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Identification and Elucidation of Novel Platelet Primers

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Research in the Faculty of Life Sciences, School of Physiology, Pharmacology and Neuroscience

September 2018

Signed:

Date:

Word count: 24711

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Abstract

Normal platelet function is important for the body to maintain haemostasis. Under pathological conditions, platelet activity may be altered by elevated circulating priming factors. Primers can enhance platelet responses to physiological agonists such as thrombin and ADP, which leads to an increased risk of thrombus formation and resistance to anti-thrombotic drugs. Previous studies have identified several platelet primers including thrombopoietin, insulin growth factor 1 and sCD40 ligands. There is evidence to suggest that more platelet primers may exist, however, the identity and underlying mechanism of novel platelet primers is still largely unknown. This project therefore aims to identify novel primers and the underlying mechanism by which they affect platelet function. Through literature research and platelet transcriptome expression studies, a list of potential platelet primers was generated. Using platelet aggregation studies, primers that increased protease-activated receptor 1 (PAR1)-mediated platelet responses were taken for further studies. Of these primers, sphingosine-1-phosphate (S1P) was identified as the novel platelet primer to be studied in more detail. This study demonstrated that S1P exhibited a concentration dependent biphasic effect on platelet function, with low concentrations of S1P enhancing PAR1-mediated platelet responses, while inhibitory effects were observed at high concentrations of S1P. To demonstrate the mechanism of S1P biphasic effects and probe the S1P receptor subtypes expression on platelets, S1P receptors agonists and antagonists were tested in aggregation, flow cytometry, immunoblotting, TxB2 ELISA, Ca^{2+} mobilization assay and platelet spreading experiments. Current results suggest that the S1P's priming effect was mediated via activation of phosphoinositide 3-kinase (PI3K) signalling pathway, resulting in increased integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure. No significant effect of S1P on platelet calcium signalling or thromboxane A2 synthesis was observed. This study also indirectly suggests that the $S1PR_1$, $S1PR_4$ and $S1PR_5$ receptors are expressed on human platelets. The positive priming effect of S1P is mediated via activation of $S1PR_1$ receptor, while inhibitory effects are likely to be mediated via $S1PR_4$ and $S1PR_5$ receptor activation. Furthermore, this study also demonstrated that sphingosine kinases may play a role in regulating platelet function.

List of Abbreviations

AA	arachidonic acid
ABCC4	ATP-binding cassette transporter 4
AC	adenylyl cyclase
ACD	acid citrate dextrose
ADP	adenosine diphosphate
Akt/PKB	protein kinase B
ApoA-I	apolipoprotein A-I;
ApoM	apolipoprotein M
APS	ammonium persulfate
AUC	area under the curve
BSA	fatty acid free bovine serum albumin
C3aR1	complement component 3a receptor 1
CalDAG-GEFI	calcium-diacylglycerol guanine nucleotide exchange factor I
cAMP	cyclic adenosine monophosphate
CCL17	chemokine ligand 17
CCL22	chemokine ligand 22
CCR4	C-C chemokine receptor type 4.
Cdc42	cell division control protein 42 homolog
CERase	ceramidases
CHO	Chinese hamster ovary
C-MPL	thrombopoietin receptor.
COX-1/2	cyclooxygenase 1/2
CRP	collagen-related pepitide
CSF1R	colony stimulating factor 1 receptor
DAG	diacylglycerol
DHS1P	D-erythro-dihydrosphingosine-1-phosphate

DMS	N,N-dimethylsphingosine
DMSO	dimethyl sulfoxide
DST	dense tubular system
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP_3	prostaglandin EP3 receptor
ERK	extracellular signal related kinase
FACS	fluorescence-activated cell sorting
Fyn	proto-oncogene tyrosine-protein kinase Fyn
Gas6	growth arrest-specific 6
GDP	guanosine diphosphate
GPCR	G-protein coupled receptors
GPR55	G protein-coupled receptor 55
GPVI	glycoprotein VI
GTP	guanosine triphosphate
HDL	high-density lipoprotein
HEX	hexadecimal
IGF-1	insulin growth factor 1
IL-17A	interleukin-17A
IL-34	interleukin-34
IP	prostacyclin receptor
IP3R	inositol trisphosphate receptor (IP3R)
IRS1	insulin receptor substrate 1
ITAM	immune-receptor tyrosine-based activation motif
JAK2	Janus kinase 2.
JNK	c-Jun N-terminal kinase

Lyn	Lck/Yes novel tyrosine kinase
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MDC	macrophage-derived chemokine
MMP-1	metalloproteinase-1
mRNA	messenger ribonucleic acid
NO	nitric oxide
P_2Y	purinergic receptor
PAF	platelet-activating factor receptor
PAR1	protease-activated receptor 1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factors
PDK	phosphoinositide-dependent kinase.
PE	phosphoethanolamine
PGE_2	prostaglandin E2
PGG_2	prostaglandin G2
PGH_2	prostaglandin H2
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4, 5-bisphosphate.
PIP_3	phosphatidylinositol (3, 4, 5)-trisphosphate.
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLCβ	phospholipase C β
PLCγ	phospholipase C γ

PS	phosphatidylserine
PTX	pertussis toxin
Rac	Ras-related C3 botulinum toxin substrate
Rac-GAP	Rac-GTPase-activating protein
Rap1b	Ras-related protein 1b
RASA3	Ras GTPase-activating protein 3
RBC	red blood cells
Rho	Ras homolog gene family
RPKM	reads per kilobase of transcript per million mapped reads
RTK	receptor tyrosine kinase
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
sCD40	soluble cluster of differentiation 40 ligand
SDF-1	stromal cell-derived factor 1
siRNA	small interfering Ribonucleic Acid
SMase	sphingomyelinases
SPHK1/2	sphingosine kinase1/2
SPL	sphingosine-1-phosphate lyse;
SPP	sphingosine-1-phosphate phosphatase
Syk	spleen tyrosine kinase
TARC	thymus and activation regulated chemokine
TBS	Tris buffered saline
TEMED	tetramethylethylenediamine
TF	tissue factor
THC	tetrahydrocannabinol. IL-34 interleukin-34.
TP	thromboxane receptor.
TPO	thrombopoietin

TxA2	thromboxane A2
TxB2	thromboxane B2
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

Chapter 1. General introduction

1.1 Platelet physiology and function

1.1.1 Overview of platelet and thrombotic disorder.

Platelets, also known as thrombocytes, are small non-nucleated cells derived from megakaryocytes that circulating in the blood to stop vascular bleeding and maintain haemostasis[1]. Damage in the vessel can activate platelets result in blood clotting and formation of thrombus. Thrombosis is when a thrombus is formed within the vessel and cause blood flow to stop, which can lead to development of cardiovascular complications such as deep vein thrombosis, stroke or heart attack[2]. Cardiovascular disease is the major cause of death, which contribute to over 18 million of the total mortality worldwide[3]. Under normal physiological conditions, platelet function are strictly controlled and its activity is suppressed by circulating antithrombotic mediators[4]. However, disruptions in this strictly regulated process might result in elevated platelet response and lead to development of thrombotic disorders. Current therapeutic strategies to prevent thrombosis include use of anti-coagulants, thrombolysis drugs or antiplatelet agents. Besides off target effects such as gastrointestinal bleeding, certain population of patients also have shown resistance to antiplatelet drugs[5]. Genetic variations between individuals might contribute to antiplatelet resistance. However, several studies have found that the concentrations of certain bioactive molecules were elevated in these patients, resulting in altered platelet responses to physiological agonists and a higher risk of developing thrombotic disorder[6]. Bioactive molecules that cause platelet hyperactivity and resistance to antiplatelet therapy are classified as “platelet primers”. Previous studies have identified several platelet primers such as thrombopoietin (TPO), insulin growth factor 1 (IGF-1), sCD40 ligand and prostaglandin E_2 (PGE_2)[7]. Recent research suggests that more platelet primers exist. Hence, identifying novel platelet primers and determining the underlying mechanism by which they increase platelet function can provide us with a better understanding of pathological platelet function, which might lead to the development of novel therapeutic strategies to treat cardiovascular disease[8].

1.1.2 Platelet activation and signalling.

Under in vivo blood flow condition, platelets are pushed towards the vessel wall due to the small cell size and discoid shape thus circulating along the endothelial lining[9]. Healthy intact vascular endothelium constantly releases antithrombotic factors, in particular, prostacyclin (PGI_2), nitric oxide (NO) and anti-thrombin factors III to supress the flowing platelets activity and inhibit the coagulation pathway[10]. Injury or disruption in the endothelial wall cause exposure of sub-endothelial matrix, which contain several thrombogenic components include collagen, von Willebrand factor (vWF), tissue factors (TF), fibronectin and laminin (Fig. 1). These thrombogenic factors can activate and interact with receptors expressed on the platelet

surface, lead to tethering and capture of circulating platelets to the sub-endothelium. The platelet adhesion process is initiated by the interaction between vWF in the extracellular matrix and glycoprotein Ib-IX-V receptor complex expressed on platelets[11] (Fig. 1). Binding of glycoprotein VI receptors to the sub-endothelial collagen results in platelet activation and stimulates activation of integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$. These integrins are adhesion receptors that can interact with collagen, vWF and fibrinogen, resulting in firm and irreversible adhesion of platelets to the sub-endothelial matrix and formation of stable platelet-platelet aggregate[12] (Fig. 1).

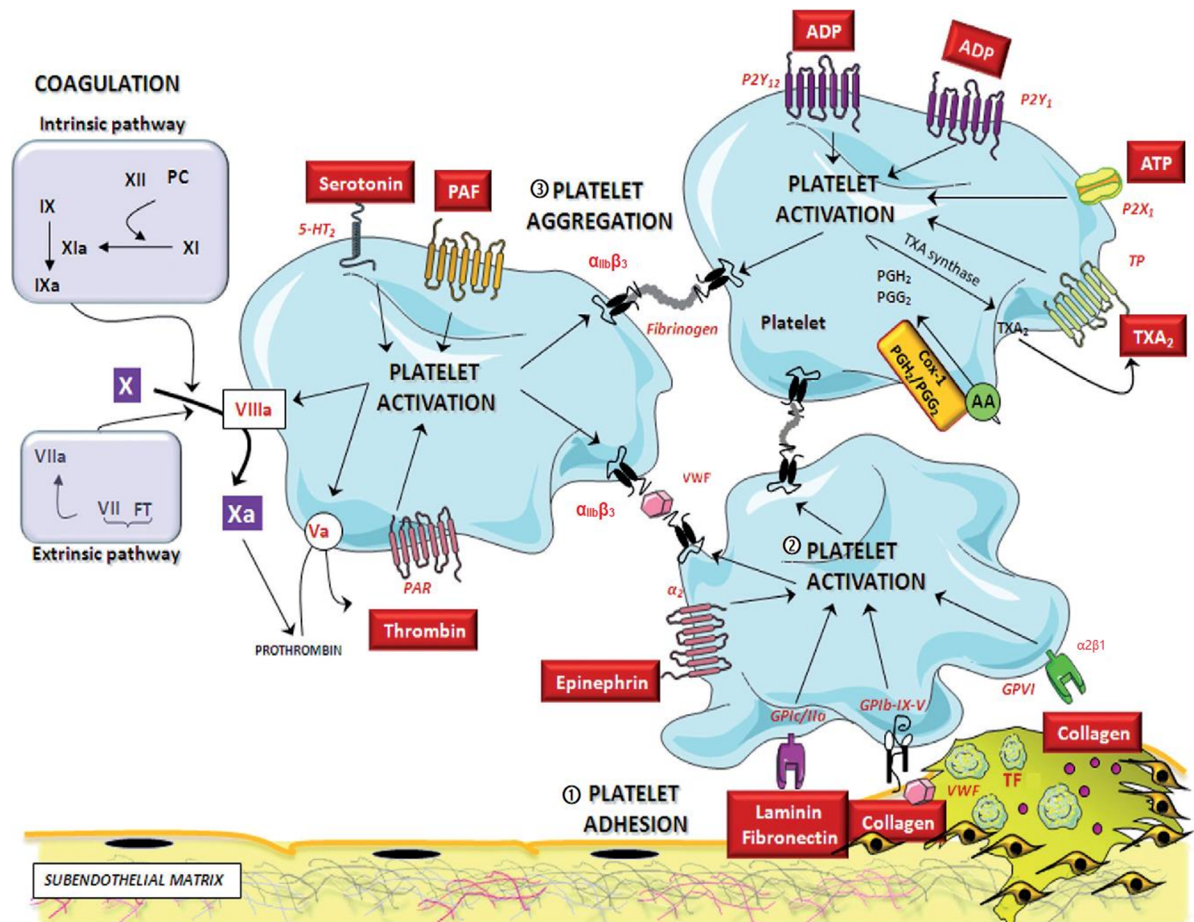


Figure 1. Schematic diagram illustrating the mechanism of vascular injury and subsequent platelet adhesion, activation and aggregation. AA: arachidonic acid; ADP: adenosine diphosphate; PGG_2: prostaglandin G2; PGH2: prostaglandin H_2; Cox-1: cyclooxygenase 1; PAF: platelet-activating factor receptor; PAR: Protease-activated receptor; TP: thromboxane receptor. (Figure adapted from Badimon et al [13])

Activation of GPVI receptor results in inside-out signalling and activation of integrins. GPVI activates phospholipase C (PLC), initiate production of diacylglycerol (DAG) and IP_3. Downstream effects of DAG and IP_3 cause of Ca^{2+} mobilisation and activation of integrin $\alpha_{IIb}\beta_3$, the effect of integrin $\alpha_{IIb}\beta_3$ will be discussed in more detail later. Activated integrins can also signal outside-in triggering the platelet degranulation process. DAG and Ca^{2+} can regulate the activity of protein kinase C (PKC), which mediate platelet granule secretion. An increase in Ca^{2+} also activates phospholipase A2 resulting thromboxane A2 (TxA2) synthesis.

Synthesized TxA2 and granule released ADP can activate G-protein coupled receptors (GPCRs) expressed on platelets. Activation of $G_{\alpha12/13}$ coupled GPCRs leads to downstream activation of the Rho family of small GTPases, which play important roles in regulating intracellular actin rearrangement and cytoskeletal dynamics[14]. Family members include RhoA, Rac1 and Cdc42 and these regulate platelet adhesion, shape change and granule secretion process via control the formation of stress fibre, lamellipodia and filopodia respectively. A study on Rac1 knock out mice demonstrated that Rac1 controls lamellipodia formation in platelets and inhibition of Rac1 significantly reduced platelet aggregate strength[15].Upon activation, platelets change shape from discoid shape to a spiculated sphere with many filopodia protrusions formed on the surface. This platelet morphology change and the spreading process greatly increase the cellular surface area, which allows further platelet-platelet interactions and aggregate formation to take place and enables subsequent granule exocytosis[16].

