobilization, including the secretion of platelet storage granules and integrin activation – both of which are critical for effective platelet aggregation (Coughlin, 2000; Coughlin, 2005).

As indicated previously, one key difference with PAR4 is in the kinetics of receptor activation and signalling. Regarding G_q-mediated signalling, it is known that PAR1 activation stimulates a rapid burst of intracellular calcium mobilization, whereas PAR4 activation elicits a slower rise, which is much more sustained over time (Covic *et al.*, 2002c). This is likely to be a result of the slower cleavage of PAR4 allowing prolonged G protein signalling. Given that mice deficient in G_q lack thrombin-dependent IP_3 and calcium responses (Gabbeta *et al.*, 1997; Offermanns *et al.*, 1997) and that PAR4 activation stimulates ongoing IP_3 and DAG, differences in calcium mobilization are likely to reflect a result of prolonged G_q signalling through PAR4 (Lau *et al.*, 1994; Covic *et al.*, 2002c). The potential outcome of this difference in signalling kinetics is discussed in more detail below, but may be important for ongoing cell signalling under conditions of prolonged agonist exposure. For example, in platelets, PAR1-mediated calcium signalling undergoes rapid desensitization but can be rescued by subsequent PAR4 activation (Falker *et al.*, 2011). In endothelial and smooth muscle cells, long-term G_q-dependent calcium signalling via PAR4 drives transcriptional and metabolic responses that may be important in vascular remodelling and proliferative processes in restenosis in response to high glucose levels (Pavic *et al.*, 2014).

Dimerization and crosstalk with other receptors

PAR4 has been shown to form both heterodimers and homodimers (Leger *et al.*, 2006; Arachiche *et al.*, 2013) (Figure 3). Specifically, PAR4 forms stable heterodimers with PAR1 and preventing this association impairs signalling downstream of either receptor (Leger *et al.*, 2006), suggesting reciprocal assistance in the cleavage and activation of PAR1 and PAR4. For example, pharmacological disruption of the PAR1–PAR4 heterodimer effectively prevents carotid artery occlusion in an *in vivo* murine thrombosis model (Leger *et al.*,

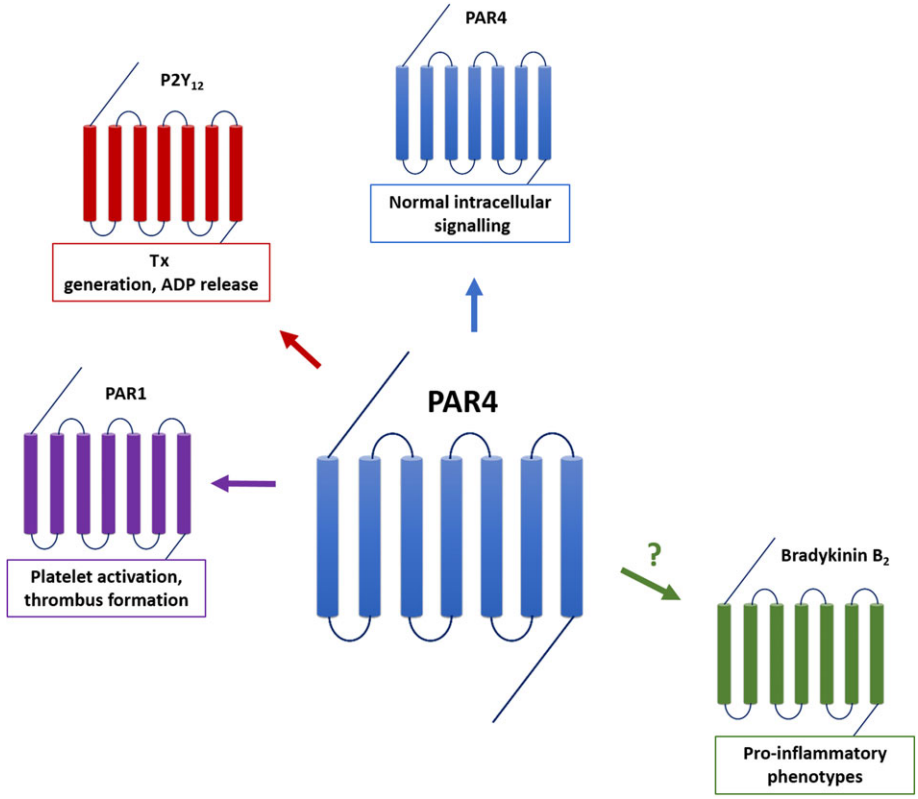


Figure 3

Protease-activated receptor 4: dimerization partners. There is direct evidence in transfected cell systems for PAR4 homodimers, as well as heterodimers with PAR1 and the $P2Y_{12}$ purinergic receptor. Each of these interactions has been shown to be important for normal intracellular signalling and have been implicated in platelet aggregation and thrombus formation. In addition, crosstalk between PAR4 and the B_2 bradykinin receptor may mediate pro-inflammatory effects, either directly or indirectly via transactivation.

2006). In addition, although direct evidence for PAR3–PAR4 heterodimers has not been reported, PAR3, at least in mouse systems, serves as a cofactor for efficient PAR4 activation (Kahn *et al.*, 1998b; Nakanishi-Matsui *et al.*, 2000), suggesting a significant level of interaction between these two PARs. PAR4 has also recently been shown to form homodimers (De La Fuente *et al.*, 2012), with the interacting region mapped to transmembrane helix 4 of the receptor. PAR4 homodimers also appear vital for normal signalling: mutation of a series of hydrophobic residues in transmembrane domain 4 reduced PAR4-mediated calcium mobilization in transfected HEK293 cells (De La Fuente *et al.*, 2012).

Other GPCRs reported to form heterodimers with PAR4 include the $P2Y_{12}$ purinergic receptor (Khan *et al.*, 2014), and functional co-operativity – potentially via receptor dimerization – has been reported with the α_{2A}-adrenoceptor (Grenegard *et al.*, 2008) and B_2 bradykinin receptor (Houle *et al.*, 2005; Mcdougall *et al.*, 2009) (Figure 3). In human platelets, PAR4 (along with PAR1) is known to work in synergy with the $P2Y_{12}$ receptor to contribute to platelet activation and aggregation events, including thromboxane (TXA_2) generation and ADP release (Holinstat *et al.*, 2006). It has been recently shown that this process is largely mediated by PAR4 and that PAR4 directly interacts with $P2Y_{12}$ receptors (Wu *et al.*, 2010; Khan *et al.*, 2014). PAR4 also interacts with the α_{2A}-adrenoceptor (Grenegard *et al.*, 2008). This interaction has been shown to cause aggregation of aspirin-treated human platelets, via cooperative signalling which eventually triggers the P2X1 ATP-gated calcium ion channel to overcome the therapeutic effects of aspirin. It has also been suggested that PAR4 communicates with the bradykinin B_2 receptor, responsible for several pro-inflammatory effects. Although the mechanisms and outcomes of this crosstalk are yet to be fully elucidated, it has been shown in models of rat paw oedema and knee joint inflammation that inflammation induced by a PAR4 activating peptide (PAR4-AP) can be inhibited by a B_2 receptor antagonist (Houle *et al.*, 2005; Mcdougall *et al.*, 2009). These various examples of receptor dimerization and/or crosstalk are indicative of the complexity of PAR4 signalling and how these events are able to affect multiple pathways to alter physiological responses.

Physiology

Tissue distribution and expression

PAR4 is broadly expressed, with gene expression by Northern blot most readily detected in the lungs, pancreas, thyroid, testis and small intestine (Xu *et al.*, 1998; Kahn *et al.*, 1998b). Moderate expression has also been detected in placenta, skeletal muscle, lymph nodes, adrenal gland, prostate, uterus and colon (Xu *et al.*, 1998; Kahn *et al.*, 1998b). In the nervous system, PAR4 protein and mRNA have been detected in rat dorsal root ganglion (DRG) non-neuronal cells (conversely, PARs 1–3 were detected in DRG neurons) (Zhu *et al.*, 2005). Despite this extensive expression profile of PAR4, most research has focused on the physiological functions of this receptor in cardiovascular and inflammatory settings. In humans, the key vascular cell types expressing PAR4 include platelets (Xu *et al.*, 1998; Kahn *et al.*, 1998b), leukocytes (Vergnolle *et al.*, 2002), endothelial cells (Kataoka *et al.*, 2003) and smooth muscle cells (Hollenberg *et al.*, 1999; Hamilton *et al.*, 2001; Vidwan *et al.*, 2010). In addition to the well-known pro-thrombotic and pro-inflammatory actions of PAR4-activating proteases, perhaps one reason for this is that several important animal models, including mice, rats, guinea pigs, rabbits, dogs and monkeys have similar PAR4 expression to humans throughout the vasculature. Platelets are one example of such conserved PAR4 expression – even in the face of variable expression of the other PARs: human platelets express PAR1 and PAR4 (Kahn *et al.*, 1998b); platelets from mice, rats and rabbits express PAR3 and PAR4 (Connolly *et al.*, 1994; Connolly *et al.*, 1996; Nakanishi-Matsui *et al.*, 2000; Khan *et al.*, 2005), while guinea pig platelets express PAR1, PAR3 and PAR4 (Andrade-Gordon *et al.*, 2001). Non-human primates are the only known animal with an identical platelet-PAR expression profile to that of humans (Derian *et al.*, 2003).Not surprisingly, this has been a significant restraint in the pursuit of PAR antagonists as anti-platelet agents. Despite this relatively conserved expression of PAR4 in vascular cells across species, there is some evidence to suggest that PAR4 may function differently between species. Specifically, PAR4 contributes to thrombin responses of mouse endothelial cells (Kataoka *et al.*, 2003) that PAR4 activation causes endothelium-dependent relaxation of rat aorta (Hollenberg *et al.*, 1999). However, in human artery preparations as well as lung fibroblasts, these responses are only elicited when treated with additional inflammatory mediators (Hamilton *et al.*, 2001; Ramachandran *et al.*, 2007). The consequences of this distinct regulation of PAR4 expression by inflammatory cytokines remain unknown but are likely to be important for future studies investigating the roles of PAR4 in inflammation.

Key physiological functions

PAR4 knockout (PAR4−/−) mice have been used to elucidate physiological functions dependent on PAR4 expression (Table 1). Sambrano *et al.* (2001) generated the first PAR4−/− mice, in which β-galactosidase was expressed in place of PAR4. Loss of PAR4 function was confirmed by platelet aggregation studies where it was shown that PAR4−/− platelets were completely unresponsive to both a PAR4-AP (AYPGKF) and thrombin (Sambrano *et al.*, 2001). PAR4−/− mice are normal in appearance, size and fertility, with no platelet, leukocyte or erythrocyte abnormalities (Sambrano *et al.*, 2001). Indeed, no spontaneous phenotype has been reported in PAR4−/− mice to date.