1.1.3 Platelet calcium mobilization, granule secretion and TxA2 production

Activation of the GPVI receptor induces intracellular calcium release and the subsequent platelet granule secretion process. Many studies have demonstrated the importance of calcium signalling in platelet activation[17]. Activation of the GPVI receptor results in phosphorylation of the immune-receptor tyrosine-based activation motif (ITAM) on the FcRγ chain, which causes activation of Src family kinases Fyn and Lyn[18] (Fig. 2). Fyn and Lyn recruit and activate spleen tyrosine kinase (Syk), phospholipase C-γ (PLCγ) and phosphoinositide 3-kinase (PI3K). PLCγ2 activation leads to IP_3 production and subsequent activation of the inositol trisphosphate receptor (IP_3R) on the dense tubular system (DST) causing calcium release. Elevated cytosolic calcium promotes subsequent platelet degranulation, thromboxane A2 production, GRCRs signalling and integrin $\alpha_{IIb}\beta_3$ activation (Fig. 2). The overall effect of these platelet responses is amplification of platelet activation and aggregation processes, resulting in the formation of stable aggregates and promoting thrombus growth. These platelet functional responses can easily be measured and quantified by experimental approaches to assess the overall platelet responsiveness.

Platelet granule secretion is mediated by PKC and Ca^{2+}. There are two types of granules in platelets, alpha granule and dense granule together contain many bioactive molecules ready to be released. Alpha granules contain cytokines and growth factors such as IGF-1, platelet-derived growth factors (PDGF). Some adhesion factors including vWF, fibrinogen and coagulation factors are also stored in alpha granules[19]. Moreover, adhesion molecules such as P-selectin, $\alpha_{IIb}\beta_3$ integrin and additional GPVI receptors are present within the alpha granules and can be translocated to the platelet cell membrane to enhance platelet adhesion and activation. The surface content of P-selectin can be measured by a fluorescent labelled antibody and used to quantify platelet granule secretion. Dense granule is another type of

granule, which releases ADP that can activate the purinergic G-protein coupled $P2Y_{12}$ ($G_{\alpha i}$) and $P2Y_1$ ($G_{\alpha q}$) receptors in both autocrine and paracrine manner[20] (Fig. 1). In addition, serotonin and histamine are released from dense granules, which contributes to stabilising thrombus formation and facilitates the coagulation pathway[21]. Furthermore, continuous granule release builds up a high local concentration of pro-coagulation factors which can counteract the effects brought by antithrombotic factor such as PGI_2 and NO[22].

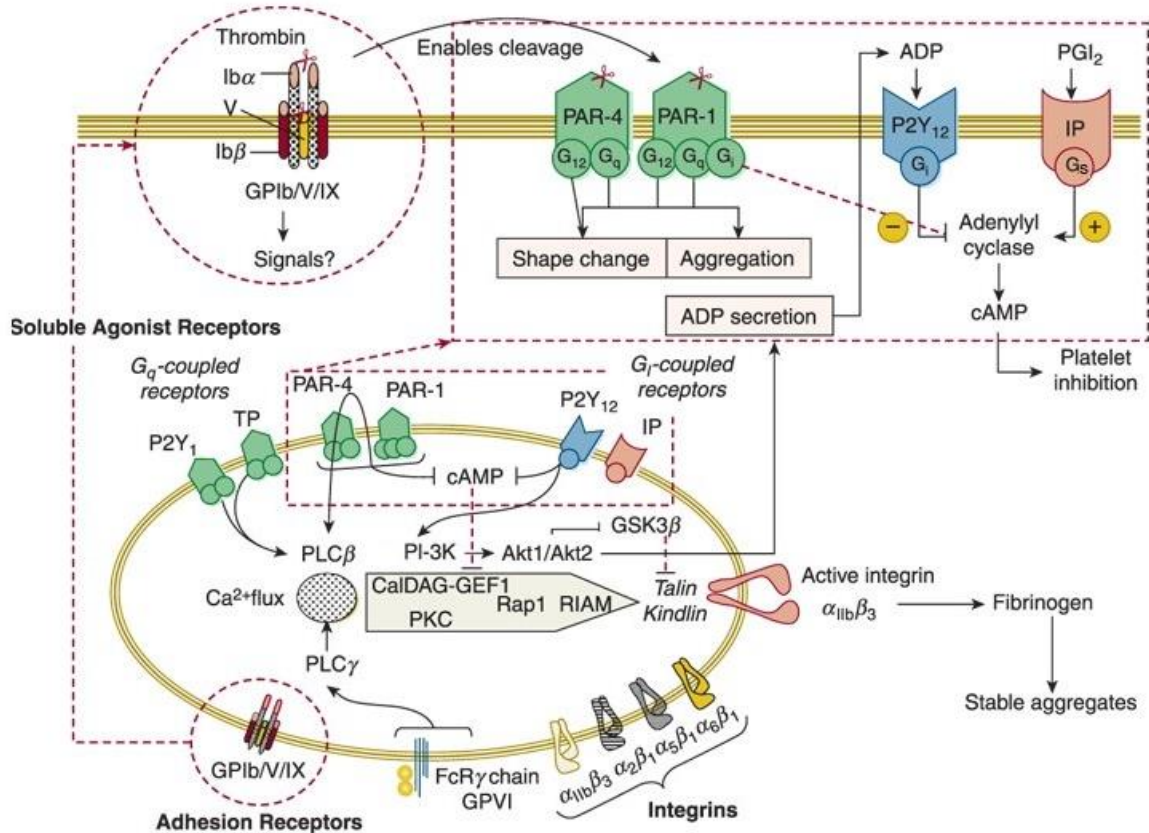


Figure 2. Schematic representation of platelet activation resulting in subsequent calcium release, GPCRs signalling and integrin activation. ADP: adenosine diphosphate; PGI_2: prostaglandin I_2; PI3K: phosphatidylinositol-4, 5-bisphosphate 3-kinase; Akt: protein kinase B. PLC: phospholipase C; PKC: protein kinase C; Rap1b: Ras-related protein 1b; RIAM: Rap1-GTP-interacting adaptor molecule (Figure adapted from Kaushansky et al[23])

In addition to granule secretion, another important platelet agonist is thromboxane A2, which is synthesized by activated platelets. A rise in intracellular calcium concentration activates the calcium dependent phospholipase A2 (PLA2), PLA2 catalyses phospholipids on the DTS surface to arachidonic acid (AA), AA is converted to prostaglandin G_2 (PGG_2) by cyclooxygenases (COX) and subsequently to prostaglandin H_2 (PGH_2) by peroxidase[24] (Fig. 1). TxA2 is generated from PGH_2 by thromboxane synthase, which can bind to the $G_{\alpha q}$ coupled thromboxane receptors resulting in further calcium release and promoting aggregation strength. TxA2 can counteract the anti-coagulate effect of prostacyclin receptors and also acts as a strong vasoconstrictor to enhance endothelial cell responses and

chemokine release[25]. Low dose aspirin exhibits anti-thrombotic effects by irreversibly blocking cyclooxygenase thereby inhibiting TxA2 synthesis.

1.1.4 GPCRs signalling and integrin $\alpha_{IIb}\beta_3$ activation in platelets

There are three major types of GPCRs involved in regulating platelet function, the P2Y family of GPCRs, protease activated receptors (PAR) and thromboxane receptors (TP)[26]. The G protein coupled purinergic receptors $P2Y_1$ and $P2Y_{12}$ can be activated by ADP released from dense granules, resulting in the recruitment and activation of surrounding platelets and the continuous stimulation of granule release. This positively feedback loop enhances the overall activation cascade and promotes platelet aggregation. Clopidogrel, Prasugrel or Ticagrelor are clinically used $P2Y_{12}$ receptor inhibitors, which reduce platelet activity and lower the risk of cardiovascular events. In addition to the P2Y family GPCR, protease-activated receptors play important roles in regulating platelet function and the aggregation consolidation process. Platelet activation causes phosphatidylserine (PS) expressed on the inner cytosolic membrane to be flipped and externalized, which creates a catalytic surface that attracts proteases and coagulation factors such as Factors Va, VIIIa and XIII. As shown in Fig. 1, Factors Va and Factors Xa together form the prothrombinase complex which cleaves prothrombin to thrombin. Thrombin is a serine protease that can cleave the amino acid motif SFLLRN on the G-protein coupled PAR receptors, resulting in self-activation of the receptor[27] (Fig. 2). Human platelets express PAR1 and PAR4 receptors and activation of the $G_{\alpha i/o}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$ coupled PAR1/4 receptors results in downstream signalling such as calcium release, granule secretion, platelet shape change and integrin $\alpha_{IIb}\beta_3$ activation. Furthermore, PAR1 signalling also activates the mitogen-activated protein kinase (MAPK) pathway, of which the downstream effector extracellular signal related kinase (ERK) can contribute to thromboxane A2 synthesis. In contrast, activating $G_{\alpha s}$ coupled prostacyclin receptor (IP) will result in cAMP increase and platelet inhibition[28].

Integrin $\alpha_{IIb}\beta_3$ is the most abundant integrin expressed in platelets, which is a heterodimer complex consists of α_{IIb} and β_3 subunit (Fig. 2). Both α and β subunit have a large extracellular domain, a transmembrane spanning region and a cytoplasmic tail[29]. In resting platelets, both subunits are in a bent form which keeps the integrin $\alpha_{IIb}\beta_3$ at a “low affinity” state. Activation of the GPVI receptor or $G_{\alpha q}$ coupled PAR1, TP or $P2Y_1$ receptor increases cytosolic calcium concentrations, resulting in activation of calcium-diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI) and Rap1b. Rap1b activates talin-1 and Kindlin resulting in a conformational change in the β_3 subunit, cause extension and exposure of the integrin head domain, which transforms integrin $\alpha_{IIb}\beta_3$ to a high-affinity state (Fig. 2). This “inside-out signalling” process activates integrin $\alpha_{IIb}\beta_3$ and allows binding of fibrinogen and vWF between different $\alpha_{IIb}\beta_3$ integrin receptors on platelets. The formation of fibrinogen bridges cross links neighbouring platelets resulting in the formation of a stable aggregate (Fig. 2). The final

platelet aggregate consolidation process is mediated by thrombin cleavage of fibrinogen resulting in a fibrin network[30]. As previously described, thrombin was synthesized from enzymatic cleavage of prothrombin by Factor Xa and Factors Va complex. Thrombin can activate Factors XIII that cleave fibrinogen to produce insoluble fibrin. Fibrin polymerises and binds platelets to form a fibril mesh[31]. Activated Factors XIII also cross links the fibrils-platelets aggregate mesh, resulting in stabilisation of the platelet plug. Furthermore, outside-in signalling via $\alpha_{IIb}\beta_3$ integrins results in clot retraction and strengthening of the clot. Therefore, targeting the $\alpha_{IIb}\beta_3$ integrins receptors can disrupt the fibrin network formation process which reduces platelet aggregate formation. Abciximab, Tirofiban and Eptifibatide are integrin $\alpha_{IIb}\beta_3$ receptor inhibitors used in the clinic for coronary intervention and prevention of thrombotic disorders[32].

1.2 Priming of platelet function

Platelet primers are bioactive molecules that do not induce platelet response by themselves but increase platelet responses to physiological agonists such as thrombin and ADP[33]. Diseases such as chronic inflammation, diabetes and cardiovascular disease have elevated circulating concentrations of certain growth factors, glycoprotein hormones, and cytokines or promote their receptor expression levels. For example, studies have shown that CCL17, CCL22 and CXCL12 enhance platelet responses to ADP, while elevated plasma concentrations of these chemokines were found in patient with chronic inflammation or myocardial infarction[34]. IGF-1 and TPO are identified platelet primers that can cause platelet hyperactivity and increased levels of these primers were found in patients with thrombotic diseases. In addition, studies have shown that elevated IGF-1 and TPO levels contribute to antiplatelet resistance, with the therapeutic effects of aspirin being compromised in the presence of these primers[7]. The priming effects of these two primers are mediated via activation of PI3K pathway. As shown in Fig. 3, thrombin cleavage results in activation of G-protein coupled PAR1 receptors, which then signals downstream via the PLC pathway to activate CalDAG-GEFI and Rap1b resulting in integrin activation. DAG also activates PKC causing ERK signalling. Activation of ERK and Rap1b results in the production of thromboxane A2 and integrin $\alpha_{IIb}\beta_3$ activation. Activation of PAR1 also signals through the PI3 Kinase pathway resulting in the production of PIP_3 and subsequent PKB activation. RASA3 is a GTPase-activating protein (GAP) that counteract the effect of CalDAG-GEFI, RASA3 can convert Rap1b to a GDP bind state resulting in Rap1b inhibition (Fig.3). A study by Stefanini et al[35] demonstrated that activation of PI3K result in PIP_3 production and that PIP_3 can recruit RASA3 to the plasma membrane and inhibit its activity. Inhibition of RASA3 results in elevated Rap1b activity and increased integrin activation. Furthermore, PIP_3 can also cause ERK activation and subsequent thromboxane A2 synthesis in platelets. Binding of TPO to C-MPL receptor and IGF-1 to IGF-R result in activation of JAK2 and IRS1 respectively, which also activates the PI3K pathway (Fig. 3). Cross-talk between the signalling cascades is a common

biological phenomenon, as different receptors expressed on the cell may share the same signalling components.

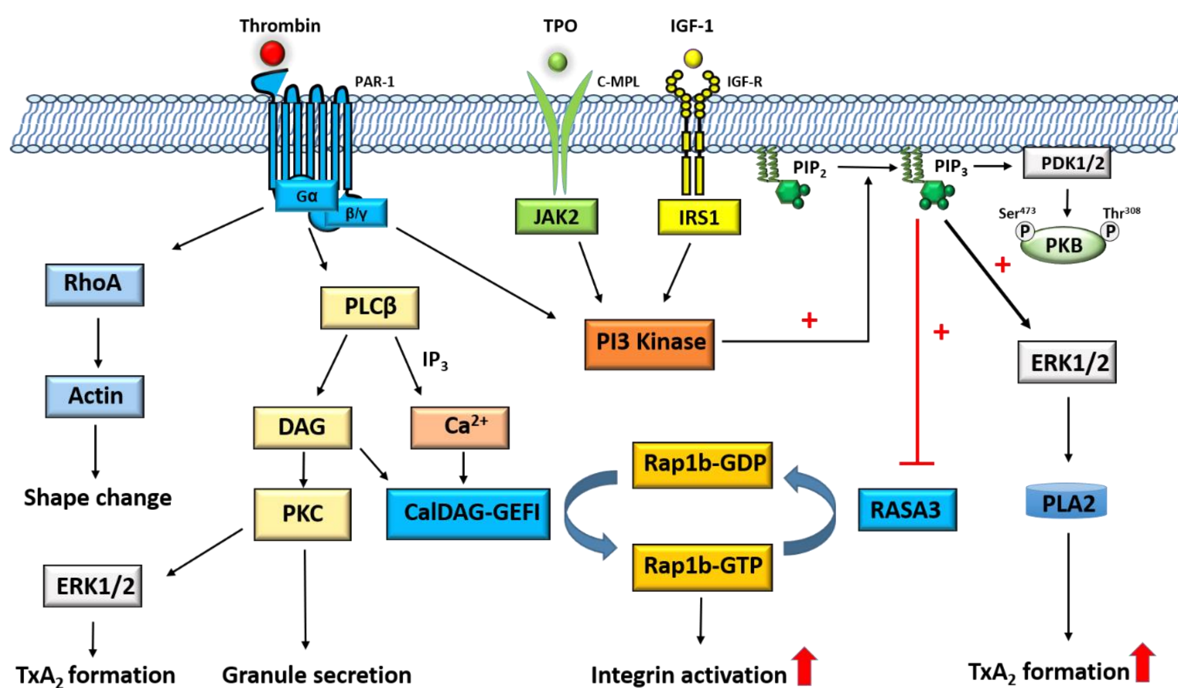


Figure 3. Schematic diagram showing the signalling mechanism of IGF-1 and TPO in priming PAR1-AP mediated platelet activation. JAK2: Janus kinase 2. IRS1: Insulin receptor substrate 1 PIP_2: phosphatidylinositol 4, 5-bisphosphate. PIP_3: phosphatidylinositol (3, 4, 5)-trisphosphate. PDK: phosphoinositide-dependent kinase. PKB: protein kinase B. ERK: extracellular signal–regulated kinases. PLA2: phospholipase A2. PLCβ: phospholipase C β. Rap-1b: Ras-related protein. DAG: diacylglycerol. RASA3: Ras GTPase-activating protein 3. CalDAG-GEFI: diacylglycerol regulated nucleotide exchange factor. GTP: guanosine triphosphate; GDP: guanosine triphosphate.