Protection against thrombosis. The first phenotype reported in PAR4−/− mice was protection against thrombosis, with mild associated bleeding (Sambrano *et al.*, 2001). Specifically, it was shown that platelets from PAR4−/− mice were completely unresponsive to thrombin, thus creating a clean genetic model in which to test for the overall importance of thrombin-mediated platelet activation for thrombosis *in vivo* (Sambrano *et al.*, 2001). Initially, ferric chloride-induced injury of mouse mesenteric arterioles was utilized. Here, the time to vessel occlusion after injury was prolonged about three-fold in PAR4−/− mice (Sambrano *et al.*, 2001). Subsequent studies have shown a similar protection against thromboplastin-

Table 1

Reported phenotypes in PAR4-deficient (PAR4−/−) mice

	Phenotype	References
Thrombosis and haemostasis	PAR4−/− mice are protected against arterial/arteriolar thrombosis.	Sambrano *et al.*, 2001; Weiss *et al.*, 2002; Lee *et al.*, 2012a, 2012b
	PAR4−/− mice are protected against pulmonary embolism.	Hamilton *et al.*, 2004
	PAR4−/− mice display increased tail bleeding times.	Sambrano *et al.*, 2001; Weiss *et al.*, 2002; Hamilton *et al.*, 2004; Lee *et al.*, 2012b
	PAR4−/− mice are protected from cerebral infarct after cerebral artery occlusion.	Mao *et al.*, 2010
Inflammation and vascular protection	PAR4−/− mice are protected from tissue oedema.	Busso *et al.*, 2008
	PAR4−/− mice have diminished vascular remodelling in the setting of diabetes.	Pavic *et al.*, 2014
Hepatotoxicity	PAR4−/− mice are protected against acetaminophen-induced liver injury.	Miyakawa *et al.*, 2015

induced pulmonary embolism (Weiss *et al.*, 2002), laser-induced endothelial cell ablation in mesenteric arterioles (Vandendries *et al.*, 2007) and electrolytic-induced (Lee *et al.*, 2012a) and trauma-induced (Lee *et al.*, 2012b) injury of the carotid artery in PAR4−/− mice. Bone marrow transplantation studies confirmed that the anti-thrombotic effects observed in PAR4−/− mice were due to deficiency of PAR4 in platelets (Hamilton *et al.*, 2004). Intriguingly, it appears that the protection against thrombosis associated with PAR4 deficiency leaves other haemostatic responses intact (Vandendries *et al.*, 2007). PAR4 deficiency in mice is also associated with a mild bleeding phenotype. This haemostatic effect has been most commonly assessed via tail bleeding time, where PAR4−/− mice are consistently shown to have prolonged tail bleeding times compared with wild-type mice (Sambrano *et al.*, 2001; Weiss *et al.*, 2002). Despite this commonly reported effect upon active limb trauma, there is no evidence for spontaneous bleeding in PAR4−/− mice. PAR4 deficiency also has protective benefits in a mouse model of stroke (transient middle cerebral artery occlusion, in which PAR4−/− mice exhibited lower cerebral infarct volume, improved neurological and motor function and reduced blood brain barrier disruption and cerebral oedema, compared with wild-type animals (Mao *et al.*, 2010). Additional models of atherosclerotic plaque formation in ApoE−/− have indicated that PAR4 probably does not play a protective role in this setting (Hamilton *et al.*, 2009). However, diabetic mice also deficient in PAR4 exhibit protective vascular remodelling phenotypes (Pavic *et al.*, 2014), indicating that PAR4 may be functionally important in certain diabetic vasculopathies.

Inflammation. PAR4 has also been linked to inflammation and neuropathic pain pathways. This is perhaps not surprising given that serine proteases capable of activating PAR4 (i.e. thrombin from coagulation activation, cathepsin G released from neutrophils and trypsin released from mast cells) are well known to drive many pro-inflammatory effects. Most studies investigating the role of PAR4 in inflammatory settings have examined gain of function phenotypes, most commonly in response to exogenous PAR4 agonists. One of the earliest studies implicating PAR4 in pro-inflammatory reactions found that PAR4 activation mimics the effects of thrombin on leukocyte rolling and adhesion and increases leukocyte migration (Vergnolle *et al.*, 2002). Specifically, superfusion of rat mesenteric venules with either thrombin or a PAR4-AP (but not a PAR1-AP), significantly increased the number of rolling leukocytes, as well as leukocyte recruitment to the peritoneal cavity, suggesting that thrombin-induced effects on leukocytes during inflammation may be mediated largely by PAR4 (Vergnolle *et al.*, 2002). In subsequent studies, PAR4 activation mediated the formation of oedema in rat paws (Hollenberg *et al.*, 2004; Houle *et al.*, 2005; Mcdougall *et al.*, 2009) via neutrophils and the kallikrein–kinin system (Houle *et al.*, 2005). In support of these observations, PAR4−/− mice develop significantly reduced paw swelling in response to tissue-factor-initiated inflammation (Busso *et al.*, 2008).

Nociception. PAR4 expression is up-regulated in DRG neurons during nociceptor activation and neurogenic pain (Dattilio *et al.*, 2005; Chen *et al.*, 2013) and in sensory neurons of the colon (Auge *et al.*, 2009), suggesting a potential role for PAR4 in nociception associated with inflammatory pathologies of the viscera, such as inflammatory bowel disease. In addition, PAR4 may be important in transmitting the signals for neurogenic itch (Tsujii *et al.*, 2008; Papoiu *et al.*, 2015; Patricio *et al.*, 2015). PAR4 activation is thought to attenuate nociception via sensory neurons by inhibiting nociceptive signals (Asfaha *et al.*, 2007). These analgesic effects of PAR4 activation have been modelled *in vivo*, where injections of PAR4-agonist peptides in rat paws evoke analgesia in response to both mechanical and thermal stimuli (Asfaha *et al.*, 2007). Further, in the setting of inflammatory bowel disease in mice, colonic delivery of PAR4 agonists reduced basal visceral pain and visceral hypersensitivity caused by PAR2 (Auge *et al.*, 2009). For a detailed review on PAR4 in pain and inflammatory pathologies, see Fu *et al.* (2015). Together, these studies help identify PAR4 as a likely mediator of

multiple pathological processes and provide rationale for the development of pharmacological reagents targeting PAR4.

Pharmacology

Agonists

Current agonists of PAR4 consist of amidated peptides based on the native tethered ligand sequence of the receptor (Table 2). A hexapeptide matching the sequence immediately downstream of the thrombin cleavage site of the receptor, GYPGQV-NH_2 (Figure 1) selectively activates PAR4, although prohibitively high concentrations of ~500 μM are required for effective activation (Faruqi *et al.*, 2000). The native tethered ligand sequence of mouse PAR4, GYPGKF-NH_2, activates both human and mouse PAR4 with slightly greater potency (Kahn *et al.*, 1998b). However, an alanine scan of this sequence undertaken to identify residues critical for receptor activation inadvertently uncovered a more potent peptide agonist, AYPGKF-NH_2, which has become the gold standard PAR4 activator. This peptide has an EC_{50} of between 5 and 100 μM, depending on the setting (Table 2) and is highly specific for PAR4 (Faruqi *et al.*, 2000; Hollenberg *et al.*, 2004).

Antagonists

Research into the physiological function of PAR4 relies on the development of specific and effective antagonists. The unique structure and activation mechanism of PARs has long posed a problem for the development of such inhibitors. In particular, activation by proteolytic cleavage is a highly efficient and irreversible system, while the tethered ligand-based activation mechanism requires antagonism of the binding of an agonist intrinsic to the receptor and presumably with considerable steric advantage. These issues have hindered the development of PAR4 antagonists, although recent efforts have begun to overcome these challenges, with several distinct antagonist classes emerging (Table 2).

Peptidomimetics. The first approach taken to identify potential inhibitors of PAR4 was the generation of numerous peptide analogues based on the tethered ligand sequences of human, mouse and rat PAR4. The peptidomimetic approach yielded two sequences based on mouse PAR4 and modified by the addition of a *trans*-cinnamoyl (tc) group (Hollenberg and Saifeddine, 2001). The resulting compound, tc-YPGKF-NH_2, appeared to bind but not activate PAR4 and abolished PAR4-AP-induced aggregation of rat platelets and significantly reduced thrombin-induced platelet aggregation, at least at high concentrations (400 μM) (Hollenberg and Saifeddine, 2001). tc-YPGKF-NH_2 also inhibited thrombin-induced platelet aggregation in human platelets (Ma *et al.*, 2005), although there is limited other evidence of this agent inhibiting human PAR4 (Hollenberg *et al.*, 2004).

Low MW compounds. Low MW PAR4 antagonists have also been developed via screening of various heterocyclic structures. The first compound identified to selectively inhibit PAR4 was an indazole derivative known as YD-3 [1-benzyl-3 (ethoxycarbonylphenyl)-indazole] (Lee *et al.*, 2001; Wu *et al.*, 2002). YD-3 inhibited thrombin-induced platelet aggregation in rabbits with an IC_{50} of 28 μM but, in humans, it only partly inhibited platelet aggregation in response to thrombin concentrations lower than 0.5 nM (Wu *et al.*, 2002, 2003). Several studies have aimed to increase the efficacy of YD-3, and have produced several derivatives (Huang *et al.*, 2006; Chen *et al.*, 2008), including the N2-(substituted benzyl)-3-(4-methylphenyl)-*2*H-indazoles, compounds numbered 19, 25, and 31(Huang *et al.*, 2006). The IC_{50} of these compounds in HL-60 cell lines was reported to be greater than 36 μM (Huang *et al.*, 2006). The newest low MW PAR4 inhibitor, ML354, is a substituted indole-based derivative (Young *et al.*, 2013; Wen *et al.*, 2014). This compound inhibited PAR4-AP-induced integrin activation with an IC_{50} of 140 nM, but also PAR1-AP-induced integrin activation with an IC_{50} of 10 μM. Initial studies using these recently developed low MW PAR4 antagonists have helped to generate interest in PAR4 as a novel therapeutic target for anti-angiogenic drugs (Huang *et al.*, 2006). A clinical candidate for PAR4 inhibition, BMS-986120, is currently being evaluated in phase I clinical trials for safety and tolerability for the prevention and/or treatment of thromboembolic disorders (NCT02208882).

Pepducins. Pepducins are a distinct class of PAR4 inhibitors that mimic the region of the receptor that binds G proteins and essentially work by blocking the interactions between the receptor and effector G protein (Covic *et al.*, 2002a). Specifically, peptides with a sequence corresponding to the third intracellular loop of a given receptor (the region responsible for interaction with G proteins) are conjugated to an N-terminal palmitate (pal) that anchors the peptide to the lipid membrane of a cell. Once anchored within the membrane, it is proposed that pepducins act by binding G proteins and thus prevent downstream signalling. PARs were used as the target receptors for initial proof-of-concept studies, and pepducins exist against both PAR1 and PAR4 (Covic *et al.*, 2002a). The anti-PAR4 pepducin, P4pal-10 (pal-SGRRYGHALR-NH_2) inhibited ~85% of thrombin-induced aggregation of both human and mouse platelets at a concentration of 3 μM and is effective *in vivo*, as assessed by increased tail bleeding time in mice (Covic *et al.*, 2002a, 2002b). However, the specificity of P4pal-10 remains debatable, as it displays a level of cross-reactivity with PAR1-AP-induced platelet activation (Covic *et al.*, 2002b), as well as collagen and TXA_2 (Stampfuss *et al.*, 2003). A subsequent pepducin (P4pal-i1) targeted against the first intracellular loop (i1) was synthesized to address the issue of specificity and has been shown to inhibit PAR4-AP-induced platelet aggregation without affecting PAR1-AP-induced platelet aggregation (Leger *et al.*, 2006).

Function-blocking antibodies. Several function-blocking anti-PAR4 antibodies have also been developed and proven to be useful PAR4 antagonists. The first anti-PAR4 function-blocking antibody was a rabbit polyclonal antibody against the thrombin cleavage site of human PAR4. This antibody inhibited thrombin-induced aggregation of human platelets in the presence of concomitant PAR1 inhibition at a concentration of 1 $mg{\cdot}mL^{-1}$ (Kahn *et al.*, 1999). More recently, a strategy was employed by Mumaw *et al.* (2014) to

Table 2

PAR4 agonists and antagonists

Agonists						
Class	**Compound**	**Target**	**EC_{50}**	**Receptor**	**Cell**	**Reference**
Amidated peptides	GYPGQV-NH_2	TLS binding site	500 μM	Human	Transfected mouse fibroblasts	Faruqi *et al.*, 2000
	GYPGKF-NH_2	TLS binding site	100 μM	Human	Transfected mouse fibroblasts	Kahn *et al.*, 1999
			40 μM	Rat	Platelets	Hollenberg *et al.*, 2004
	AYPGKF-NH_2	TLS binding site	5 μM	Human	Transfected mouse fibroblasts	Faruqi *et al.*, 2000
			13 μM	Rat	Platelets	Hollenberg *et al.*, 2004
			11 μM	Rat	Aorta	
Antagonists						
Class	**Compound**	**Target**	**IC_{50}[a]**	**Receptor**	**Cell type**	**Reference**
Peptidomimetics	tc-YPGKF-NH_2	TLS binding site	100 μM	Rat, human	Platelets	Hollenberg *et al.*, 2001; Hollenberg *et al.*, 2004; Ma *et al.*, 2005
Low MW compounds	YD-3	TLS binding site	28 μM	Rabbit, rat, human	Platelets, smooth muscle cells	Wu *et al.*, 2002; Wu *et al.*, 2003; Peng *et al.*, 2004
	19, 25, 31	TLS binding site	>36 μM	Human	Platelets	Huang *et al.*, 2006
	ML354	TLS binding site	140 nM[b]	Human	Platelets	Young *et al.*, 2013; Wen *et al.*, 2014
Pepducins	P4pal-10	Third intracellular loop	1 μM	Mouse, human	Platelets	Covic *et al.*, 2002a, b
	P4pal-i1	First intracellular loop	5 $\mu mol \cdot L^{-1}$[c]	Guinea pigs, human	Platelets	Leger *et al.*, 2006
Function-blocking antibodies	Rabbit polyclonal	Thrombin cleavage site	1 $mg \cdot mL^{-1}$[c]	Human, rat	Platelets, fibroblasts	Kahn *et al.*, 1999
	Rabbit polyclonal (Can12)	Anionic region	10 $ng \cdot mL^{-1}$	Mouse, human	Platelets	Mumwaw *et al.*, 2014
	Mouse monoclonal (14H6, 5 F10)	Thrombin cleavage site	50 $\mu g \cdot mL^{-1}$[c] partial inhibition	Human	Platelets, HEK-293s	Mumwaw *et al.*, 2015

[a]IC_{50} values correspond to inhibition of thrombin-induced PAR4 activation.
[b]IC_{50} values correspond to inhibition of PAR4 agonist peptide-induced activation.
[c]TLS, tethered ligand sequence; Values are concentrations reported to inhibit thrombin-induced PAR4 activation.

raise anti-PAR4 antibodies against the anionic region sequence of PAR4 (C^{54}ANDSDTLTLPD), just downstream of the thrombin cleavage site. The lead antibody to emerge from these studies, CAN12, not only inhibits thrombin-induced aggregation of human platelets but also inhibits aggregation induced by PAR4-AP, ADP or collagen (Mumaw *et al.*, 2014), suggesting major issues with specificity. CAN12 was shown to inhibit arterial thrombosis in the Rose Bengal carotid artery thrombosis model at 0.5 $mg \cdot kg^{-1}$ (Mumaw *et al.*, 2014), indicating cross-reactivity with murine PAR4. Beneficial effects were seen when CAN12 was infused both 10 min pre-injury and 15 min post-injury. A new series of monoclonal antibodies from the same group has been very recently reported and appear to bind either to the anionic region or to the thrombin cleavage site of PAR4 and partially inhibit thrombin-induced cleavage of the receptor in expression systems (Mumaw *et al.*, 2015).

This diverse set of PAR4 antagonists, developed over recent years, have been invaluable for investigations into the physiological roles of PAR4 and have also served to promote targeting of this receptor as a novel therapeutic approach. However, there still remains significant work to be done to develop PAR4 antagonists suitable for potential clinical utility.

Clinical significance

Cardiovascular disease remains a major clinical problem. Increasingly prevalent conditions such as diabetes, hypertension and coronary artery disease eventuate in diseased and pro-thrombotic vasculature and a common endpoint of these, often co-existing, conditions is the formation of pathological arterial thrombosis leading to myocardial infarction or ischaemic stroke. Together, such conditions account for ~40% of all deaths in Western countries (Mozaffarian *et al.*, 2015). Given that platelets are the key cellular component of arterial thrombi, anti-platelet agents are the primary therapy for the prevention of myocardial infarction and ischaemic stroke. There are several anti-platelet drugs on the market, but a lack of efficacy and safety of these existing drugs has fuelled a need for improved therapies. Given the importance of PARs for platelet activation, PAR antagonists represent one of the leading classes of new anti-platelet drugs.

Targeting PAR4 as an anti-platelet approach?

Ever since the discovery of PAR1 as the major platelet thrombin receptor, there was strong interest in developing PAR1 antagonists as novel anti-platelet agents. This led to the development of the first PAR1 antagonist, vorapaxar (trade name Zontivity), which was recently granted FDA approval for use in the USA for the prevention of myocardial infarction and peripheral artery disease. Vorapaxar was assessed in two large-scale phase III clinical trials, of which one was terminated early due to a significant increase in intracranial haemorrhage in the vorapaxar-treated cohort (Scirica *et al.*, 2012; Tricoci *et al.*, 2012). Indeed, throughout these trials, vorapaxar was associated with reduction of ischaemic outcomes in some subgroups, whereas in others was associated with adverse bleeding events (French *et al.*, 2015). Subsequent studies examining blood from patients receiving vorapaxar have confirmed that PAR4-mediated platelet responses remain intact (Judge *et al.*, 2014). As a result, there is growing interest in the clinical potential of PAR4 antagonists as novel anti-platelet agents.

Clinically significant variants of PAR4

Further support for the targeting of PAR4 as an anti-platelet approach comes from recent studies demonstrating racial differences in the expression and reactivity of platelet PAR4 (Edelstein *et al.*, 2013; Edelstein *et al.*, 2014; Tourdot *et al.*, 2014). Significantly, these differences appear to associate with resistance to current anti-platelet drugs and overall poorer cardiovascular outcomes (Edelstein *et al.*, 2013; Edelstein *et al.*, 2014; Tourdot *et al.*, 2014). Specifically, platelets from healthy Black subjects were hyper-responsive to PAR4-AP when compared with platelets from non-Black subjects. This increased sensitivity persisted in the face of PAR1 antagonism (vorapaxar) or aspirin treatment. This work led to the identification of a genetic variant of PAR4, $Ala^{120}Thr$, which is associated with greater PAR4 reactivity and occurs at a higher frequency in Black subjects than in non-Black subjects. This racial difference may have important clinical implications and potential therapeutic impact on specific population responses, and therefore clinical outcomes, to anti-platelet therapy, and further supports the idea of targeting platelet PAR4 for improved anti-thrombotic therapy.

Conclusions

Despite the discovery of PAR4 over 15 years ago, comparatively little is known about the function of this receptor. Generation and characterization of PAR4−/− mice has yielded phenotypes relating to thrombosis and haemostasis, as well as inflammation and vascular protection – prompting recent interest in the field and leading to the development of improved inhibitors for investigations into PAR4 function. This important ground work now allows several key remaining questions to be addressed. For instance, apart from thrombosis, inflammation, and pain, what physiological functions are there for PAR4? How can PAR4 be best inhibited pharmacologically? What is the importance of PAR4 expression and reactivity differences observed in genetically distinct patient groups and how will this affect treatment options for people with cardiovascular and/or inflammatory diseases? In addition, the limited number of molecular pharmacology studies of PAR4 leaves several other unanswered questions: how important is receptor dimerization for specific cell signalling events? Does the importance of PAR3/PAR4 co-factoring in mouse platelets extend to other receptors in other settings? Does biased signalling occur for PAR4, as it does for both PAR1 and PAR2? The recent increase in interest in PAR4 will, we hope, culminate in the development of improved pharmacological reagents and further molecular pharmacology experiments that will provide the tools to address these questions in the coming years.

Acknowledgements

This work was supported by grants to J.R.H. from the National Health & Medical Research Council of Australia (491143 and 1047295). J.R.H. is an Australian Research Council Future Fellow. The authors are indebted to George C. Coventry for providing important insights.

Conflict of interest

The authors declare no conflicts of interest.

References

Alexander SPH, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE, *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: G Protein-Coupled Receptors. Br J Pharmacol 172: 5744–5869.

Alexander SPH, Peters JA, Kelly E, Marrion N, Benson HE, Faccenda E, *et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Ligand-gated ion channels. Br J Pharmacol 172: 5870–5903.

Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE, *et al.* (2015c). The Concise Guide to PHARMACOLOGY 2015/16: Enzymes. Br J Pharmacol 172: 6024–6109.

Andrade-Gordon P, Derian CK, Maryanoff BE, Zhang H-C, Addo MF, Cheung W-M, *et al.* (2001). Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J Pharmacol Exp Ther 298: 34–42.

Arachiche A, Mumaw MM, De La Fuente M, Nieman MT (2013). Protease-activated receptor 1 (PAR1) and PAR4 heterodimers are required for PAR1-enhanced cleavage of PAR4 by alpha-thrombin. J Biol Chem 288: 32553–32562.

Asfaha S, Cenac N, Houle S, Altier C, Papez MD, Nguyen C, *et al.* (2007). Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. Br J Pharmacol 150: 176–185.