However, under pathological conditions, increased circulating TPO and IGF-1 levels or over expression of their receptors may result in amplification of the PI3K signalling. This synergises with physiological agonists to induce more downstream signalling causing enhanced TxA_2 production and integrin $\alpha_{IIb}\beta_3$ activation, resulting in platelet hyperactivity and promoting aggregate formation (Fig. 3). Moreover, certain platelet primers also enhance the production and release of platelets from megakaryocytes resulting in thrombocythemia. Priming of platelet function not only increases the risk of developing thrombotic complications, but also reduces the efficacy of antiplatelet agents making it more difficult to treat the disease as high dosage or chronic usage of antiplatelet drugs might increase the risk of bleeding[36]. A novel approach to treat patients with thrombotic disorders and other pathological complications could be to target platelet primers in combination with traditional anti-platelet therapy. Therefore, it has great clinical importance to identify potential platelet primers and exam the underlying mechanism of their actions, which might lead to development of novel anti-thrombotic strategies.

1.3 Aims and Objectives

Platelet primers can enhance platelet responses to physiological agonists resulting in platelet hyperactivity and increased risk of developing thrombotic disorders. Previously, research have identified several platelet primers, but evidence suggests there might be more bioactive molecules that can act as novel platelet primers. However, the identity and underlying mechanism by which these primers increase platelet function is still largely unknown. This project aims to first identify potential novel platelet primers through literature research and platelet transcriptome studies, then evaluate their priming effects on platelet aggregation. One of these identified novel platelet primers will subsequently be studied in further detail, to gain in depth insight into the underlying mechanism of how novel platelet primer may enhance platelet function. Finally, this project will evaluate the clinical significance of the primers identified and conclude potential therapeutic implications.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Agonists, antagonists and inhibitors

Macrophage colony-stimulating factor, interleukin-17A, interleukin-34, chemokine ligand 17 (CCL17), chemokine ligand 22 (CCL22), Platelet derived growth factor (PDGF-AA/BB) were purchased from PeproTech (London, UK). Sulprostone, insulin-like growth factor 1 and thrombopoietin were from Research and Diagnostic Systems (Oxfordshire, UK). Sphingosine-1-phosphate, FTY720, selective $S1PR_1$ agonist SEW2871, $S1PR_2$ agonist CYM5520, $S1PR_3$ agonist CYM5541, $S1PR_4$ agonist CYM50260 and $S1PR_5$ agonist A971432. $S1PR_1$ antagonists Ex 26, W146, $S1PR_2$ antagonist TY52156, $S1PR_3$ antagonist JTE-013, $S1PR_4$ antagonist CYM50358 hydrochloride, sphingosine kinase 1 inhibitor PF543 hydrochloride, non-selective sphingosine kinases inhibitor N,N-Dimethylsphingosine (DMS) and non-selective PI3K inhibitor wortmannin were all purchased from Tocris Bioscience (Bristol, UK). D-erythro-Dihydrosphingosine-1-phosphate (DHS1P), GSK575594A, apyrase, indomethacin, prostaglandin E1 were purchased from Sigma-Aldrich (Poole, UK). FTY720-Phosphate were purchased from Insight Biotechnology (Wembley, UK). The target, EC_{50} and IC_{50} of S1P related agonists and antagonists were summarised in **Table 1**.

S1PR pan agonists	Receptor	EC50
Sphingosine 1-phosphate	$S1PR_{1-5}$	6-64 nM
Dihydro-S1P	$S1PR_{1-5}$	10-230 nM
FTY720-phosphate	$S1PR_{1,3,4,5}$	0.3 – 3 nM
S1PRs agonists	**Receptor**	**EC50**
SEW 2871	$S1PR_1$	13 nM
CYM 5520	$S1PR_2$	480 nM
CYM 5541	$S1PR_3$	100 nM
CYM 50260	$S1PR_4$	45 nM
A 971432	$S1PR_5$	5 nM

Antagonists/inhibitors	Target	IC50
Ex 26	$S1PR_1$	0.93 nM
JTE 013	$S1PR_2$	17.6 nM
TY 52156	$S1PR_3$	110 nM
CYM 50358 hydrochloride	$S1PR_4$	25 nM
AID 503497 (not available)	$S1PR_5$	10 μM
PF543 hydrochloride	SphK1	2 nM
N,N-Dimethylsphingosine (DMS)	SphK1/2	500 nM

Table 1. Summary of S1P related agonists, antagonist and inhibitors used in this study with indications of their target receptors and potency. (Data were collected from IUPHAR/BPS guide to pharmacology[37])

2.1.2 Immunoblotting solution and antibodies

30% acrylamide mix (37.5:1), 1.0 M Tris pH 6.8 solution and 1.5 M Tris pH 8.8 solution were from National Diagnostics (Nottingham, UK). Immobilon-FL PVDF Membrane (0.45-micron filter) were from Merck Millipore (Hertfordshire, UK). Sodium dodecyl sulphate (SDS) were from GE health care worldwide (Hatfield, UK). Tetramethylethylenediamine (TEMED), 10% SDS solution and precision plus protein pre-stained standards were from Bio-Rad Laboratories (Hertfordshire, UK). Dithiothreitol (DTT) were obtained from Apollo Scientific (Bredbury, England). Odyssey® Blocking Buffer were from LI-COR Biosciences (Cambridge, UK). Primary antibodies include phospho-AKt (Ser473) antibody, phospho-p44/42(Erk 1/2)

MAPK (Thr202/Tyr204) antibody, phospho-VASP (Ser239) antibody, phospho-Talin (Ser425) antibody, phospho-serine PKC substrate antibody were purchased from Cell Signalling Technology (Hertfordshire, UK). Alexa Fluor® 680 AffiniPure Donkey Anti-Goat IgG (H+L) and Alexa Fluor® 680 AffiniPure Donkey Anti-Rabbit IgG (H+L) were the secondary antibody used and purchased from Jackson Immunoresearch (Cambridgeshire, UK)

2.1.3 Reagents for flow cytometry, calcium assay and spreading experiment

FITC-PAC1 and PE-CD62P antibody were from BD Bioscience (Berkshire, UK). Phosphate buffered saline (PBS), Fura-2 AM cell permeant calcium indicator and ActinGreen 488 ReadyProbes Reagent was purchased from Thermo Fisher Scientific (Loughborough, UK). Ethylenediaminetetraacetic acid (EDTA) and Triton X-100 were from Sigma-Aldrich (Poole, UK). Fatty acid free bovine serum albumin (BSA) and FluorSaver mounting medium were from Calbiochem (Hertfordshire, UK).

2.1.4 Solutions and dilutions

Trisodium citrate, acid citrate dextrose (ACD), D-glucose, ammonium persulfate (APS), sodium hydroxide, NuPAGE LDS Sample Buffer (4X), Tris buffered saline (TBS) and polysorbate 20 (Tween 20) were from Thermo Fisher Scientific (Loughborough, UK). Dimethyl sulfoxide (DMSO) were obtained from VWR international (Leicestershire, UK). HEPES Tyrode's (10 mM HEPES, 1 mM Magnesium, 0.5 mM monosodium phosphate, 145 mM sodium chloride, 3 mM potassium chloride. pH 7.2), 2-butanol, ethanol, methanol was from Sigma-Aldrich (Poole, UK). Trisodium citrate were dissolved in Milli-Q water to 4% w/v. PAR1-AP, D-glucose and apyrase were diluted in HEPES Tyrode's buffer. S1P were dissolved in methanol. Indomethacin, wortmannin, FTY720, FTY720 phosphate, SEW 2871, CYM 5520, CYM 5541, CYM 50260, A971432, Ex 26, TY 52156, JTE 013, CYM 50358 hydrochloride, PF543 hydrochloride, N,N-Dimethylsphingosine and Fura-2 AM were prepared in DMSO. Sulprostone were pre-dissolved in methyl acetate. IGF-1 were made in 0.1 M HAC and 0.1% BSA. All peptide ligands (m-CSF, IL-17A/34, CCL17/22 and PDGF-AA/BB) were dissolved in 0.1% BSA solution. All stock solutions were kept in -20°C freezer. Antibodies were diluted in Odyssey Blocking Buffer with TBST (v/v 1:1) to optimal concentrations and kept at +4°C in fridge.

2.2 Methods

2.2.1 Isolation and preparation of human platelet

Venous blood was collected from healthy volunteers under the regulation of local ethics committee. Signed consent form in accordance with the Declaration of Helsinki was obtained from each volunteer. Blood samples were mixed with 1 in 9 volumes of 4% trisodium citrate solution to be anticoagulated and then acidified with 1 in 7 ratio of acid citrate dextrose. Anticoagulated blood sample were then centrifuged in a Heraeus Megafuge 11 Centrifuge

(Thermo Fisher Scientific, UK) at 1000 revolutions per minute (rpm) for 17 minutes which then platelet rich plasma (PRP) were obtained. PRP were collected and supplemented with 0.02 U · mL^{-1} apyrase and 140 nM prostaglandin E1, then centrifuged at 1700 rpm for 10 minutes. The upper layer of platelet poor plasma was removed and resulted platelets pellet was resuspended with 2 mL modified HEPES Tyrode's (HEPES Tyrode's buffer, 0.1% D-glucose and 0.02 U · mL^{-1} apyrase). Platelets were diluted as 1:2000 ratio in 10 mL isoton dilution agent, then the density of the platelets was determined by a Z1 coulter particle counter (Beckman Coulter, London, UK). Platelets were resuspended with modified HEPES Tyrode's to a final density of 4×10^{8} mL^{-1} and rest for 30 minutes in a 30°C water bath before experimentations. Platelets used in FACS, calcium mobilization and immunoblotting experiments were isolated and rested in the presence of 10 μM indomethacin and 0.02 U · mL^{-1} apyrase instead.

2.2.2 Platelet aggregation

Rested platelets (4×10^{8} mL^{-1}) were diluted to 2×10^{8} mL^{-1} using modified HEPES Tyrode's buffer, a volume of 250 μL of platelets were added into transparent aggregation cuvettes each contain a Teflon coated mini magnetic stir bar. Platelet were pre-incubated with vehicle or primers for 5 minutes in prior to the addition of PAR1-AP at indicated concentration. Platelet aggregation was performed on a Chrono-log model 490 aggregometer (Labmedics, Oxfordshire, UK) under 1200 rpm stirring speed with a constant temperature of 37°C. Light transmission changes were measured and recorded as aggregation traces. Platelets samples were used within 3 hours after the resting and all wastes were decontaminated by adding disinfectants overnight.

2.2.3 Protein extraction, SDS-PAGE electrophoresis and immunoblotting

Rested human platelets (4×10^{8} mL^{-1}) were pre-incubated with primers for 5 minutes. Subsequently, PAR1-AP were added into the platelet to react for 5 minutes. Platelets sample were lysed by adding 4X LDS NuPAGE sample buffer (62.5 mM Tris-HCl, 10% glycerol, 1% LDS and 0.005% bromophenol blue) supplemented with 50 mM dithiothreitol. Samples were kept at - 20°C before use. Platelets protein extract was completed by heating the platelet lysate samples at 75°C for 5 minutes. 4 μL pre-stained protein and 25 μL platelets protein extract were loaded separately onto a 5% Bis-Tris polyacrylamide stacking gel shaped with 15 wells. Subsequently, the protein electrophoresis was carried out on 8% Bis-Tris polyacrylamide resolving gels at 100 volts constant for 90 minutes based in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Proteins were transferred to a PDVF membrane using Transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 100 V for 60 minutes. Subsequently, the PVDF membrane with protein transferred was blocked using Odyssey® blocking buffer in TBS-T (volume ratio 1:1, 50 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 hour. The PDVF membranes were incubated with primary antibodies (1:500 dilution)

overnight at + 4°C. Membranes were then washed with TBS-T for 3 times with a 5 minutes interval. The membrane was then incubated with the secondary antibodies (1:5000) for an hour at room temperature. Subsequently, the membranes were washed extensively for 5 times with TBS-T and scanned by an Odyssey® CLx Imaging System (LI-COR Biosciences, UK).

2.2.4 Fluorescence-activated cell sorting flow cytometry

Rested platelets were diluted to 2×10^7 mL^{-1} in a modified HEPES Tyrode's buffer, platelet was incubated with vehicle control or primers for 10 minutes. A master mix of FITC-PAC1 and PE-CD62P antibody (v/v, 2:1) were loaded into a 96 well plate and mixed with vehicle control or increasing concentrations of PAR1-AP. Platelets pre-treated with primers were loaded into each well and incubated for 10 minutes to allow platelet activation and binding of antibodies. Platelet sample were fixed by adding 2% paraformaldehyde and read on a BD Accuri™ C6 Plus flow cytometer (BD Bioscience). Total of 10000 events were collected from each well.

2.2.5 Calcium mobilization assay

Plate rich plasm was isolated as described previously, then Fura-2 AM calcium indicator was added to the PRP (1:1000 dilution, working concentration 4 μM) allow 60 minutes incubation at 30 °C. Subsequently, platelets were isolated and resuspended to 4×10^8/mL final concentration as describe before. Washed human platelets were resting for 30 minutes in dark at 30 °C to allow complete de-esterification of intracellular acetoxymethyl esters. Platelets were diluted to 2×10^8 mL^{-1} in a modified HEPES Tyrode's buffer supplemented with 2 mM calcium, then incubated with vehicle or primers for 5 minutes at 37 °C. Subsequently, platelets samples were loaded into a 96 wells plate and read on a Tecan Infinite M200 multimode plate reader (Männedorf, Switzerland) allow 10 cycles to establish the baseline. Then PAR1-AP were added, and responses were recorded for the next 40 cycles. In the last 10 cycles, Triton X-100, EDTA and TRIS were added into the system for calibration purpose.

2.2.6 Measurement of TxA2 synthesis (Competitive TxB2 ELISA)

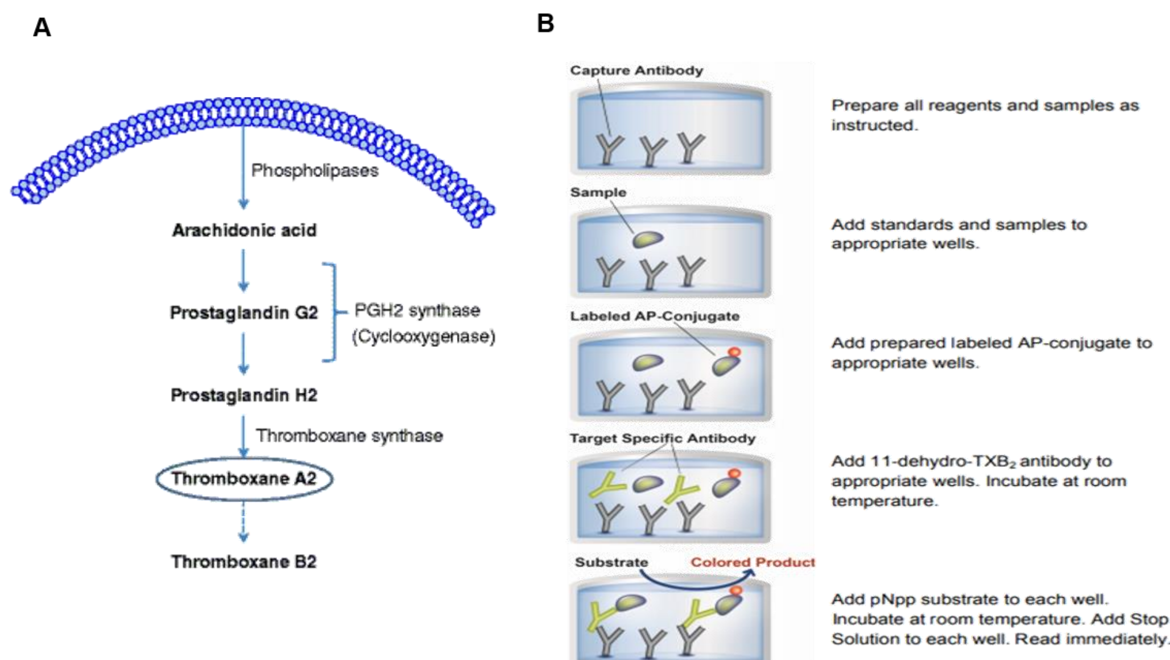


Figure 4. Thromboxane A2 synthesis in human platelet and summary of TxB2 ELISA. (A) Arachidonic acid (AA) is generated from phospholipid by phospholipase A2, AA then get converted to prostaglandin G2 by cyclooxygenases then subsequently to prostaglandin H2, TxA2 is generated from prostaglandin H2 then shortly being metabolised to thromboxane B2. **(B) Schematic diagram summarises the TxB2 ELISA process**. Supernatant collected from lysed human platelets were added to the ELISA wells coated with goat capture antibody, then TxB2 conjugate with alkaline-phosphatase were added to the well. Any unbound TxB2 or APL conjugate was washed off and para-Nitrophenylphosphate (pNpp) substrate was added for incubation. (Figure adapted from Abcam Instruction[38])

TxA2 plays an important role in platelets function and thrombus formation, however it has a very short half-life of 37 seconds that cannot be measured directly[39] (Fig. 4A). Thromboxane B2 (TxB2) is the inactive product of TxA2 which has a half-life of 7 minutes, while its stable metabolic product 11-dehydro-TxB2 has a much longer half-life of 1 hour[40]. Hence, 11-dehydro-TxB2 can be used to indirectly measure the TxA2 production in platelets. Human platelets (4×10^8/mL) were incubated with vehicle control or primer for 5 minutes in 37 °C, then stimulated with PAR1-AP. Reaction were stopped by adding 5 mM EDTA and 200 μM Indomethacin. Samples were span at 12000 g and + 4 °C for 3 minutes, subsequently the supernatant were removed and stored in -80°C. TxB2 ELISA kits were acquired from Enzo Life Sciences (Exeter, UK). A set of TxB2 standard samples were prepared to generate a logarithmic TxB2 standard curve. The supernatant sample was diluted 1:50 with assay buffer provided then loaded onto a 96 wells plate coated with goat antibody. Subsequently, TxB2 conjugate with alkaline-phosphatase was added to each well, followed by addition of 11-dehydro-TxB2 antibody (Fig. 4B). The plate was sealed and incubated at 500 rounds per minute on an orbital shaker at room temperature for 2 hours. After the incubation, the well contents were emptied and washed three times using a diluted wash buffer. A non-proteinaceous chromogenic substrate para-Nitrophenylphosphate (pNpp) substrate were

added to each well and the plate was incubated in the dark for 45 minutes. Reaction were stopped by adding stop solution and the plate was read at optical density (OD) of 405 nm with reference correction at 570 nm on Tecan Infinite M200 multimode plate reader (Männedorf, Switzerland). The TxB2 concentration for each sample were calculated from the TxB2 standard curve.

2.2.7 Human platelet spreading

A 96 wells glass-bottom plate were coated by 100 µg/mL BSA (control) or 100 µg/mL fibrinogen for 1 hour, subsequently the uncoated area of the well was blocked by 3% BSA for 30 minutes. Platelets were diluted to 7×10^{7} mL^{-1} in modified HEPES Tyrode's buffer supplemented with 2 mM calcium, then incubated with vehicle control or primers for 10 minutes. Treated platelets were incubated in the wells for 60 minutes at 37°C to allow cell spread and adhere onto the fibrinogen coated surface. Subsequently, platelets were fixed by adding 4% paraformaldehyde and washed using phosphate buffered saline. Platelets were permeabilised for fluorescent stain entry by addition of 0.1% Triton X-100, then washed by PBS and incubated with ActinGreen 488 reagent for 30 minutes. Each well was washed by PBS buffer before loading with Fluro-keeper mounting medium solution and covered by a 5-millimetre glass coverslip. The plate was set in a dark overnight and images were captured using Leica DMI6000 B fluorescent microscope (Leica Camera AG, Germany).

2.2.8 Statistics and software

Platelet aggregation data were recorded on Chrono-log Aggrolink software (Chronolog Corporation, Pennsylvania, USA). Calcium mobilization and TxB2 ELISA data were collected from an I-control™ microplate reader software. Platelet spreading images were analysed using Leica application suite X and ImageJ. Immunoblotting data were analysed by ImageStudio™ Lite 5.2 (LI-COR Biosciences, UK). Data were collected from at least 3 independent observations and plotted on Histograms graphs with mean ± SEM arrow bars using GraphPad Prism 7.0 software (GraphPad Software, California, USA). Statistical test used to analyse aggregation data was Student paired t-test, assume data points were normally distributed. Two-way ANOVA with Bonferroni post hoc multiple comparisons were used to analyse FACS, Calcium mobilization and TxA2 ELISA data points on the curves. EC_{50} value of the curves were compared using Student paired t-test. One-way ANOVA with Dunnett's test were used to analyse immunoblotting and platelet spreading data compare to the vehicle value. Statistical significances were indicated with asterisks on the graphs. NS, no statistical significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 3. Identification and pre-selection of novel platelet primers

3.1 Introduction

3.1.1 Overview of platelet primers

Elevated platelet activity was found in patients with coronary artery disease and these patients also show resistance to current antiplatelet treatment. It is well established that increased circulating primers contribute to platelet hyperactivity[41]. There are several platelet primers have previously been identified, including IGF-1, growth arrest-specific 6 (Gas6), matrix metalloproteinase-1 (MMP-1), sCD40L, TPO, stromal cell-derived factor 1 (SDF-1), vascular endothelial growth factor (VEGF), adrenaline, histamine and serotonin[42]. These primers can be released from activated endothelial cells and platelets, or naturally be present in the circulating plasma. Hence, the priming effects can be mediated via both autocrine and paracrine manner, resulting in positive feedback of platelet responses and promotion of thrombus growth. Most of the known existing primers are cytokines, chemokines, hormones or neurotransmitters, the *in vitro* effects of these primers include increased platelet aggregation or integrin activation to physiological agonists such as ADP, thrombin and collagen-related peptide (CRP). Moreover, primers such as IGF-1 and VEGF also play important roles in regulating vascular integrity, modulating immune response and promoting cell growth[43]. In addition, primers also exhibit pathological roles in diabetes, chronic inflammations and cancer development. It is likely that there is a whole array of platelet primers contributing to enhanced platelet function in disease states and this project therefore aims to identify novel platelet primers and their underlying mechanism of action.

3.1.2 Using human platelet transcriptome to identify potential primers

To identify potential novel platelet primers, we focussed on candidates that meet the following criteria. Firstly, corresponding receptors should be expressed on platelets according to proteomic/transcriptomic/western blotting data, and activation of the receptors by candidate molecules should not inhibit platelet function. Secondly, candidate primers and/or expression level of corresponding receptors are elevated under pathological conditions. Thirdly, the concentration of candidate primer used to evoke priming effects should ideally be in a physiological or pathological relevant range. In addition, there should ideally be literature to suggest candidate primer can alter platelet response, but it has not already been well characterised and therefore remains relatively “novel” in the research field. Finally, this is not a strict criterion, but we are also interested in any candidate platelet primer that can promote platelet formation from megakaryocytes which might result in thrombocythemia. Using TPO and IGF-1 as an example, activation of certain cytokine receptor or receptor tyrosine kinases (RTKs) can amplify platelet signalling via amplification of the PI3K pathway. Many identified primers also mediate their effects via these two types of receptor. Hence, RTK and cytokine receptors can be potential targets to study. Moreover, ligands that activate GPCRs on

platelets might be interesting to study. As previous described, activation of GPCRs such as PAR receptors or thromboxane receptor can directly trigger platelet response, while activation of $G_{\alpha i/o}$ coupled $P2Y_{12}$ receptor or $G_{\alpha q}$ coupled $P2Y_1$ receptor can amplify platelet aggregation[44]. Studies also demonstrated activation of $G_{\alpha 12/13}$ coupled receptor can result in cytoskeleton changes in platelets[45]. In contrast, activation of $G_{\alpha s}$ coupled prostacyclin receptor by PGI_2 will inhibit platelet aggregation, while knock out of prostacyclin receptor result in an increased risk of atherosclerosis[46]. Activation of $G_{\alpha s}$ coupled GPCR elevates intracellular cyclic AMP levels resulting in platelet inhibition, therefore, any ligands activating $G_{\alpha s}$ GPCR will be excluded from this study.

Prior to selecting potential platelet primers, it is important to determine whether their corresponding receptors expressed or not. Although platelets have lost their nuclear DNA genome, they still retain cytoplasmic mRNA to synthesize protein[47]. Using transcriptomics technologies, we can exam the total number of RNA transcripts (or transcriptome) for every protein in human platelet. The transcriptome level can be used to indicate the relative expression level of certain proteins or receptors[48]. Work done by Rowley et al[49] have characterized both mouse and human platelet transcriptome using next generation RNA sequencing techniques. The transcriptome level of GPCRs and RTKs in human platelets were summarised and ranked in Fig. 5. For instance, PAR1 receptor and $P2Y_{12}$ receptor have the highest transcriptome level among all GPCRs expressed in human platelets, which measured a mean RPKM of 72.7 and 52.5 respectively (Fig. 5A). The chemokine receptors CCR4 and CXCR4 also have relative high transcriptome expression levels of 4.48 and 15.1 respectively.

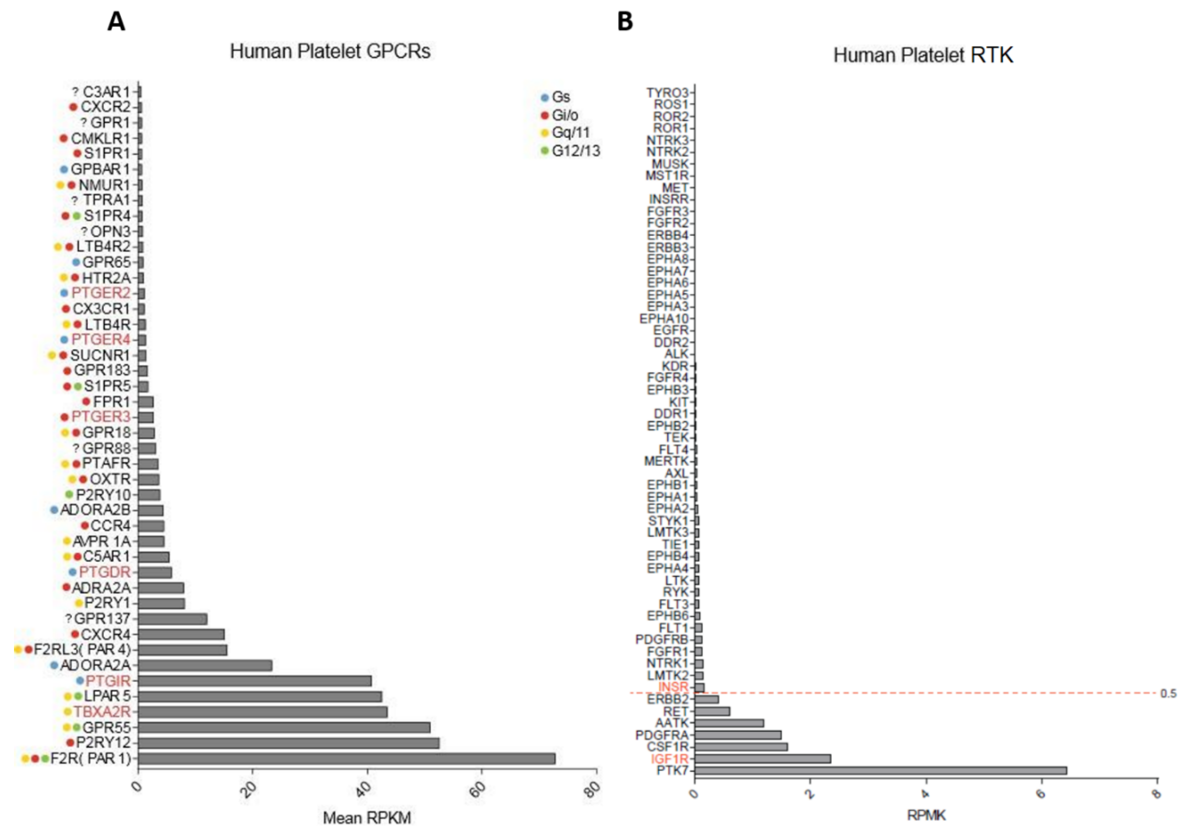


Figure 5. Ranked RNA transcript expression level of GPCRs and RTKs in human platelets. (A) Transcript expression level of GPCRs in human platelets. **(B)** Transcript expression RTKs in human platelets. RPKM: Reads Per Kilobase of transcript per Million mapped reads. Figure was generated by R.W.Hunter using transcriptome data from work by Rowley et al[49]. G-protein coupling information was extracted from the IUPHAR database by R.W.Hunter.

In contrast, 5-HT$_{2A}$ is a serotonin receptor expressed at a low level in platelets and can enhance platelet aggregation. 5-HT$_{2A}$ receptors have a total transcriptome of 0.91 (HTR2A), therefore any GPCRs transcriptome below this level should raise doubts whether the receptor is expressed or not. The absolute minimum is the transcriptome level for complement component 3a receptor 1 (C3aR1, RPKM=0.0867), which is detectable in human platelets. In contrast, the platelet RTK expression levels are much lower in general, therefore only receptors that have a RPMK above 0.5 will be considered in this study (Fig. 5B). However, the transcriptome level is not necessarily positively correlated to the total protein synthesized. As small differences between the translational features encoded in the mRNA sequence can lead to large differences in the total amount protein expressed[50]. Moreover, there might be false positives or negative results from the transcriptome data and it should therefore not be used as an absolute judgement.