Auge C, Balz-Hara D, Steinhoff M, Vergnolle N, Cenac N (2009). Protease-activated receptor 4 (PAR4): a role as inhibitor of visceral pain and hypersensitivity. Neurogastroenterol Motil 21: 1189–e107.

Ayala YM, Cantwell AM, Rose T, Bush LA, Arosio D, Di Cera E (2001). Molecular mapping of thrombin–receptor interactions. Proteins Struct Funct Bioinform 45: 107–116.

Busso N, Chobaz-Peclat V, Hamilton J, Spee P, Wagtmann N, So A (2008). Essential role of platelet activation via protease activated receptor 4 in tissue factor-initiated inflammation. Arthritis Res Ther 10: R42.

Chen D, Wang Z, Zhang Z, Zhang R, Yu L (2013). Capsaicin up-regulates protease-activated receptor-4 mRNA and protein in primary cultured dorsal root ganglion neurons. Cell Mol Neurobiol 33: 337–346.

Chen HS, Kuo SC, Teng CM, Lee FY, Wang JP, Lee YC, *et al.* (2008). Synthesis and antiplatelet activity of ethyl 4-(1-benzyl-1H-indazol-3-yl)benzoate (YD-3) derivatives. Bioorg Med Chem 16: 1262–1278.

Connolly AJ, Ishihara H, Kahn ML, Farese RV, Coughlin SR (1996). Role of the thrombin receptor in development and evidence for a second receptor. Nature 381: 516–519.

Connolly T, Condra C, Feng D, Cook J, Stranieri M, Reilly C, *et al.* (1994). Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. Thromb Haemost 72: 627–633.

Cottrell GS, Amadesi S, Grady EF, Bunnett NW (2004). Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. J Biol Chem 279: 13532–13539.

Coughlin SR (2000). Thrombin signalling and protease-activated receptors. Nature 407: 258–264.

Coughlin SR (2005). Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 3: 1800–1814.

Covic L, Gresser AL, Talavera J, Swift S, Kuliopulos A (2002a). Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. Proc Natl Acad Sci U S A 99: 643–648.

Covic L, Misra M, Badar J, Singh C, Kuliopulos A (2002b). Pepducin-based intervention of thrombin–receptor signaling and systemic platelet activation. Nat Med 8: 1161–1165.

Covic L, Singh C, Smith H, Kuliopulos A (2002c). Role of the PAR4 thrombin receptor in stabilizing platelet–platelet aggregates as revealed by a patient with Hermansky–Pudlak syndrome. Thromb Haemost 87: 722–727.

Cunningham MR, Mcintosh KA, Pediani JD, Robben J, Cooke AE, Nilsson M, *et al.* (2012). Novel role for proteinase-activated receptor 2 (PAR2) in membrane trafficking of proteinase-activated receptor 4 (PAR4). J Biol Chem 287: 16656–16669.

Dabek M, Ferrier L, Annahazi A, Bezirard V, Polizzi A, Cartier C, *et al.* (2011). Intracolonic infusion of fecal supernatants from ulcerative colitis patients triggers altered permeability and inflammation in mice: role of cathepsin G and protease-activated receptor-4. Inflamm Bowel Dis 17: 1409–1414.

Dabek M, Ferrier L, Roka R, Gecse K, Annahazi A, Moreau J, *et al.* (2009). Luminal cathepsin G and protease-activated receptor 4: a duet involved in alterations of the colonic epithelial barrier in ulcerative colitis. Am J Pathol 175: 207–214.

Dattilio A, Vizzard MA (2005). Up-regulation of protease-activated receptors in bladder after cyclophosphamide induced cystitis and colocalization with capsaicin receptor (VR1) in bladder nerve fibers. J Urol 173: 635–639.

De La Fuente M, Noble DN, Verma S, Nieman MT (2012). Mapping human protease-activated receptor 4 (PAR4) homodimer interface to transmembrane helix 4. J Biol Chem 287: 10414–10423.

Derian CK, Damiano BP, Addo MF, Darrow AL, D'andrea MR, Nedelman M, *et al.* (2003). Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. J Pharmacol Exp Ther 304: 855–861.

Dorsam RT, Kim S, Jin J, Kunapuli SP (2002). Coordinated signaling through both G12/13 and Gi pathways is sufficient to activate GPIIb/IIIa in human platelets. J Biol Chem 277: 47588–47595.

Duvernay M, Young S, Gailani D, Schoenecker J, Hamm HE (2013). Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets. Mol Pharmacol 83: 781–792.

Edelstein LC, Simon LM, Lindsay CR, Kong X, Teruel-Montoya R, Tourdot BE, *et al.* (2014). Common variants in the human platelet

PAR4 thrombin receptor alter platelet function and differ by race. Blood 124: 3450–3458.

Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, *et al.* (2013). Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. Nat Med 19: 1609–1616.

Falker K, Haglund L, Gunnarsson P, Nylander M, Lindahl TL, Grenegard M (2011). Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets. Biochem J 436: 469–480.

Faruqi TR, Weiss EJ, Shapiro MJ, Huang W, Coughlin SR (2000). Structure–function analysis of protease-activated receptor 4 tethered ligand peptides. Determinants of specificity and utility in assays of receptor function. J Biol Chem 275: 19728–19734.

French SL, Arthur JF, Tran HA, Hamilton JR (2015). Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. Blood Rev 29: 179–189.

Fu Q, Cheng J, Gao Y, Zhang Y, Chen X, Xie J (2015). Protease-activated receptor 4: a critical participator in inflammatory response. Inflammation 38: 886–895.

Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK (1997). Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. Proc Natl Acad Sci U S A 94: 8750–8755.

Grenegard M, Vretenbrandt-Oberg K, Nylander M, Desilets S, Lindstrom EG, Larsson A, *et al.* (2008). The ATP-gated P2X1 receptor plays a pivotal role in activation of aspirin-treated platelets by thrombin and epinephrine. J Biol Chem 283: 18493–18504.

Hamilton JR, Cornelissen I, Coughlin SR (2004). Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. J Thromb Haemost 2: 1429–1435.

Hamilton JR, Cornelissen I, Mountford JK, Coughlin SR (2009). Atherosclerosis proceeds independently of thrombin-induced platelet activation in ApoE−/− mice. Atherosclerosis 205: 427–432.

Hamilton JR, Frauman AG, Cocks TM (2001). Increased expression of protease-activated receptor-2 (PAR2) and PAR4 in human coronary artery by inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. Circ Res 89: 92–98.

Holinstat M, Voss B, Bilodeau ML, Mclaughlin JN, Cleator J, Hamm HE (2006). PAR4, but not PAR1, signals human platelet aggregation via Ca2+ mobilization and synergistic P2Y12 receptor activation. J Biol Chem 281: 26665–26674.

Hollenberg MD, Saifeddine M (2001). Proteinase-activated receptor 4 (PAR4): activation and inhibition of rat platelet aggregation by PAR4-derived peptides. Can J Physiol Pharmacol 79: 439–442.

Hollenberg MD, Saifeddine M, Al-Ani B, Gui Y (1999). Proteinase-activated receptor 4 (PAR4): action of PAR4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR1 and PAR2. Can J Physiol Pharmacol 77: 458–464.

Hollenberg MD, Saifeddine M, Sandhu S, Houle S, Vergnolle N (2004). Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists in vivo. Br J Pharmacol 143: 443–454.

Houle S, Papez MD, Ferazzini M, Hollenberg MD, Vergnolle N (2005). Neutrophils and the kallikrein–kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. Br J Pharmacol 146: 670–678.

Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF (1993). Internalization and recycling of activated thrombin receptors. J Biol Chem 268: 13756–13763.

Huang LJ, Shih ML, Chen HS, Pan SL, Teng CM, Lee FY, *et al.* (2006). Synthesis of N2-(substituted benzyl)-3-(4-methylphenyl) indazoles as novel anti-angiogenic agents. Bioorg Med Chem 14: 528–536.

Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, *et al.* (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 386: 502–506.

Jacques S, Kuliopulos A (2003). Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. Biochem J 376: 733–740.

Jacques SL, Lemasurier M, Sheridan PJ, Seeley SK, Kuliopulos A (2000). Substrate-assisted catalysis of the PAR1 thrombin receptor enhancement of macromolecular association and cleavage. J Biol Chem 275: 40671–40678.

Judge HM, Jennings LK, Moliterno DJ, Hord E, Ecob R, Tricoci P, *et al.* (2014). PAR1 antagonists inhibit thrombin-induced platelet activation whilst leaving the PAR4-mediated response intact. Platelets 26: 236–242.

Kahn ML, Hammes SR, Botka C, Coughlin SR (1998a). Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. J Biol Chem 273: 23290–23296.

Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 103: 879–887.

Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, *et al.* (1998b). A dual thrombin receptor system for platelet activation. Nature 394: 690–694.

Kataoka H, Hamilton JR, Mckemy DD, Camerer E, Zheng YW, Cheng A, *et al.* (2003). Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. Blood 102: 3224–3231.

Kelso EB, Lockhart JC, Hembrough T, Dunning L, Plevin R, Hollenberg MD, *et al.* (2006). Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. J Pharmacol Exp Ther 316: 1017–1024.

Khan A, Li D, Ibrahim S, Smyth E, Woulfe DS (2014). The physical association of the P2Y12 receptor with PAR4 regulates arrestin-mediated Akt activation. Mol Pharmacol 86: 1–11.

Khan TA, Bianchi C, Voisine P, Sandmeyer J, Feng J, Sellke FW (2005). Aprotinin inhibits protease-dependent platelet aggregation and thrombosis. Ann Thorac Surg 79: 1545–1550.

Lau LF, Pumiglia K, Cote YP, Feinstein MB (1994). Thrombin–receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. Biochem J 303 (Pt 2): 391–400.

Lee FY, Lien JC, Huang LJ, Huang TM, Tsai SC, Teng CM, *et al.* (2001). Synthesis of 1-benzyl-3-(5′-hydroxymethyl-2′-furyl)indazole analogues as novel antiplatelet agents. J Med Chem 44: 3746–3749.

Lee H, Sturgeon SA, Mountford JK, Jackson SP, Hamilton JR (2012a). Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. Br J Pharmacol 166: 2188–2197.

Lee H, Sturgeon SA, Jackson SP, Hamilton JR (2012b). The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. Thromb Haemost 107: 328.

Leger AJ, Jacques SL, Badar J, Kaneider NC, Derian CK, Andrade-Gordon P, *et al.* (2006). Blocking the protease-activated receptor 1–4 heterodimer in platelet-mediated thrombosis. Circulation 113: 1244–1254.

Lohman RJ, Cotterell AJ, Suen J, Liu L, Do AT, Vesey DA, *et al.* (2012). Antagonism of protease-activated receptor 2 protects against experimental colitis. J Pharmacol Exp Ther 340: 256–265.

Ma L, Perini R, Mcknight W, Dicay M, Klein A, Hollenberg MD, *et al.* (2005). Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. Proc Natl Acad Sci U S A 102: 216–220.