3.1.3 Potential novel platelet primers included in this study

Based on the literature and transcriptomic data, a list of candidate platelet primers was generated. The normal plasma concentration in healthy human individuals, corresponding receptor, receptor type and the receptor transcriptome expression level of the candidate primers are summarised in Table 2. IGF-1 and TPO are well defined platelet primers that will

be used as positive controls in this study. Thymus and activation regulated chemokine (TARC, CCL17) and macrophage-derived chemokine (MDC, CCL22) are chemokines that predominately expressed in thymus. The two chemokines share a very similar structure and have more than 1/3 of identical amino acid sequence[51]. Both chemokines can activate the $G_{\alpha i/o}$ coupled chemokine receptor CCR4, which has a relative high transcriptome expression level in platelet (RPKM=4.48). It was demonstrated these two chemokines increase platelet responses to ADP stimulation[34]. Elevated plasma concentrations of CCL17 and CCL22 are found in patients with asthma, dementia and chronic inflammation[60]. Prostaglandin E2 (PGE_2) is a prostaglandin that released from endothelial cell and activated platelets, PGE_2 can induce concentration dependent biphasic effects on platelet function. At low concentrations PGE_2 mainly activates the $G_{\alpha i}$ coupled prostaglandin EP3 receptor to enhance thrombin-mediated aggregation[52]. Sulprostone is the synthetic analogue of the PGE_2 to be tested in this study[53]. S1P is a signalling sphingolipid that activate S1P receptors, which primarily coupled to $G_{\alpha i}$ and $G_{12/13}$. Elevated S1P concentration have been found in obesity, cancer and renal failure patients, and S1P has been reported to promote whole blood aggregation and induce platelet activation[54]. GSK5755494A is a synthetic lysophosphatidylinositol that activates $G_{12/13}$ coupled GPR55 receptor, and is a novel cannabinoid receptor agonist reported to have blood pressure lowering effects[55].

Moreover, m-CSF (as known as CSF-1) and IL-34 are cytokines reported to activate CSF1R and cause elevated risk of coronary artery disease[56]. Platelet derived growth factor (PDGF) is released from activated platelets, however the effects of PDGF on platelets are not clear. As some studies suggests PDGF can inhibit platelet function[57], while others found it can enhance[58]. Increased plasma PDGF level was found in patients with hypertension and cardiovascular diseases[76]. Moreover, interleukin 17A is a cytokine that have been reported in the literature to promote thrombosis and atherosclerosis formation in mice models[59].

Candidate primer	Normal plasma concentration	Target Receptor	Receptor Type	Transcriptome (mean RPKM)
CCL17 (TARC)	32 to 52 pg/mL[60, 61]	CCR4	GPCR-$G_{\alpha i/o}$	4.48
CCL22 (MDC)	105 to 437 pg/mL[62, 63]	CCR4	GPCR-$G_{\alpha i/o}$	4.48
Sulprostone	PGE_2: 10.3 pg/mL [64]	EP3	GPCR-$G_{\alpha i/o}$	2.68
S1P	0.4 to 1.0 μM [65]	S1PR	GPCR-$G_{\alpha i/o}$ GPCR-$G_{\alpha 12/13}$	$S1PR_{1,4,5}$ 0.63, 0.69, 1.72
GSK575594A	THC: 98 ng/mL [66]	GPR55	GPCR-$G_{\alpha 12/13}$	50.9
IGF-1	100 to 200 ng/mL [67, 68]	IGF1R	RTK	2.34
IL-34	29 to 120 pg/mL [69, 70]	CSF1R	RTK	1.58
m-CSF	107 to 282 pg/mL [71, 72]	CSF1R	RTK	1.58
PDGF-AA	34 to 85 pg/mL [73, 74]	PDGFR-αα	RTK	1.48
PDGF-BB	32 to 101 pg/mL [75, 76]	PDGFR-αα PDGFR-αβ PDGFR-ββ	RTK	α: 1.48 β: 0.115
IL-17A	40 to 105 pg/mL [77, 78]	IL-17R	Cytokine receptor	4.14
TPO	52 to 121 pg/ml [79, 80]	c-MPL	Cytokine receptor	17.4

Table 2. List of candidate primers included in this study, their physiological plasma concentration, target receptor, receptor type and transcriptome level [60-80]. TARC: thymus and activation regulated chemokine. MDC: macrophage derived chemokine. PGE_2: prostaglandin E2. S1P: sphingosine-1-phosphate. THC: Tetrahydrocannabinol. IL-34: interleukin-34. M-CSF: macrophage colony-stimulating factor. PDGF-AA/BB: platelet derived growth factor AA/BB. IL-17A: interleukin 17A; TPO: thrombopoietin; CCR4: C-C chemokine receptor type 4. EP3: prostaglandin EP3 receptor GPR55: G protein-coupled receptor 55. CSF1R: colony stimulating factor 1 receptor. C-MPL: thrombopoietin receptor.

Candidate primers listed in Table 2 will initially be tested in platelet aggregation studies. Those primers that enhance platelet aggregation will be taken for further signalling and functioning studies. The ligand that consistently promotes platelet function throughout the experiments and is relatively “novel” according to the literature will be selected as the novel primer to take forward for further in-depth studies.

3.1.4 Aims

Platelet primers can enhance platelet responses to physiological agonists resulting in platelet hyperactivity and evidence suggest that more primers exist. A list of potential platelet primers was generated through literature research and platelet transcriptome studies. However, the effects of these candidate primers on platelet function is unknown. The aims of this part of the project are therefore:

1. Identify and evaluate the priming effects of novel platelet primers from selected ligands using functional platelet assays.
2. Decide which novel primer will be taken for further study using defined criteria.

3.2 Results

3.2.1 CCL17, CCL22, sulprostone and S1P enhance PAR1-AP mediated platelet aggregation.

To investigate whether candidate primers that are agonists at GPCRs can enhance platelet function, platelet aggregation experiments were carried out by stimulating human platelets with subthreshold concentrations of PAR1-AP (0.6-1.0 μM) in the presence or absence of candidate primers. Aggregation data indicated that CCL17, CCL22, sulprostone and S1P can enhance PAR1-mediated platelet aggregation (Fig. 6A-Di). In the presence of 1.0 μg/mL CCL17, the maximal aggregation significantly increased from 7.44 ± 1.6 % to 63.4 ± 4.1 % (Fig. 6Aii). CCL17 enhanced the total platelet aggregation by 216%, measured as the area under the curve (AUC) (Fig. 6Aiii). Similarly, another CCR4 receptor ligand CCL22 significantly increased the maximal platelet aggregation (Fig. 6Bii). CCL22 enhanced the total platelet aggregation by 204%, measured by the AUC (Fig. 6Biii). The enhancing effects of CCL17 and CCL22 on PAR1-AP mediated aggregation can also be observed at lower concentrations starting at 0.2 μg/mL and saturated at 1.0 μg/mL. In the presence of 0.1 μg/mL sulprostone, the maximal aggregation significantly increased from 10 ± 3.21% to 72.1 ± 3.52% (Fig. 6Cii) The suboptimal PAR1-AP induced aggregation was significantly increased from 13.1 ± 2.57 % to 46.5 ± 6.29 % in the presence of 100 nM S1P (Fig. 6Dii), and a 118% increase in the AUC was observed (Fig. 6Diii). No significant effect on platelet aggregation was observed in the presence of 1 μM GSK575594A (Fig. 6Ei-iii).

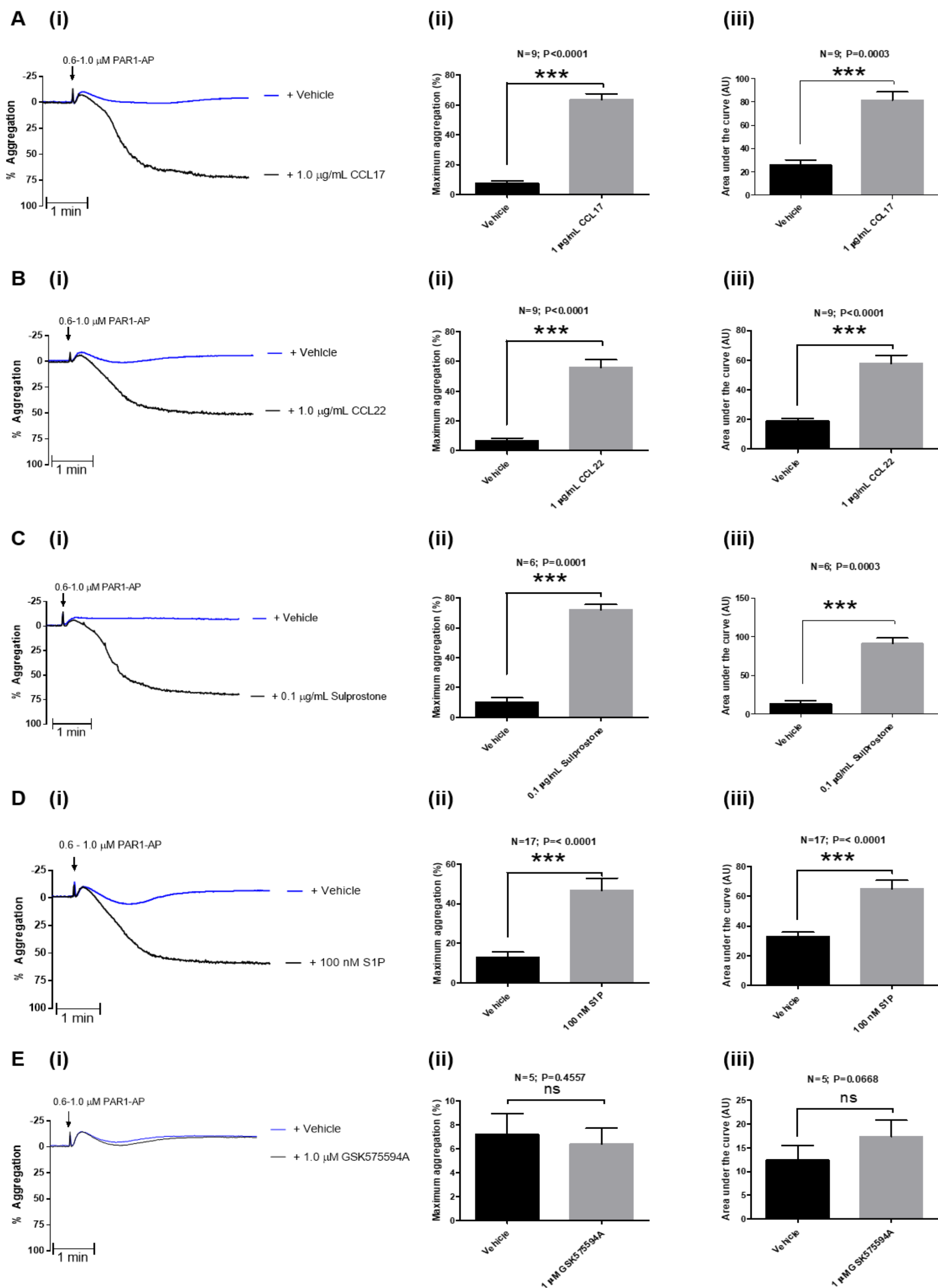


Figure 6. The effect of GPCR agonists on PAR-mediated platelet aggregation. Washed human platelets (2×10^{8}/mL) were treated with vehicle or candidate primers for 5 minutes. Platelet aggregation was induced by a subthreshold concentration (0.6-1.0 µM) of PAR1-AP and recorded for 5 minutes. **(A-E) (i)** Representative traces of PAR1-AP-mediated platelet aggregation in the absence or presence of 1.0 µg/mL CCL17, 1.0 µg/mL CCL22, 0.1 µg/mL sulprostone, 100 nM S1P or 1.0 µM GSK575594A. **(ii)** Histograms of percentage maximum aggregation induced by PAR1-AP in the absence or presence of primer. **(iii)** Histograms of AUC. Data are mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; *** P < 0.001.

3.2.2 IGF-1, IL-34 and PDGF-AA enhance PAR1-AP mediated platelet aggregation.

To investigate whether candidate primers that are agonists at RTKs can enhance platelet function, platelet aggregation experiments were carried out by stimulating human platelets with subthreshold concentrations of PAR1-AP. Aggregation data indicated that IGF-1, IL34, and PDGF-AA enhance platelet aggregation induced by subthreshold concentrations of PAR1-AP (Fig. 7Ai, Bi, Di). In the presence of 0.1 μg/mL IGF-1, the maximal aggregation induced by subthreshold concentrations of PAR1-AP significantly increased from 9.33 ± 6.57 % to 44.7 ± 8.09 % (Fig.7Aii). IGF-1 also enhanced the total platelet aggregation by 246%, measured as the AUC (Fig. 7Aiii). IL-34 significantly increased the maximal platelet aggregation from 8.40 ± 1.63 % to 38.2 ± 12.5 % (Fig. 7Bii). 1.0 μg/mL IL-34 also increased the AUC as shown in (Fig. 7Biii). There was no significant difference between the maximal aggregation induced by PAR1-AP alone or in the presence of 1 μg/mL m-CSF (Fig. 7Ci, Cii). In the presence of 1 μg/mL PDGF-AA, PAR1-AP induced platelet aggregation was significantly increased from 11 ± 3.06 % to 72 ± 7.21% (Fig.7Dii) The priming effect of PDGF-AA could be observed at lower concentrations (0.2 μg/mL, data not shown). There was no significant difference between the maximal aggregation induced by PAR1-AP alone or in the presence of 1 μg/mL PDGF-BB (Fig.7E).