Mao Y, Zhang M, Tuma RF, Kunapuli SP (2010). Deficiency of PAR4 attenuates cerebral ischemia/reperfusion injury in mice. J Cereb Blood Flow Metab 30: 1044–1052.

Mathews I, Padmanabhan K, Ganesh V, Tulinsky A, Ishii M, Chen J, *et al.* (1994). Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. Biochemistry 33: 3266–3279.

Mcdougall JJ, Zhang C, Cellars L, Joubert E, Dixon CM, Vergnolle N (2009). Triggering of proteinase-activated receptor 4 leads to joint pain and inflammation in mice. Arthritis Rheum 60: 728–737.

Miyakawa K, Joshi N, Sullivan BP, Albee R, Brandenberger C, Jaeschke H, *et al.* (2015). Platelets and protease-activated receptor-4 contribute to acetaminophen-induced liver injury in mice. Blood 126: 1835–1843.

Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, *et al.* (2012). Vorapaxar in the secondary prevention of atherothrombotic events. New Engl J Med 366: 1404–1413.

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, *et al.* (2015). Heart disease and stroke statistics–2015 update: a report from the American Heart Association. Circulation 131: e29–322.

Mumaw M, La Fuente M, Noble D, Nieman M (2014). Targeting the anionic region of human protease activated receptor 4 (PAR4) inhibits platelet aggregation and thrombosis without interfering with hemostasis. J Thromb Haemost. 12: 1331–1341.

Mumaw MM, De La Fuente M, Arachiche A, Wahl JK 3rd, Nieman MT (2015). Development and characterization of monoclonal antibodies against protease activated receptor 4 (PAR4). Thromb Res 135: 1165–1171.

Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR (2000). PAR3 is a cofactor for PAR4 activation by thrombin. Nature 404: 609–613.

Nystedt S, Emilsson K, Larsson AK, Strömbeck B, Sundelin J (1995). Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. Eur J Biochem 232: 84–89.

Offermanns S, Laugwitz K-L, Spicher K, Schultz G (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc Natl Acad Sci U S A 91: 504–508.

Offermanns S, Toombs CF, Hu Y-H, Simon MI (1997). Defective platelet activation in Gαq-deficient mice. Nature 389: 183–186.

Papoiu AD, Chaudhry H, Hayes EC, Chan YH, Herbst KD (2015). TriCalm((R)) hydrogel is significantly superior to 2% diphenhydramine and 1% hydrocortisone in reducing the peak intensity, duration, and overall magnitude of cowhage-induced itch. Clin Cosmet Investig Dermatol 8: 223–229.

Patricio ES, Costa R, Figueiredo CP, Gers-Barlag K, Bicca MA, Manjavachi MN, *et al.* (2015). Mechanisms underlying the scratching behavior induced by the activation of proteinase-activated receptor-4 in mice. J Invest Dermatol 135: 2484–2491.

Pavic G, Grandoch M, Dangwal S, Jobi K, Rauch BH, Doller A, *et al.* (2014). Thrombin receptor protease-activated receptor 4 is a key regulator of exaggerated intimal thickening in diabetes mellitus. Circulation 130: 1700–1711.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SPH, Buneman OP, *et al.* , NC-IUPHAR(2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–D1106.

Peng CY, Pan SL, Guh JH, Liu YN, Chang YL, Kuo SC, *et al.* (2004). The indazole derivative YD-3 inhibits smooth muscle cell proliferation and attenuates intimal thickening after balloon injury. Thromb Haemst 92: 1232–1239.

Ramachandran R, Sadofsky LR, Xiao Y, Botham A, Cowen M, Morice AH, *et al.* (2007). Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4. Am J Physiol Lung Cell Mol Physiol 292: L788–L798.

Sambrano GR, Weiss EJ, Zheng Y-W, Huang W, Coughlin SR (2001). Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 413: 74–78.

Scirica BM, Bonaca MP, Braunwald E, De Ferrari GM, Isaza D, Lewis BS, *et al.* (2012). Vorapaxar for secondary prevention of thrombotic events for patients with previous myocardial infarction: a prespecified subgroup analysis of the TRA 2 P-TIMI 50 trial. Lancet 380: 1317–1324.

Shapiro MJ, Trejo J, Zeng D, Coughlin SR (1996). Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. J Biol Chem 271: 32874–32880.

Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR (2000). Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. J Biol Chem 275: 25216–25221.

Soh UJ, Dores MR, Chen B, Trejo J (2010). Signal transduction by protease-activated receptors. Br J Pharmacol 160: 191–203.

Stampfuss JJ, Schror K, Weber AA (2003). Inhibition of platelet thromboxane receptor function by a thrombin receptor-targeted pepducin. Nat Med 9: 1447–1448.

Tourdot BE, Conaway S, Niisuke K, Edelstein LC, Bray PF, Holinstat M (2014). Mechanism of race-dependent platelet activation through the protease-activated receptor-4 and Gq signaling axis. Arterioscler Thromb Vasc Biol 34: 2644–2650.

Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van De Werf F, *et al.* (2012). Thrombin–receptor antagonist vorapaxar in acute coronary syndromes. New Engl J Med 366: 20–33.

Tsujii K, Andoh T, Lee JB, Kuraishi Y (2008). Activation of proteinase-activated receptors induces itch-associated response through histamine-dependent and -independent pathways in mice. J Pharmacol Sci 108: 385–388.

Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC (2007). Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. Proc Natl Acad Sci U S A 104: 288–292.

Vergnolle N, Derian CK, D'andrea MR, Steinhoff M, Andrade-Gordon P (2002). Characterization of thrombin-induced leukocyte rolling

and adherence: a potential proinflammatory role for proteinase-activated receptor-4. J Immunol 169: 1467–1473.

Vidwan P, Pathak A, Sheth S, Huang J, Monroe DM, Stouffer GA (2010). Activation of protease-activated receptors 3 and 4 accelerates tissue factor-induced thrombin generation on the surface of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 30: 2587–2596.

Voss B, Mclaughlin JN, Holinstat M, Zent R, Hamm HE (2007). PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. Mol Pharmacol 71: 1399–1406.

Vu T-KH, Hung DT, Wheaton VI, Coughlin SR (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64: 1057–1068.

Weiss EJ, Hamilton JR, Lease KE, Coughlin SR (2002). Protection against thrombosis in mice lacking PAR3. Blood 100: 3240–3244.

Wen W, Young SE, Duvernay MT, Schulte ML, Nance KD, Melancon BJ, *et al.* (2014). Substituted indoles as selective protease activated receptor 4 (PAR-4) antagonists: discovery and SAR of ML354. Bioorg Med Chem Lett 24: 4708–4713.

Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Lee CY, *et al.* (2002). Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. Thromb Haemost 87: 1026–1033.

Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Teng CM (2003). The role of PAR4 in thrombin-induced thromboxane production in human platelets. Thromb Haemost 90: 299–308.

Wu CC, Wu SY, Liao CY, Teng CM, Wu YC, Kuo SC (2010). The roles and mechanisms of PAR4 and P2Y12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. Br J Pharmacol 161: 643–658.

Xu W-F, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, *et al.* (1998). Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci U S A 95: 6642–6646.

Young SE, Duvernay MT, Schulte ML, Lindsley CW, Hamm HE (2013). Synthesis of indole derived protease-activated receptor 4 antagonists and characterization in human platelets. PLoS One 8, e65528.

Zhu WJ, Yamanaka H, Obata K, Dai Y, Kobayashi K, Kozai T, *et al.* (2005). Expression of mRNA for four subtypes of the proteinase-activated receptor in rat dorsal root ganglia. Brain Res 1041: 205–211.

Appendix III

Drugs targeting protease-activated receptor-4 improve the anti-thrombotic therapeutic window

Shauna L. French, Justin R. Hamilton

Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

Correspondence to: Justin Hamilton. Australian Centre for Blood Diseases, Monash University, L2, AMREP Building, The Alfred Commercial Rd, Melbourne, Victoria, 3004, Australia. Email: Justin.Hamilton@monash.edu.

Provenance: This is an invited Editorial commissioned by the Section Editor Dr. Hongcheng Zhu, MD, PhD (Department of Radiation Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China).

Comment on: Wong PC, Seiffert D, Bird JE, *et al.* Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding. Sci Transl Med 2017;9(371). pii: eaaf5294.

Submitted Sep 01, 2017. Accepted for publication Sep 07, 2017.
doi: 10.21037/atm.2017.09.10
View this article at: http://dx.doi.org/10.21037/atm.2017.09.10

Introduction

Antiplatelet agents are the main pharmacotherapy for arterial thrombosis prevention and are central in the management of cardiovascular conditions such as myocardial infarction, transient ischaemic attack, and coronary and peripheral artery diseases. Yet despite their long history and extensive clinical use, antiplatelet agents appear to have reached a disappointingly low therapeutic ceiling-predominantly due to the narrow therapeutic window afforded by strategies targeting platelet function. Platelets are critical for normal hemostasis as well as pathological thrombosis. Inhibiting platelet function for protective benefit without causing unwanted bleeding limits the efficacy of current antiplatelet drugs. Aspirin and the thienopyridine class of drugs (e.g., clopidogrel, prasugrel, ticagrelor) are by far the most commonly prescribed antiplatelet agents, yet prevent just 15 and 17% of lethal cardiovascular events respectively (1,2). Combination therapy provides a marginal increase in efficacy (~7%), but also increases the risk of bleeding (2). More potent antiplatelet drugs, such as the glycoprotein $\alpha_{IIb}\beta_3$ inhibitors, carry even more bleeding risk and are thereby limited to acute use settings such as periprocedural percutaneous coronary intervention (3,4). Therefore, the search to identify antiplatelet drugs that increase the therapeutic window of antithrombotic therapy continues. A recent study by Wong *et al.* (5) provides compelling evidence that targeting the platelet thrombin receptor, PAR4, may achieve this goal.

In the setting of thrombosis, platelets are activated by a combination of endogenous agonists, some of which are blocked by existing drugs. For example, aspirin prevents production of the platelet activator thromboxane A_2 while the thienopyridines block the major platelet ADP receptor, $P2Y_{12}$. Thrombin is the most potent platelet activator, which it achieves predominantly via two cell surface GPCRs, protease-activated receptor 1 (PAR1) and PAR4. PAR1 has greater affinity for thrombin than PAR4 and has therefore been the focus of drug development targeting thrombin-induced platelet activation. The first PAR1 antagonist, vorapaxar, was approved by the US FDA in 2014 for the prevention of thrombotic events in patients with a history of myocardial infarction or peripheral artery disease. Yet because it must be administered in addition to standard-of-care antiplatelet therapy (aspirin and/or a thienopyridine), vorapaxar provides only limited therapeutic benefit to a small group of patients without significantly increasing major bleeding (6,7). In line with the clinical experience of other combination antiplatelet therapies, the narrow therapeutic window of vorapaxar in the presence of standard-of-care antiplatelet drugs has translated to limited clinical utility. As a result, there has been much renewed interest in targeting the 'second' platelet thrombin receptor, PAR4, for antithrombotic therapy. Although previous studies have rationalised PAR4 as a viable antithrombotic target (8-11), the work

Table 1 Characteristics of the PAR4 antagonist BMS-986120

Test	Experimental system	Parameter measured	Value for BMS-986120
Binding	HEK293T membranes	K_d	0.098 nM
		K_{on}	0.12 $nM^{-1}.min^{-1}$
		K_{off}	0.008 min^{-1}
Specificity	HEK293T	Calcium mobilisation IC_{50} *vs.* PAR4-AP	0.56 nM
	HEK293T	Calcium mobilisation IC_{50} *vs.* PAR1-AP	>5,000 nM
	CHO	Calcium mobilisation IC_{50} *vs.* PAR2-AP	>42,000 nM
Efficacy (inhibition of PAR4 signaling)	HEK293T	IC_{50}: activation of $G_{\alpha 11}$	3.4 nM
		IC_{50}: activation of $G_{\alpha q}$	3.9 nM
		IC_{50}: activation of $G_{\alpha 14}$	31 nM
		IC_{50}: β-arrestin 2 recruitment	7.2 nM
		IC_{50}: ERK1/2 activation	47 nM
Efficacy (inhibition of platelet aggregation)	Platelet-rich plasma (human)	IC_{50} *vs.* γ-thrombin	7.3 nM
	Whole blood (human)	IC_{50} *vs.* PAR4-AP	9.5 nM
	Whole blood (monkey)	IC_{50} *vs.* PAR4-AP	2.1 nM
Efficacy (prevention of *in vivo* thrombosis)	Monkey	Carotid artery occlusion time (fold-increase *vs.* vehicle)	2.7×, 0.2 mg/kg; 3×, 0.5 mg/kg; no occlusion, 1 mg/kg
		Thrombus weight (% reduction *vs.* vehicle)	36%, 0.2 mg/kg; 50%, 0.5 mg/kg; 82%, 1 mg/kg
Safety (impact on *in vivo* hemostasis)	Monkey	Kidney bleeding time (fold increase *vs.* vehicle)	1.4×, 0.2 mg/kg; 1.9×, 0.5 mg/kg; 2.2×, 1 mg/kg
		Mesenteric bleeding time (fold increase *vs.* vehicle)	1.4×, 0.2 mg/kg; 1.7×, 0.5 mg/kg; 1.8×, 1 mg/kg

PAR4, protease-activated receptor-4.

by Wong and colleagues expands on this to describe the development of a potent and specific small molecule PAR4 antagonist with a markedly improved therapeutic window over one standard antiplatelet drug (clopidogrel) in a preclinical model.

Discovery of a potent and specific small molecule PAR4 antagonist

In proof-of-concept work that supports previous studies (8-11), the team at BMS first used function-blocking anti-PAR4 antibodies in a guinea pig model to show *in vivo* anti-thrombotic efficacy and relative safety of selective PAR4 blockade. To shift to the highly desired small molecule approach, they then embarked on an impressive drug discovery program. The unique activation mechanism of PARs has provided a major hurdle for the development of efficacious antagonists. Thrombin cleavage of PARs reveals an endogenous tethered ligand which then binds to and self-activates the receptor. Therefore, antagonists must overcome an agonist that is intrinsic to the receptor and presumably has considerable steric advantage. Wong and colleagues screened a library of over 1 million compounds to identify a lead candidate that was then subject to iterative rounds of medicinal chemistry and testing to result in BMS-986120—a potent and selective PAR4 antagonist with impressive oral bioavailability and antithrombotic efficacy (*Table 1*).

Selective inhibition of PAR4 over PAR1 has been elusive in previous efforts developing small molecule PAR4 antagonists. For example, the indazole-derivative YD-3 (12) and its derivative ML354 (13) exhibit cross-

reactivity toward PAR1 (12,13). Here though, BMS-986120 demonstrated specificity in both HEK293 cells transfected with PAR4 and human platelets, with no effect observed on platelet activation by a PAR1 activating peptide, collagen, ADP, or thromboxane A_2 (*Table 1*). To demonstrate efficacy of BMS-986120, the authors examined inhibitory profiles of platelet aggregation against two isoforms of thrombin—α and γ, thought to preferentially activate PAR1 and PAR4 respectively. BMS-986120 effectively suppressed platelet aggregation in response to γ-thrombin but required concomitant PAR1 inhibition to do so against α-thrombin. Whether complete blockade of thrombin-induced platelet activation will be required for effective antithrombotic therapy, or whether partial inhibition will be sufficient, remains to be determined.

For PAR antagonists to be efficacious against endogenous enzymatic activation of the receptor by thrombin, they must exhibit strong binding affinity. Yet in the clinical context it is highly desirable for an anti-platelet agent to have the potential to be rapidly reversed should any unwanted bleeding challenges occur. Wong *et al.* used (^{3}H)-BMS-986120 binding to PAR4-expressing cell membranes to reveal that BMS-986120 is a high affinity and reversible binder of PAR4 (*Table 1*). In studies performed on platelets isolated from monkeys dosed with BMS-986120, this translated to normalised aggregation 24 h after a single dose of 0.2 mg/kg. A more detailed time course will be required to determine the half-life of BMS-986120. Although the dissociation constant is relatively fast, competition studies would be useful to demonstrate whether there was potential for an antidote if required. Regardless, the pharmacodynamic profile is considerably advantageous in comparison to other antiplatelet drugs. For example, platelet inhibition by the PAR1 antagonist vorapaxar is retained 4–8 weeks after a single loading dose in humans (14) while aspirin and clopidogrel are both irreversible protein modifiers with long-term effects.

Most importantly though, BMS-986120 appeared to provide an impressive therapeutic window, with a single oral dose of BMS-986120 providing marked antithrombotic effects and a low bleeding profile in a series of *in vivo* models in the cynomolgus monkey (*Table 1*). Appropriate examination of *in vivo* platelet PAR function is limited to primates since the traditional small animal models (e.g., mice, rats, guinea-pigs, rabbits, dogs) have a different PAR expression profile to that of humans. Therefore, Wong and colleagues used an electrolytic model of carotid artery thrombosis in the cynomolgus monkey for preclinical evaluation of their PAR4 antagonist. BMS-986120 alone prevented occlusive thrombus formation at the highest dose (1 mg/kg) and significantly reduced thrombosis at lower doses (0.2–0.5 mg/kg). Hemostasis, measured either by kidney or mesenteric bleeding time, was increased by just over 2-fold at the 1 mg/kg dose and much less at the lower doses. Importantly, when this dose-response of BMS-986120 on hemostasis and thrombosis was compared directly with that of clopidogrel, there was a clear separation provided by BMS-986120 that was not evident with clopidogrel. At doses of these two agents that caused equivalent anti-thrombotic effects, markedly more bleeding was observed with clopidogrel compared with BMS-986120. For example, at a dose that caused a 50% reduction in thrombus weight, clopidogrel induced a 7.3- to 8.1-fold increase in bleeding compared with a 1.7- to 1.9-fold increase for BMS-986120. This was more pronounced at doses that caused a near 100% reduction in thrombus weight, with clopidogrel inducing a >10-fold increase in bleeding versus a 1.8- to 2.2-fold increase for BMS-986120. Given this stark difference, it would be interesting to known how the therapeutic window changed when used in combination with aspirin, $P2Y_{12}$ antagonists and/or vorapaxar.

A potential mechanism for an improved therapeutic window

How is it that PAR4 inhibition provides such strong separation between impacting on thrombosis and hemostasis? One clue comes from recent work indicating that PAR4 performs distinct functions to other key platelet receptors. PAR4 activation elicits a slower, but significantly more sustained, intracellular calcium response than that elicited by PAR1 (15). This prolonged calcium signal mediates later-stage platelet activation events, such as the platelet procoagulant response involving phosphatidylserine exposure on the platelet membrane and consequent assembly of coagulation factors leading to thrombin generation and fibrin formation. Indeed, selective inhibition of PAR4 but not PAR1 significantly inhibits thrombin activity and fibrin deposition in human thrombi *ex vivo* (8). One explanation for the improved therapeutic window of BMS-986120 reported by Wong *et al.* is that PAR4 inhibition is blocking platelet function at a distinct time and place to all existing approaches.

What does the future hold for PAR4 antagonists?

BMS-986120 was evaluated in a phase 1 dosing study, yet despite efficacy and a lack of adverse events no phase 2 studies of this compound were undertaken. Rather, BMS are investigating the related compound, BMS-986141, which also underwent a phase 1 study (NCT02341638) and a subsequent phase 2 trial for the prevention of mini-stroke (NCT02671461). The trial (A Phase 2, Placebo Controlled, Randomized, Double-Blind, Parallel-Arm Study to Evaluate Efficacy and Safety of BMS-986141 For the Prevention of Recurrent Brain Infarction in Subjects Receiving Acetylsalicylic Acid Following Acute Ischemic Stroke or Transient Ischemic Attack) had a primary efficacy endpoint of a composite of symptomatic ischemic stroke or unrecognized brain infarction, and a primary safety endpoint of a composite of adjudicated major bleeding and adjudicated clinically relevant non-major bleeding during the treatment period. It was completed in April 2017 but has not yet been reported.

It is far too early to predict the likely clinical success and/or usefulness of PAR4 antagonists, and several key questions remain. How well will PAR4 antagonism combine with current standard-of-care agents? This is a central point, since any trial will be conducted in the presence of standard-of-care, which frequently involves dual antiplatelet therapy. With the PAR1 antagonist vorapaxar, for example, the increased bleeding observed is believed to be due to poor compatibility with clopidogrel. Indeed, sub-study analyses show no additional bleeding in patients receiving aspirin plus vorapaxar versus those receiving aspirin alone (6,16). Here, it is interesting to note that BMS chose to investigate a patient group being treated with aspirin alone in its first phase 2 trial of its lead PAR4 antagonist.

What specific indications will be best served by a PAR4 antagonist? Again, sub-study analyses of the vorapaxar trials may provide pointers. These trials showed the most efficacy in reducing the rate of spontaneous myocardial infarction as well as in prevention of vascular complications associated with peripheral artery disease. This is perhaps unsurprising given the well-known role of thrombin generation in acute myocardial infarction, particularly in patients with a background of unstable angina and/or coronary artery disease (17). Whether PAR4 antagonism will similarly demonstrate superior efficacy in these clinical situations where thrombin-induced platelet activation are implicated is an obvious place to start for future clinical trials.

Finally, one emerging issue for PAR4 antagonism is that of population genetics. Recent studies have revealed a commonly expressed genetic variant of PAR4 (rs773902; encoding either Ala120 or Thr120) that appears to significantly alter receptor pharmacology. Specifically, the Thr120 PAR4 variant, expressed in 20–80% of people depending on the population, renders the receptor hyper-sensitive to agonists and hypo-sensitive to antagonists (18,19). The mechanism behind this change in PAR4 pharmacology remains unknown, as does whether all PAR4 antagonists, including BMS-986120 and BMS-986141, will be similarly affected. Studies directly addressing these points will be critical in determining whether the approach proposed by Wong *et al.* will afford consistent antithrombotic benefit across the population.