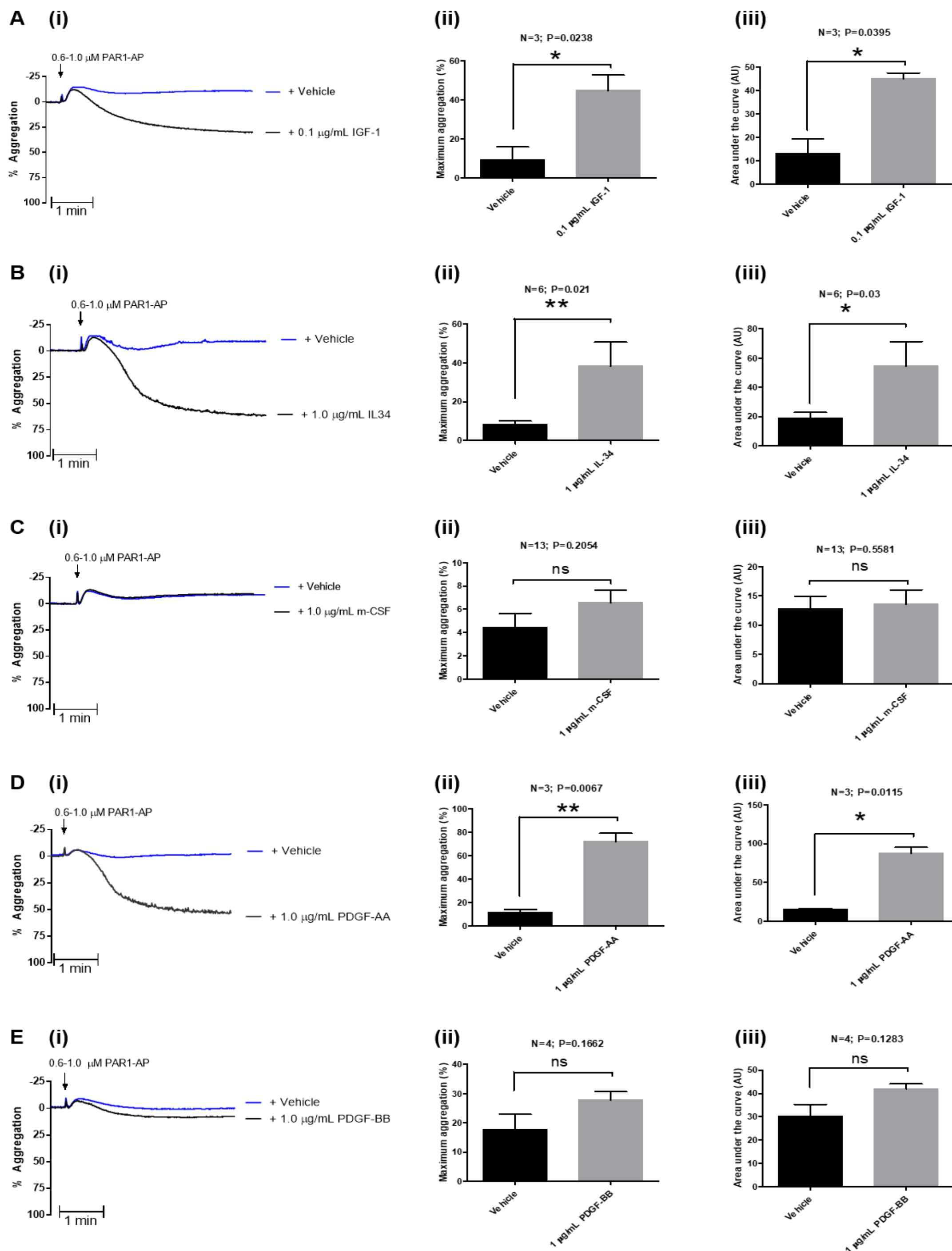


Figure 7. The effect of RTKs agonists on PAR-mediated platelet aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle or candidate primers for 5 minutes. Platelet aggregation were induced by subthreshold concentrations (0.6-1.0 μM) of PAR1-AP and recorded for 5 minutes. **(A-E) (i)** Representative traces of subthreshold concentrations PAR1-AP induced platelet aggregation in the absence or presence of 0.1 μg/mL IGF-1, 1.0 μg/mL IL-34, 1.0 μg/mL m-CSF, 1.0 μg/mL PDGF-AA or 1.0 μg/mL PDGF-BB. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or in presence of candidate primers. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or in the presence of candidate primers. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; * P < 0.05, ** P < 0.01.

3.2.3 Confirmation of TPO priming but no effect of IL-17A observed on PAR1-AP mediated platelet aggregation.

To investigate whether candidate primers that target cytokine receptors can enhance platelet function, platelet aggregation experiments were carried out by stimulating human platelets with suboptimal concentrations of PAR1-AP. Aggregation data confirmed that TPO enhances platelet aggregation induced by subthreshold concentrations of PAR1-AP[7] (Fig. 8A). In the presence of 0.1 μg/mL TPO, the maximal aggregation induced by subthreshold concentrations of PAR1-AP significantly increased from 13 ± 2.52 % to 72.7± 2.60 % (Fig.8Aii). TPO enhanced the total platelet aggregation by 381% as data shown in Figure 8Aiii. There was no significant difference between the maximal aggregation induced by PAR1-AP alone or in the presence of 1 μg/mL IL-17A as data shown in Fig. 8B.

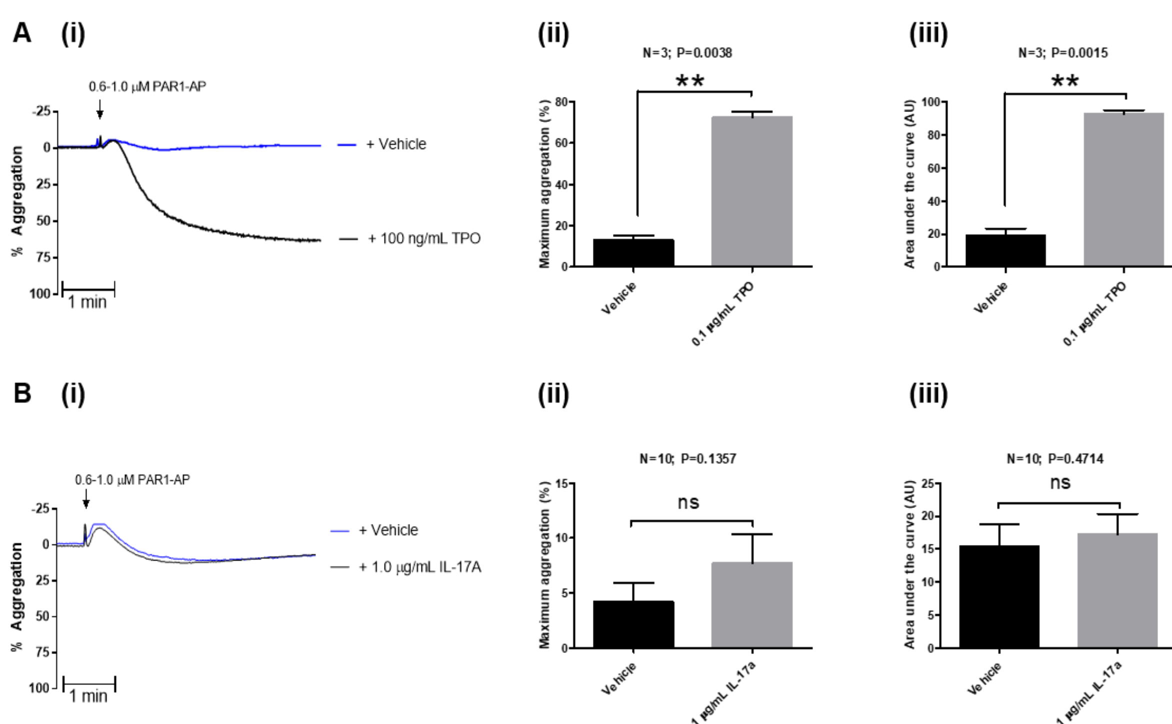


Figure 8. The effect cytokine receptor agonists on PAR-mediated platelet aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle control or candidate primers for 5 minutes. Platelet aggregation were was induced by subthreshold concentrations (0.6-1.0 μM) of PAR1-AP and recorded for 5 minutes. **(A-B) (i)** Representative traces of subthreshold concentrations of PAR1-AP induced platelet aggregation in the absence or presence of 0.1 μg/mL TPO or 1.0 μg/mL IL-17A respectively. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or in presence of candidate primers. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or in the presence of candidate primers. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; ** $P < 0.01$.

3.2.4 Primers alone do not induce platelet aggregation

Previous work demonstrated that primers such as IGF-1 and TPO do not induce platelet aggregation by themselves[42]. To confirm that all candidate primers tested cannot induce platelet aggregation by themselves, candidate primers were added directly to washed human platelets and aggregation recorded for 5 minutes. None of the candidate primers induced platelet aggregation by themselves (Fig. 9).

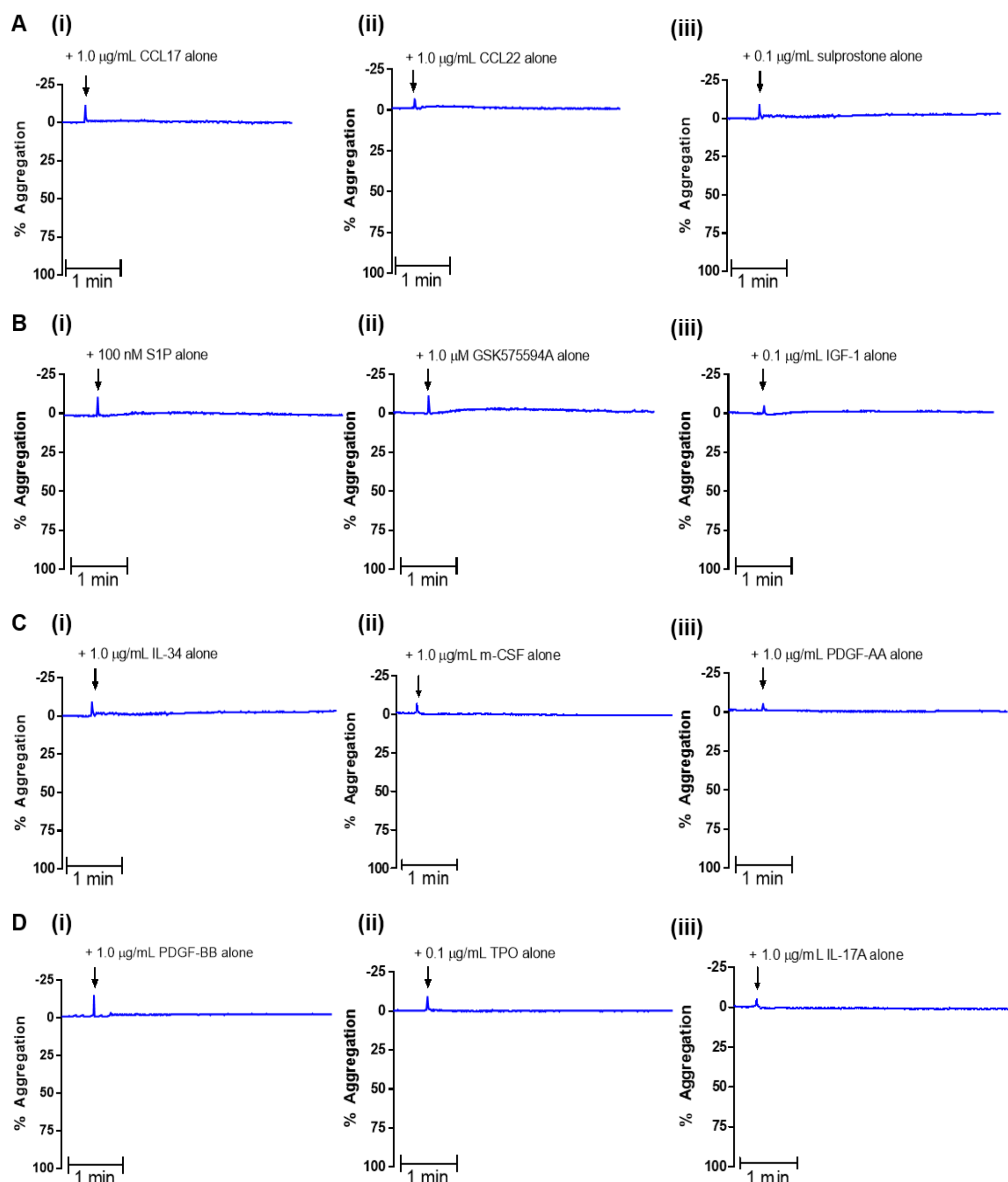


Figure 9. Candidate primers do not induce platelet aggregation. Washed human platelets (2×10^8/mL) were treated with candidate primers alone and recorded for 5 minutes. **(A-D)** Representative traces of platelet aggregation induced by CCL17, CCL22, sulprostone, S1P, GSK575594A, IGF-1, IL-34, m-CSF, PDGF-AA, PDGF-BB, TPO and IL-17A. N=2

3.3 Discussion

From this part of the study several candidate primers include CCL17, CCL22, sulprostone, S1P, IGF-1, IL-34, PDGF-AA and TPO were found to potentiate PAR-mediated platelet aggregation. In contrast, no significant effect on PAR-mediated platelet aggregation was observed in the presence of GSK575594A, m-CSF, PDGF-BB or IL-17A. In addition, no platelet aggregation was observed in the presence of candidate primer alone.

3.3.1 Confirmation that IGF-1 and TPO prime platelet aggregation

Previously it was demonstrated that IGF-1 and TPO can potentiate platelet function[34]. My data confirmed that both IGF-1 and TPO significantly increase platelet aggregation. However, the vehicle response induced by subthreshold PAR1-AP and the amplitude of priming effects were bigger than in previous works. This might be due to platelets being isolated in the presence of 140 nM PGE_1 and 0.02 U/mL apyrase in this study, in contrast to using 10 μM indomethacin and 0.02 U/mL apyrase in previous work. Future work on platelet primers may want to consider using PGE_1 and apyrase treatment, since indomethacin will block primer-mediated TxA2 production[7].

3.3.2 CCL17, CCL22 and S1P prime platelet function via GPCR signalling

Aggregation data confirmed that CCL17 and CCL22 can significantly increase PAR1-AP induced platelet aggregation. This is in agreement with Gear et al[34], who demonstrated that CCL17 and CCL22 can enhance low dose ADP and thrombin-mediated platelet aggregation. Interestingly, at the same concentration of 1.0 μg/mL, CCL17 induced slightly bigger increases in platelet aggregation than CCL22 did. However, CCR4 competitive binding assays performed by Imai et al[51] suggested that CCL22 has a higher binding affinity (IC_{50}=0.65 nM) for CCR4 receptor than CCL17 (IC_{50}= 2.1 nM). The priming effects of both chemokines can start to be observed at lower concentration (0.2 μg/mL), while several studies have found 5 to 10 folds increase in plasma concentration of CCL17 and CCL22 under pathological condition such as asthma, atopic dermatitis and Hodgkin Lymphoma. However, the concentration to produce a priming effect is still thousand times higher than the physiological plasma concentration. In addition, my data indicated that CCL17 and CCL22 alone did not induce aggregation of washed human platelets, which is in accordance with Shenkman et al[81]. In contrast, Sylvie et al[82] suggested CCL17 or CCL22 alone can directly induce platelet aggregation in PRP. In the clinic, inhibiting the CCR4 receptor by monoclonal antibody Mogamulizumab is used to treat cutaneous T-cell lymphoma[83]. In line with literature on the effect of PGE_2 and sulprostone on platelet function, aggregation data confirmed that sulprostone at 0.1 μg/mL concentration potentiated PAR1-AP induced platelet aggregation[52]. Sulprostone is the synthetic analogue of PGE_2 which can activate the $G_{\alpha i/o}$ coupled prostaglandin EP_3 receptor (EP_3), resulting in cAMP inhibition and potentiation of platelet function. In comparison to PGE_2, sulprostone did not induce a concentration dependent

biphasic effect due to its high specificity on EP_3 receptors over the $G_{\alpha s}$ coupled EP_2 and EP_4 receptors[53]. However, it is worth to noting that the concentration evoking the priming effect is supraphysiological, even though elevated PGE_2 concentration were found in patients with asthma or diabetes. Sulprostone is currently used in the clinic for abortion or stopping postpartum hemorrhage, and it is in development with other synthetic prostaglandin analogues to treat gastrointestinal bleeding[84]. The effect of S1P on platelet function is unclear. It was reported S1P can directly induce platelet aggregation in PRP[85]. However, no similar effect was observed in the washed human platelet system in this project. My data suggest that PAR1-AP mediated platelet aggregation is enhanced in the presence of 100 nM S1P. Later experiments also found non-physiological concentrations of S1P inhibit platelet aggregation, which agrees with work done by Onuma et al[85].

3.3.3 IL-34 and PDGF-AA prime platelet function via RTK signalling

IL-34 and m-CSF both are functional ligands of CSF1 receptor but induced different effects on platelet aggregation. 1.0 µg/mL IL-34 (19.1 nM, kDa of 52.6) significantly enhanced PAR1-AP mediated aggregation, whereas no obvious effect was found with 1.0 µg/mL m-CSF (27.2 nM, kDa of 36.8) addition. A possible explanation for this discrepancy could be the structural differences between IL-34 and m-CSF which affect binding to and activation of CSF1R, since these two ligands do not share any similarity in the amino acid sequence. A study by Chihara et al[86] suggests that IL-34 exhibits a 9 to 13-fold higher affinity than m-CSF for binding to CSF1R. Moreover, it was reported that IL-34 binding can produce stronger phosphorylation of downstream tyrosine kinases compare to m-CSF. In addition, studies on macrophages suggest IL-34 can increase the production and release of certain chemokines than m-CSF, in particular, IL-34 can elevate the synthesis of monocyte chemoattractant protein 1 (MCP1)[87]. MCP1 can bind to CCR4, which is the same receptor that CCL17 and CCL22 bind to and potentially further enhance platelet aggregation. Hence, the differences between amino acid sequence, receptor binding affinity, downstream mediator and chemokine release might result in different biological effects induced by IL-34 and m-CSF on human platelets. A previous study demonstrated that elevated local production of m-CSF contributes to the onset of atherosclerosis[88], m-CSF also has been shown to enhance platelet recovery in patients that had chemotherapy[89]. Although one study found increased serum concentration of IL-34 in patients with coronary artery disease, to my knowledge there is no study have been conducted to investigate the effect of IL-34 on platelet function. My data indicates therefore for the first time that IL-34 has a priming effect on human platelets. Future studies on the effect of IL-34 on platelet function should be conducted. Research by Selheim et al[57] suggested that platelet-derived-growth factor inhibits platelet activation via blocking platelet-derived micro-particle formation. However, my aggregation results indicated that PDGF-AA significantly potentiates platelet aggregation by over 400%. In contrast, PDGF-BB slightly increased platelet aggregation, although this did not reach significance. Studies have shown

that PDGF-AA only binds to the PDGF-αα receptor, while PDGF-BB primarily binds to the PDGF-ββ receptor, but also can activate PDGF-αα or the PDGF-αβ heterodimer receptor. However, the transcriptome data indicates possible PDGF-α isomer (RPKM=1.48) but not PDGF-β (RPKM=0.115) expression in human platelets. Moreover, there is no literature evidence support PDGF-αβ and ββ receptor expression in platelets Therefore, PDGF-αα might be the only PDGF receptor expressed in human platelets. In addition, Lin et al[90] using receptor binding assays indicated that PDGF-AA can bind to PDGF-αα receptor with higher affinity (K_d = 134 nM) than PDGF-BB (K_d = 420 nM). The latter may explain the smaller effect of PDGF-BB on platelet function. Furthermore, transcriptome data indicates high levels of PDGF-AA over PDGF-BB expression (RPKM of 15.5 vs 0.136), which is in line with a study that showed activated platelets release PDGF-AA/AB but not PDGF-BB[91]. Hence, it is possible that endogenous PDGF-AA released from activated platelets is competing with PDGF-BB on binding PDGF-αα receptor, resulting in a reduction of the effect evoked by PDGF-BB. It was reported that PDGF can enhance platelet production and recovery in thrombocytopenia mice[92], but future studies are needed to investigate the mechanisms by which PDGF affects platelet function. In the clinic, recombinant human PDGF-BB has been shown to help cure stomach ulcer and counteract the side effects induced by aspirin treatment[93].

3.3.4 Activation of GPR55 or the IL-17A receptor has no effect on platelet function

It was found that Delta-9-Tetrahydrocannabinol (THC) enhanced platelet function by increasing integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure levels[94]. Recently it was reported that THC can also activate the $G_{\alpha q}$ coupled GPR55 receptor causing concentration dependent increases in cytosolic calcium concentration[95]. We have tested the synthetic selective GPR55 receptor agonist GSK575594A (EC_{50}=158 nM) using a concentration range from 10 nM up to 100 µM. However, no effect on PAR1-AP mediated platelet aggregation nor any effect of GSK575594A alone was observed, despite high GPR55 transcriptome expression levels in human platelets (RPKM=50.9) and expression of a functional receptor[96]. Similarly, activation of IL-17A receptor by interleukin 17A showed no effect on PAR-mediated platelet function, although previous studies showed that elevated IL-17A plasma concentrations correlate to the development of acute coronary syndrome, and IL-17A promoted ADP induced platelet aggregation[97]. Future investigations might be necessary to fully probe the effect of GPR55 and IL-17A receptors on platelets.

3.3.5 Identification and evaluation of the platelet primer to take for further study.

The effect and potential underlying mechanism of the candidate primers has been discussed in previous paragraphs. To gain more insight into the mechanism of novel primers, one primer will be selected for much a focused study. PDGF-BB, m-CSF, GSK575594A and IL-17A are

excluded from the study since no effect was observed. IGF-1, TPO and sulprostone (or PGE_2) have already been well characterized, thus will be used as positive control throughout the project. Although CCL17, CCL22, PDGF-AA and IL-34 have shown priming effects, the concentration used to evoke responses is far higher than physiological or even pathological concentrations. For instance, the concentration of CCL17 and CCL22 used to evoke a maximal priming effect was 1.0 μg/mL, which is equal to 125 nM CCL17 (kDa of 8) or 100 nM CCL22 (kDa of 10). However, the concentration used is hundred times higher than the EC_{50} concentration of the CCR4 receptor (0.65 and 2.1 nM respectively). In addition, these concentrations are much higher than the picogram range of physiological concentration. Hence, any priming effect induced by supraphysiological concentrations should be viewed with caution, as it could potentially be a non-physiological or an off-target effect. However, we cannot rule out that the *in vitro* environment might affect cellular responses. For example, isolation of platelets might cause receptor loss or desensitization, thus higher concentrations of the ligands are required to produce similar responses. Also, it is likely in an *in vivo* environment that the concentration of multiple platelet primers could be raised and therefore a combination of primers at lower concentrations are needed to detect a functional effect[42]. However, another problem of studying these primers is the lack of pharmacological tools such as receptor antagonists. Furthermore, the downstream signalling pathway and the ligand binding affinity remain ambiguous, whereas the cost and instability of these protein peptide could limit the type and number of experiments being carried out. Aggregation data suggest S1P can enhance platelet aggregation in a physiological relevant concentration. In contrast to the chemokine or growth factor family primers, there are many commercially available pharmacological tools with reasonable price to be used to study S1P receptors. In addition, the G protein coupled S1P receptors downstream signalling pathway have been well established in other cell lines. Furthermore, there are only a few studies have been carried out to exam S1P effects on platelet function and the effect on platelet function is controversial. Hence, S1P was selected as the “novel platelet primer” to be carried forward for further study in this project.

Chapter 4. Investigation of sphingosine-1-phosphate mediated platelet priming

4.1 Introduction

4.1.1 Overview of sphingosine-1-phosphate

S1P is a bioactive signalling sphingolipid that was discovered in 1993. Early research suggested that S1P was a product released from thrombin-stimulated platelets. However, later studies discovered that mast cells, red blood cells and endothelial cells also contribute to the production of circulating S1P[98]. Recent research demonstrated that S1P released from red blood cells and endothelial cells is responsible for maintaining plasma S1P concentration, while activated platelets contribute to build up of local S1P concentration and facilitated the blood clotting process on injured endothelium (Fig. 10). Circulating S1P is associated with albumin or high-density lipoprotein (HDL), which limits the bioavailable S1P concentration in tissue fluid resulting in a physiological S1P gradient. Early studies recognised S1P as a cellular secondary messenger[98], however, later studies found that S1P can activate the G protein coupled lysophospholipid family S1P receptors. S1P is an important mediator in regulating vascular integrity and permeability, also play a role in immune system to control immune cells migration[99].

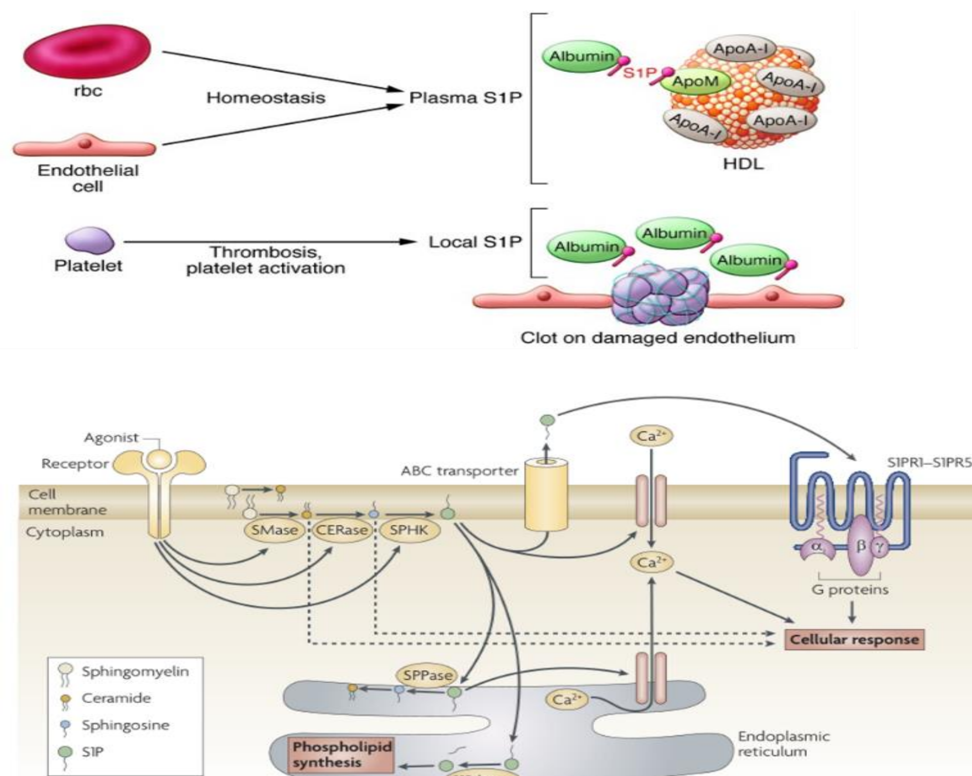


Figure 10. Schematic diagram showing the synthesis, transportation, degradation and signalling process of sphingosine-1-phosphate. RBC: red blood cells; ApoM: apolipoprotein M; ApoA-I: Apolipoprotein A-I; HDL: High-density lipoprotein; SMase: sphingomyelinases; CERase: ceramidases; SPHK: sphingosine kinase; SPP: sphingosine-1-phosphate phosphatase; SPL: sphingosine-1-phosphate lyse; ABC transporter: ATP-binding cassette transporter. Figure adapted from Rivera et al[99].

4.1.2 Sphingosine-1-phosphate synthesis and degradation

S1P synthesis is a stimulation dependent event[100]. Shear stress or activation of RTKs, cytokine receptors and GPCRs can result in activation of sphingomyelinases, ceramidases and sphingosine kinase (Fig. 10). The sphingomyelin in the cell membrane lipid bilayer is broken down by activated sphingomyelinases to ceramide, which in turn is deacylated by ceramidases to form sphingosine. Sphingosine then gets phosphorylated by the sphingosine kinase to generate sphingosine-1-phosphate. Synthesized S1P can be transported out of the cell via ABC transporters, then bind to apolipoprotein M rich HDL or albumin and entering the circulatory system[100]. The intracellular and circulating S1P concentration is tightly regulated by two enzymes: sphingosine-1-phosphate phosphatase (SPP) and sphingosine-1-phosphate lyase (SPL). SPP can dephosphorylate S1P to sphingosine, which in turn is resynthesized to ceramide by ceramide synthase, subsequently sphingomyelin synthase covert the ceramide to sphingomyelin (Fig. 10). Alternatively, S1P can be irreversibly broken down by SPL to form hexadecimal (hex) and phosphoethanolamine (PE) for the synthesis of other phospholipids[98]. The expression level of SPP and SPL are inversely related to the local concentration of S1P. For instance, the average S1P concentration in tissue is about 100 nM due to high SPL expression level, in contrast, the serum S1P concentration is much higher at about 0.5 to 1.4 µM[101]. Studies also indicated that RBC and endothelial cells can store S1P and contribute to the high circulating S1P concentration, due to SPL not being expressed in RBC, and both the SPL and SPP expression are suppressed in endothelial cell[102]. Platelets lack of both SPP and SPL and the concentration of S1P in platelets is about 0.1 nM per $4x10^8$ cells, however, platelets only contribute to local S1P concentration upon activation. Furthermore, the S1P concentration is around 100 nM in lymph, 200 nM in blood, 550 nM in platelet poor plasma, 1.0 µM in plasma and about 1.0-1.2 µM in platelet rich plasma[101,103]. This concentration gradient of S1P is vital for several physiological processes in the body such as lymphocyte migration and angiogenesis[104]. However, the bioactive concentration of S1P in plasma is around 10 nM, because almost 1/3 of S1P is bound to albumin and 2/3 bound to HDL[105].

4.1.3 S1P signalling and its physiological implications

Circulating S1P functions as a bioactive lipid that evokes cellular responses via binding to the G-protein coupled S1P receptors on the cell surface[106]. It was reported that S1P can also act as a secondary messenger to mobilize calcium release (Fig. 10). There are five different subtypes of G-protein coupled S1P receptors ($S1PR_{1-5}$), which have S1P binding affinities in the nanomolar range (6 to 64 nM respectively). $S1PR_1$ is coupled to the G protein subunit $G_{\alpha i/o}$, whereas $S1PR_2$ and $S1PR_3$ are coupled to $G_{\alpha i/o}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$, and $S1PR_4$ and $S1PR_5$ are coupled to $G_{\alpha i/o}$ and $G_{\alpha 12/13}$[106]. Activation of S1PR evokes downstream signalling and cellular responses such as proliferation and migration (Fig. 11). Studies indicate that S1P also plays important roles in several cellular processes, including regulating cell differentiation,

survival and apoptotic process[107]. In addition, S1P contributes to angiogenesis during the embryonic development phase and the maintenance of vasculature integrity. The concentration gradients of S1P in the body also help control lymphocyte migration processes, supporting a role of S1P in inflammation and the immune system. Immuno-modulating drugs such as Fingolimod and Ozanimod that were designed to target S1PR receptors cause $S1PR_1$ internalisation to treat multiple sclerosis[108]. $S1PR_1$ was also reported to be involved in tumour cell growth. Activation of $S1PR_1$ by exogenous S1P results in downstream signalling of ERK and activation of the PI3K-Akt-Rac pathway, thereby amplifying tumour cell proliferation and metastasis[109] (Fig. 11). Activating $S1PR_1$ greatly enhanced the ability of ovarian cancer cells to invade the ovary. Moreover, the proliferative effect of S1P on endothelial cell also facilitates angiogenesis around the tumour[110].