Conclusions

The recent preclinical study by Wong *et al.* (5) details the development and preclinical evaluation of the first PAR4 antagonist to enter a clinical trial and represents a potentially important breakthrough in the treatment of arterial thrombosis. While further insights are still to be gained regarding the utility of PAR4 antagonism in clinical settings, this study has contributed an important reagent to help study this previously under-appreciated platelet activation mechanism, and has identified a potentially useful approach for the safe and effective prevention of arterial thrombosis.

Acknowledgements

This work was supported by grants to JRH from the National Health & Medical Research Council of Australia and the CASS Foundation. JR Hamilton is a Future Fellow of the Australian Research Council. The authors thank L Hodge for exceptional contributions over his career.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 2002;324:71-86.

2. French SL, Arthur JF, Tran HA, et al. Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. Blood Rev 2015;29:179-89.
3. Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists. What are the relevant issues concerning their pharmacology and clinical use? Circulation 1999;100:437-44.
4. Kastrati A, Mehilli J, Neumann FJ, et al. Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT 2 randomized trial. JAMA 2006;295:1531-8.
5. Wong PC, Seiffert D, Bird JE, et al. Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding. Sci Transl Med 2017;9(371). pii: eaaf5294.
6. Tricoci P, Huang Z, Held C, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. N Engl J Med 2012;366:20-33.
7. Morrow DA, Braunwald E, Bonaca MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. N Engl J Med 2012;366:1404-13.
8. French SL, Arthur JF, Lee H, et al. Inhibition of protease-activated receptor 4 impairs platelet procoagulant activity during thrombus formation in human blood. J Thromb Haemost 2016;14:1642-54.
9. Lee H, Sturgeon SA, Mountford JK, et al. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. Br J Pharmacol 2012;166:2188-97.
10. Sambrano GR, Weiss EJ, Zheng YW, et al. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 2001;413:74-8.
11. Covic L, Misra M, Badar J, et al. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. Nat Med 2002;8:1161-5.
12. Wu CC, Hwang TL, Liao CH, et al. Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. Thromb Haemost 2002;87:1026-33.
13. Wen W, Young SE, Duvernay MT, et al. Substituted indoles as selective protease activated receptor 4 (PAR-4) antagonists: Discovery and SAR of ML354. Bioorg Med Chem Lett 2014;24:4708-13.
14. Kosoglou T, Reyderman L, Tiessen RG, et al. Pharmacodynamics and pharmacokinetics of the novel PAR-1 antagonist vorapaxar (formerly SCH 530348) in healthy subjects. Eur J Clin Pharmacol 2012;68:249-58.
15. Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. Biochem 2000;39:5458-67.
16. Mahaffey KW, Huang Z, Wallentin L, et al. Association of aspirin dose and vorapaxar safety and efficacy in patients with non-ST-segment elevation acute coronary syndrome (from the TRACER Trial). Am J Cardiol 2014;113:936-44.
17. Merlini PA, Bauer KA, Oltrona L, et al. Persistent activation of coagulation mechanism in unstable angina and myocardial infarction. Circulation 1994;90:61-8.
18. Edelstein LC, Simon LM, Montoya RT, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. Nat Med 2013;19:1609-16.
19. Edelstein LC, Simon LM, Lindsay CR, et al. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. Blood 2014;124:3450-8.

Cite this article as: French SL, Hamilton JR. Drugs targeting protease-activated receptor-4 improve the anti-thrombotic therapeutic window. Ann Transl Med 2017. doi: 10.21037/atm.2017.09.10

References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics-2015 update: a report from the American Heart Association. *Circulation*. 2015;131(4):e29-322.

2. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *Eur Heart J*. 2016;37(3):267-315.

3. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate–receptor interactions in platelet thrombus formation under flow. *Cell*. 1998;94(5):657-66.

4. Vicente V, Kostel P, Ruggeri Z. Isolation and functional characterization of the von Willebrand factor-binding domain located between residues His1-Arg293 of the alpha-chain of glycoprotein Ib. *J Biol Chem*. 1988;263(34):18473-9.

5. White JG. Platelet Structure. In: Michelson AD, editor. Platelets. Third ed. London: Academic Press; 2013. p. 117-45.

6. Antithrombotic Trialists'Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002;324(7329):71-86.

7. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet*. 1996;348(9038):1329-39.

8. Bhatt DL, Fox KA, Hacke W, Berger PB, Black HR, Boden WE, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. *N Engl J Med*. 2006;354(16):1706-17.

9. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation*. 2003;107(23):2908-13.

10. Goodman T, Ferro A, Sharma P. Pharmacogenetics of aspirin resistance: a comprehensive systematic review. *Br J Clin Pharmacol*. 2008;66(2):222-32.

11. Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists: what are the relevant issues concerning their pharmacology and clinical use? *Circulation*. 1999;100(4):437-44.

12. Kastrati A, Mehilli J, Neumann F-J, Dotzer F, ten Berg J, Bollwein H, et al. Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT 2 randomized trial. *JAMA*. 2006;295(13):1531-8.

13. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000;407(6801):258-64.

14. Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, et al. A dual thrombin receptor system for platelet activation. *Nature*. 1998;394(6694):690-4.

15. Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991;64(6):1057-68.

16. Xu W-f, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, et al. Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A*. 1998;95(12):6642-6.

17. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, et al. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. 1997;386(6624):502-6.

18. Howells GL, Macey MG, Chinni C, Hou L, Fox MT, Harriott P, et al. Proteinase-activated receptor-2: expression by human neutrophils. *J Cell Sci*. 1997;110(7):881-7.

19. Bono F, Lamarche I, Herbert J. Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochem Biophys Res Commun*. 1997;241(3):762-4.

20. Hamilton JR, Chow JM, Cocks TM. Protease-activated receptor-2 turnover stimulated independently of receptor activation in porcine coronary endothelial cells. *Br J Pharmacol*. 1999;127(3):617-22.

21. Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, et al. Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. *Circ Res*. 2000;86(10):1054-61.

22. Sabri A, Short J, Guo J, Steinberg SF. Protease-Activated Receptor-1–Mediated DNA Synthesis in Cardiac Fibroblast Is via Epidermal Growth Factor Receptor Transactivation Distinct PAR-1 Signaling Pathways in Cardiac Fibroblasts and Cardiomyocytes. *Circ Res*. 2002;91(6):532-9.

23. Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, et al. Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem*. 1997;272(7):4043-9.

24. Camerer E, Huang W, Coughlin SR. Tissue factor-and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci U S A*. 2000;97(10):5255-60.

25. Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem*. 2000;275(34):26333-42.

26. Scarborough R, Naughton M, Teng W, Hung D, Rose J, Vu T, et al. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem*. 1992;267(19):13146-9.

27. Offermanns S. Activation of platelet function through G protein–coupled receptors. *Circ Res*.. 2006;99(12):1293-304.

28. Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase–mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol*. 1999;144(4):745-54.

29. Offermanns S, Toombs CF, Hu Y-H, Simon MI. Defective platelet activation in Gαq-deficient mice. *Nature*. 1997;389(6647):183-6.

30. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Investig*. 1999;103(6):879-87.

31. Liu L-W, Vu T, Esmon C, Coughlin S. The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. *J Biol Chem*. 1991;266(26):16977-80.

32. Nieman MT, Schmaier AH. Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. *Biochemistry*. 2007;46(29):8603-10.

33. Jacques SL, LeMasurier M, Sheridan PJ, Seeley SK, Kuliopulos A. Substrate-assisted catalysis of the PAR1 thrombin receptor, enhancement of macromolecular association and cleavage. *J Biol Chem*. 2000;275(52):40671-8.

34. Covic L, Singh C, Smith H, Kuliopulos A. Role of the PAR4 thrombin receptor in stabilizing platelet-platelet aggregates as revealed by a patient with Hermansky-Pudlak syndrome. *Thromb Haemost.* 2002;87(4):722-7.

35. Jacques S, Kuliopulos A. Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem J*. 2003;376:733-40.

36. Derian CK, Damiano BP, Addo MF, Darrow AL, D'Andrea MR, Nedelman M, et al. Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule

antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. *J Pharmacol Exp Ther*. 2003;304(2):855-61.

37. Sambrano GR, Weiss EJ, Zheng Y-W, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature*. 2001;413(6851):74-8.

38. Hollenberg MD, Saifeddine M. Proteinase-activated receptor 4 (PAR4): activation and inhibition of rat platelet aggregation by PAR4-derived peptides. *Can J Physiol Pharmacol*. 2001;79(5):439-42.

39. Khan TA, Bianchi C, Voisine P, Sandmeyer J, Feng J, Sellke FW. Aprotinin inhibits protease-dependent platelet aggregation and thrombosis. *Ann Thorac Surg*. 2005;79(5):1545-50.

40. Andrade-Gordon P, Maryanoff BE, Derian CK, Zhang HC, Addo MF, Darrow AL, et al. Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci U S A*. 1999;96(22):12257-62.

41. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. *Blood*. 2002;100(9):3240-4.

42. Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature*. 2000;404(6778):609-13.

43. Hamilton J, Cornelissen I, Coughlin S. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. *J Thromb Haemost*. 2004;2(8):1429-35.

44. Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. *Proc Natl Acad Sci U S A*. 2007;104(1):288-92.

45. Lee H, Sturgeon S, Mountford J, Jackson S, Hamilton J. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. *Br J Pharmacol*. 2012;166(7):2188-97.

46. Lee H, Sturgeon SA, Jackson SP, Hamilton JR. The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. *Thromb Haemost*. 2012;107(2):328-37.

47. Mao Y, Zhang M, Tuma RF, Kunapuli SP. Deficiency of PAR4 attenuates cerebral ischemia/reperfusion injury in mice. *J Cereb Blood Flow Metab*. 2010;30(5):1044-52.

48. Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van de Werf F, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *N Engl J Med*. 2012;366(1):20-33.

49. Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366(15):1404-13.

50. Mahaffey KW, Huang Z, Wallentin L, Storey RF, Jennings LK, Tricoci P, et al. Association of aspirin dose and vorapaxar safety and efficacy in patients with non–ST-segment elevation acute coronary syndrome (from the TRACER Trial). *Am J Cardiol*. 2014;113(6):936-44.

51. Duerschmied D, Bode C. Vorapaxar expands antiplatelet options. Which patients may benefit from thrombin receptor antagonism? *Hamostaseologie*. 2011;32(3):221-7.

52. Leonardi S, Tricoci P, White HD, Armstrong PW, Huang Z, Wallentin L, et al. Effect of vorapaxar on myocardial infarction in the thrombin receptor antagonist for clinical event reduction in acute coronary syndrome (TRA.CER) trial. *Eur Heart J*. 2013;34(23):1723-31.

53. Merlini PA, Bauer KA, Oltrona L, Ardissino D, Cattaneo M, Belli C, et al. Persistent activation of coagulation mechanism in unstable angina and myocardial infarction. *Circulation*. 1994;90(1):61-8.

54. Szczeklik A, Dropinski J, Radwan J, Krzanowski M. Persistent generation of thrombin after acute myocardial infarction. *Arterioscler Thromb Vasc Biol*. 1992;12(5):548-53.

55. Huisse M-G, Lanoy E, Tcheche D, Feldman LJ, Bezeaud A, Anglès-Cano E, et al. Prothrombotic markers and early spontaneous recanalization in ST-segment elevation myocardial infarction. *Thromb Haemost*. 2007;98(2):420.

56. Bonaca MP, Scirica BM, Creager MA, Olin J, Bounameaux H, Dellborg M, et al. Vorapaxar in Patients With Peripheral Artery Disease Results From TRA2° P-TIMI 50. *Circulation*. 2013;127(14):1522-9.

57. Kahn ML, Hammes SR, Botka C, Coughlin SR. Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *J Biol Chem*. 1998;273(36):23290-6.

58. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. *Nature*. 1991;353(6345):674-7.

59. Mathews I, Padmanabhan K, Ganesh V, Tulinsky A, Ishii M, Chen J, et al. Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry*. 1994;33(11):3266-79.

60. Zhu WJ, Yamanaka H, Obata K, Dai Y, Kobayashi K, Kozai T, et al. Expression of mRNA for four subtypes of the proteinase-activated receptor in rat dorsal root ganglia. *Brain Res*. 2005;1041(2):205-11.

61. Vergnolle N, Derian CK, D'Andrea MR, Steinhoff M, Andrade-Gordon P. Characterization of thrombin-induced leukocyte rolling and adherence: a potential proinflammatory role for proteinase-activated receptor-4. *J Immunol*. 2002;169(3):1467-73.

62. Kataoka H, Hamilton JR, McKemy DD, Camerer E, Zheng YW, Cheng A, et al. Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood*. 2003;102(9):3224-31.

63. Hollenberg MD, Saifeddine M, Al-Ani B, Gui Y. Proteinase-activated receptor 4 (PAR4): action of PAR4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR1 and PAR2. *Can J Physiol Pharmacol*. 1999;77(6):458-64.

64. Hamilton JR, Frauman AG, Cocks TM. Increased expression of protease-activated receptor-2 (PAR2) and PAR4 in human coronary artery by inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. *Circ Res*. 2001;89(1):92-8.

65. Vidwan P, Pathak A, Sheth S, Huang J, Monroe DM, Stouffer GA. Activation of protease-activated receptors 3 and 4 accelerates tissue factor-induced thrombin generation on the surface of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2010;30(12):2587-96.

66. Ramachandran R, Sadofsky LR, Xiao Y, Botham A, Cowen M, Morice AH, et al. Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(3):L788-98.

67. Cottrell GS, Amadesi S, Grady EF, Bunnett NW. Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. *J Biol Chem*. 2004;279(14):13532-9.

68. Ayala YM, Cantwell AM, Rose T, Bush LA, Arosio D, Di Cera E. Molecular mapping of thrombin-receptor interactions. *Proteins: Struct Funct Bioinf*. 2001;45(2):107-16.

69. Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry*. 2000;39(18):5458-67.

70. Faruqi TR, Weiss EJ, Shapiro MJ, Huang W, Coughlin SR. Structure-function analysis of protease-activated receptor 4 tethered ligand peptides. Determinants of specificity and utility in assays of receptor function. *J Biol Chem*. 2000;275(26):19728-34.

71. Voss B, McLaughlin JN, Holinstat M, Zent R, Hamm HE. PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. *Mol Pharmacol*. 2007;71(5):1399-406.

72. Offermanns S, Laugwitz K-L, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci U S A*. 1994;91(2):504-8.

73. Dorsam RT, Kim S, Jin J, Kunapuli SP. Coordinated signaling through both G12/13 and Gi pathways is sufficient to activate GPIIb/IIIa in human platelets. *J Biol Chem*. 2002;277(49):47588-95.

74. Dabek M, Ferrier L, Annahazi A, Bezirard V, Polizzi A, Cartier C, et al. Intracolonic infusion of fecal supernatants from ulcerative colitis patients triggers altered permeability and inflammation in mice: role of cathepsin G and protease-activated receptor-4. *Inflamm Bowel Dis*. 2011;17(6):1409-14.

75. Duvernay M, Young S, Gailani D, Schoenecker J, Hamm HE. Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets. *Mol Pharmacol*. 2013;83(4):781-92.

76. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost*. 2005;3(8):1800-14.

77. Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. *Proc Natl Acad Sci U S A*. 1997;94(16):8750-5.

78. Lau LF, Pumiglia K, Cote YP, Feinstein MB. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem J*. 1994;303 (Pt 2):391-400.

79. Falker K, Haglund L, Gunnarsson P, Nylander M, Lindahl TL, Grenegard M. Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets. *Biochem J*. 2011;436(2):469-80.

80. Shapiro MJ, Trejo J, Zeng D, Coughlin SR. Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J Biol Chem*. 1996;271(51):32874-80.

81. Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem*. 2000;275(33):25216-21.

82. Ma L, Perini R, McKnight W, Dicay M, Klein A, Hollenberg MD, et al. Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. *Proc Natl Acad Sci U S A*. 2005;102(1):216-20.

83. Hollenberg MD, Saifeddine M, Sandhu S, Houle S, Vergnolle N. Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists in vivo. *Br J Pharmacol*. 2004;143(4):443-54.

84. Covic L, Gresser AL, Talavera J, Swift S, Kuliopulos A. Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci U S A*. 2002;99(2):643-8.

85. Leger AJ, Jacques SL, Badar J, Kaneider NC, Derian CK, Andrade-Gordon P, et al. Blocking the protease-activated receptor 1-4 heterodimer in platelet-mediated thrombosis. *Circulation*. 2006;113(9):1244-54.

86. Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Lee CY, et al. Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. *Thromb Haemost*. 2002;87(6):1026-33.

87. Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Teng CM. The role of PAR4 in thrombin-induced thromboxane production in human platelets. *Thromb Haemost*. 2003;90(2):299-308.

88. Peng CY, Pan SL, Guh JH, Liu YN, Chang YL, Kuo SC, et al. The indazole derivative YD-3 inhibits thrombin-induced vascular smooth muscle cell proliferation and attenuates intimal thickening after balloon injury. *Thromb Haemost*. 2004;92(6):1232-9.

89. Huang LJ, Shih ML, Chen HS, Pan SL, Teng CM, Lee FY, et al. Synthesis of N2-(substituted benzyl)-3-(4-methylphenyl)indazoles as novel anti-angiogenic agents. *Bioorg Med Chem*. 2006;14(2):528-36.

90. Young SE, Duvernay MT, Schulte ML, Lindsley CW, Hamm HE. Synthesis of indole derived protease-activated receptor 4 antagonists and characterization in human platelets. *PLoS One*. 2013;8(6):e65528.

91. Wen W, Young SE, Duvernay MT, Schulte ML, Nance KD, Melancon BJ, et al. Substituted indoles as selective protease activated receptor 4 (PAR-4) antagonists: Discovery and SAR of ML354. *Bioorg Med Chem Lett*. 2014;24(19):4708-13.

92. Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med*. 2002;8(10):1161-5.

93. Mumaw MM, de la Fuente M, Noble DN, Nieman MT. Targeting the anionic region of human protease-activated receptor 4 inhibits platelet aggregation and thrombosis without interfering with hemostasis. *J Thromb Haemost*. 2014;12(8):1331-41.

94. Mumaw MM, de la Fuente M, Arachiche A, Wahl JK, 3rd, Nieman MT. Development and characterization of monoclonal antibodies against Protease Activated Receptor 4 (PAR4). *Thromb Res*. 2015;135(6):1165-71.

95. Stampfuss JJ, Schror K, Weber AA. Inhibition of platelet thromboxane receptor function by a thrombin receptor-targeted pepducin. *Nat Med*. 2003;9(12):1447-8.

96. Lee FY, Lien JC, Huang LJ, Huang TM, Tsai SC, Teng CM, et al. Synthesis of 1-benzyl-3-(5'-hydroxymethyl-2'-furyl)indazole analogues as novel antiplatelet agents. *J Med Chem*. 2001;44(22):3746-9.

97. Chen HS, Kuo SC, Teng CM, Lee FY, Wang JP, Lee YC, et al. Synthesis and antiplatelet activity of ethyl 4-(1-benzyl-1H-indazol-3-yl)benzoate (YD-3) derivatives. *Bioorg Med Chem*. 2008;16(3):1262-78.

98. Wong PC, Seiffert D, Bird JE, Watson CA, Bostwick JS, Giancarli M, et al. Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding. *Sci Transl Med*. 2017;9(371): eaaf5294.

99. Tourdot BE, Conaway S, Niisuke K, Edelstein LC, Bray PF, Holinstat M. Mechanism of race-dependent platelet activation through the protease-activated receptor-4 and Gq signaling axis. *Arterioscler Thromb Vasc Biol*. 2014;34(12):2644-50.

100. Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nat Med*. 2013;19(12):1609-16.

101. Edelstein LC, Simon LM, Lindsay CR, Kong X, Teruel-Montoya R, Tourdot BE, et al. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood*. 2014;124(23):3450-8.

102. Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost*. 2013;11(1):2-16.

103. Vretenbrant K, Ramstrom S, Bjerke M, Lindahl TL. Platelet activation via PAR4 is involved in the initiation of thrombin generation and in clot elasticity development. *Thromb Haemost*. 2007;97(3):417-24.

104. Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci U S A.* 1999;96(20):11189-93.

105. Keuren JF, Wielders SJ, Ulrichts H, Hackeng T, Heemskerk JW, Deckmyn H, et al. Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler Thromb Vasc Biol*. 2005;25(7):1499-505.

106. Agbani EO, Poole AW. Procoagulant Platelets:-Generation, Function and Therapeutic Targeting in Thrombosis. *Blood*. 2017. doi: 10.1182/blood-2017-05-787259

107. Mega JL, Simon T. Pharmacology of antithrombotic drugs: an assessment of oral antiplatelet and anticoagulant treatments. *Lancet*. 2015;386(9990):281-91.

108. Arachiche A, de la Fuente M, Nieman MT. Platelet Specific Promoters Are Insufficient to Express Protease Activated Receptor 1 (PAR1) Transgene in Mouse Platelets. *PloS One*. 2014;9(5):e97724.

109. Sims P, Wiedmer T, Esmon CT, Weiss H, Shattil S. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem*. 1989;264(29):17049-57.

110. Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, et al. High-resolution crystal structure of human protease-activated receptor 1. *Nature*. 2012;492(7429):387-92.