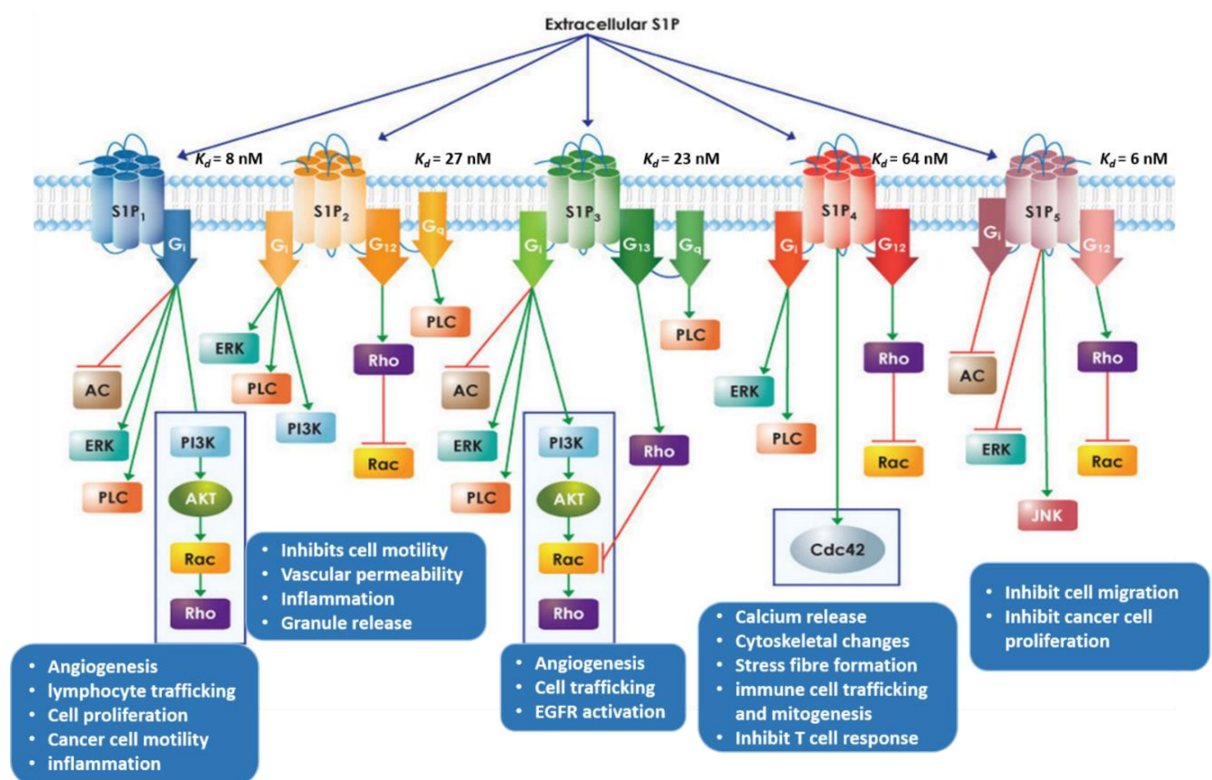


Figure 11. Schematic diagram showing downstream signalling pathways of G-protein coupled sphingosine-1-phosphate receptor subtypes. AC: adenylyl cyclase; ERK: extracellular signal-regulated kinases; PLC: phospholipase C; PI3K: phosphoinositide 3-kinase; Akt: Protein kinase B; Rac: Ras-related C3 botulinum toxin substrate; Rho: Ras homolog gene family; Cdc42: cell division control protein 42 homolog; JNK: c-Jun N-terminal kinase. Figure adapted from Isphingo[111].

Changes in circulating S1P concentrations or S1P signalling have been reported to be associated with many diseases and clinical implications. For instance, increased serum S1P concentrations were found in patients with inflammatory disease and ovarian cancer[112]. Recent research suggest that S1P also relates to the development of atherosclerosis, diabetes and neurodegeneration[113]. Due to the majority of S1P being bound to HDL, alterations in HDL level or dysfunction of HDL may disrupt the HDL-S1P interactions resulting

in increased free bioavailable S1P concentrations. Studies have found lower HDL-S1P interactions and elevated plasma S1P concentrations in patients with heart disease, diabetes and obesity[105]. Elevated bioavailable S1P can signal through the S1P receptors, which may result in amplified cellular responses. Moreover, there is evidence to indicate that S1P is involved in blood coagulation pathway, with elevated S1P concentrations stimulating endothelial cell release of coagulation Factor Xa thereby accelerating thrombin production[114]. In addition, it has been reported that elevated circulating S1P concentrations are linked to the development of angiotensin II-induced hypertension[115].

4.1.4 S1P and platelet function; many questions need to be addressed.

As previously described, intracellular cellular S1P concentrations are kept low by SPP and SPL enzymes. However, platelets lack of both SPP and SPL to degrade S1P, but also exhibit constitutive high sphingosine kinase activity. Hence, platelets can produce and store a large amount of S1P [116]. Studies have demonstrated that thrombin-mediated PAR receptor activation can induce S1P synthesis and release from platelets[117]. In addition, TxA2 and ADP also cause S1P release from platelets, which can be blocked by aspirin[118]. S1P can affect local endothelial cell responses resulting in elevated tissue factor, chemokines and growth factor release[119]. A study suggest that S1P can synergize with thrombin to promote the blood coagulation process[54]. However, there are many controversies in the literature about S1P synthesis, release by platelets and whether the endogenous S1P can affect platelet function. Urtz et al[54] showed that sphingosine kinase 2 is the major isoform of SphK in mouse platelets and responsible for platelet S1P synthesis, inhibition of SphK2 greatly reduced platelet aggregation. This is concurrent with mouse platelet transcriptome data, where SphK1 is much lower than SphK2 (RPKM= 0.18 vs 4.83). In contrast, Münzer et al[120] claims that SphK1 negatively regulates mouse platelet function and knock out SphK1 significantly promoted platelet function. The effects of Sphingosine kinases in human platelets is largely unknown, transcriptome data indicate the mean RPKM of SphK1 is about 24.7 in human platelets, which is much higher than SphK2 (RPKM = 0.5) expression levels. Furthermore, the mechanism of release of S1P from activated platelet is also controversial. Current studies propose S1P can be released in two ways: a readily exchangeable and ATP dependent way near the cytosolic membrane for the *de novo* synthesized S1P release, and an alpha granule release mediated exocytosis dependent way which requires platelet activation. Jonnalagadda et al [121] demonstrated that S1P release is primarily mediated by granule release and the SNARE complex is required, which Munc13-4 KO showed defective S1P release. However, many research groups recognise S1P is transported out of the platelet via ATP binding cassette subfamily C member 4 (ABBC4) transporter [122]. Inhibition of this ABBC transporter by staurosporine or glyburide markedly diminished S1P release from thrombin activated platelets[123]. Furthermore, it was reported that platelets also express S1P receptors, therefore, platelet synthesized S1P might be able to signal in both autocrine and paracrine manner[124].

However, the effects of S1P on platelet function and the S1P receptor subtypes expression are still largely unknown. Recent studies have demonstrated that $S1PR_1$, $S1PR_2$ and $S1PR_4$ receptors are expressed on both matured human and mouse megakaryocytes[127]. Moreover, $S1PR_1$ receptor expression on megakaryocytes is essential for the formation of pro-platelet extension and release of platelets[125]. Knock out $S1PR_1$ or $S1PR_4$ receptor on megakaryocytes significantly reduced the number of pro-platelets formed and decreased the total platelet count in mouse models[126]. Conversely, activation of $S1PR_1$ receptors on megakaryocytes increased platelet production[127]. No effect of KO $S1PR_2$ was found. Based on platelets being derived from megakaryocytes, along with literature reports and S1P receptor transcriptome expression data in platelets (Fig. 5), I hypothesize that $S1PR_1$, $S1PR_2$, $S1PR_4$ and $S1PR_5$ receptors are expressed on human platelets.

The effect of S1P on platelet function is not clear and there are many contradictions between different studies. Yamamura et al [128] suggested that S1P can directly activate platelets causing shape change and aggregation, however, other studies oppose this finding. A whole blood study conducted by Urtz et al[54] indicates that S1P produced by platelets directly controls platelet aggregation and potentiates coagulation via activation of the $S1PR_1$ receptor on the platelets. Inhibition of $S1PR_1$ reduced ADP or PAR4-AP induced aggregation. Moreover, it was reported that the $S1PR_2$ receptor is involved in S1P-induced platelet aggregation and in patients with type II diabetes[129]. In contrast, work by Onuma et al[85] demonstrated that S1P can inhibit platelet responses to CRP via the activation of $S1PR_4$. However, most studies on S1P and platelets were conducted using supraphysiological concentrations of S1P (> 10 μM), which makes the relevance of the findings questionable. Previous work by García-Pacios et al[130] indicated S1P exhibits an amphiphilic nature in Langmuir balance studies, in which S1P can be dissolved in aqueous buffer with a critical micelle concentration of 12 μM. Hence, S1P is the only sphingolipid that can dissociate into the cytosol from the phospholipid bilayer[130]. Theoretically, the highest S1P concentration can be delivered into an *in vitro* system is around 12 to 14 μM, which further challenge current *in vitro* S1P studies using 20 to 40 μM non-physiological concentrations. Moreover, there are no studies conducted on $S1PR_5$ receptor expression on platelets. Furthermore, as a drug target, S1PRs agonist FTY720 (Fingolimod) has been approved by FDA and MEA in 2011 to treat multiple sclerosis, yet its potential effect on platelets is still not clear[131]. In order to clarify the effect of S1P on platelet function and probe the S1P receptor expression and signalling in platelets, along with discussions in Chapter 3, S1P was selected as the “novel platelet primer” to be carried forward for further studies in this project.

4.1.5 Aims

Previous experiments have found S1P can enhance PAR1-AP mediated platelet aggregation, however, the underlying mechanism of S1P priming of platelet function is still not clear. The aims of this part of the projects are therefore

1. Investigate the effect of S1P on human platelets using functional and signaling assays.
2. Demonstrate the signaling cascade and elucidate the mechanism of S1P priming.
3. Identify S1P receptor subtypes expression on platelets and determine the effect of activating or inhibiting S1PRs.
4. Examine the role of sphingosine kinases in platelet function
5. Clarify certain controversies in the literature about S1P effects on platelets.

4.2 Results

4.2.1 A low concentration of S1P can enhance PAR-mediated aggregation

To investigate S1P priming of platelet function, aggregation experiments were carried out by incubating human platelets in the presence or absence of 100 nM S1P for five minutes before stimulation with subthreshold (0.6-1.0 μM) or maximal concentrations (1.5-2.0 μM) of PAR1-AP. Aggregation data indicated that 100 nM S1P can enhance platelet aggregation induced by low concentrations of PAR1-AP (Fig. 12A). The maximal aggregation was significantly increased from 13.1 ± 2.57% with PAR1-AP alone to 46.5 ± 6.29% in the presence of 100 nM S1P (Fig. 12Aii), also a 118% increase in the AUC was observed (Fig. 12Aiii). Note that Fig. 12 A is the same figure shown in Fig. 6 D and has been included here for clarity. In contrast, high concentration PAR1-AP (1.5-2.0 μM)-induced maximal aggregation was only slightly increased in the presence 100 nM S1P (Fig. 12Bi-ii). No significant difference was found between maximum aggregations induced by PAR1-AP alone 60.7 ± 4.07% or in the presence of 100 nM S1P 64.1 ± 4.32% (Fig.12Bii). There was a 2% increase in the total AUCs (Fig.12Biii). In addition, no effect on platelet aggregation was observed with vehicle alone (0.2% methanol, data not shown). These data demonstrate that a low concentration of S1P can enhance PAR-mediated platelet aggregation.

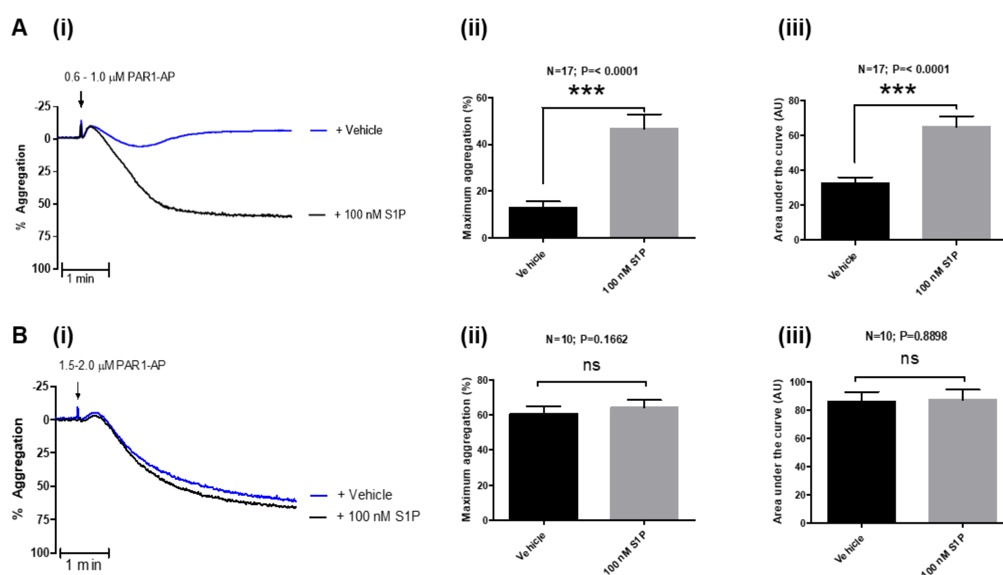


Figure 12. S1P enhances PAR1-AP induced platelet aggregation. Washed human platelets (2 × 10^8/mL) were pre-incubated with vehicle (0.2% methanol) or 100 nM S1P for 5 minutes. Platelet aggregation were induced by subthreshold (0.6-1.0 μM) or maximal (1.5-2.0 μM) concentrations of PAR1-AP and recorded for 5 minutes. **(A-B) (i)** Representative traces of subthreshold and maximal aggregation induced by PAR1-AP in the absence or presence of 100 nM S1P. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or with the presence of S1P. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or with the presence of S1P. AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. *** P < 0.001

4.2.2 High concentrations of S1P inhibit PAR-mediated aggregation

Studies by Onuma et al[85] found S1P can inhibit CRP-induced platelet aggregation at 10 to 40 µM concentrations. To investigate the effect of higher concentrations of S1P, aggregation was induced by stimulating human platelets with a high concentration of PAR1-AP (1.5-2.0 µM) in the absence and presence of increasing concentrations of S1P. In the presence of 1 µM S1P, a small reduction platelet aggregation was observed (Fig. 13A). However, no statistical significance was found between maximum aggregation induced by PAR1-AP alone or in the presence of 1 µM S1P (Fig.13Aii), nor by measure the AUC (Fig. 13Aiii). Platelet aggregation was however significantly reduced in the presence of 10 µM and 30 µM S1P (Fig. 13B-C). These findings indicate that high concentrations of exogenous S1P can suppress platelet aggregation.

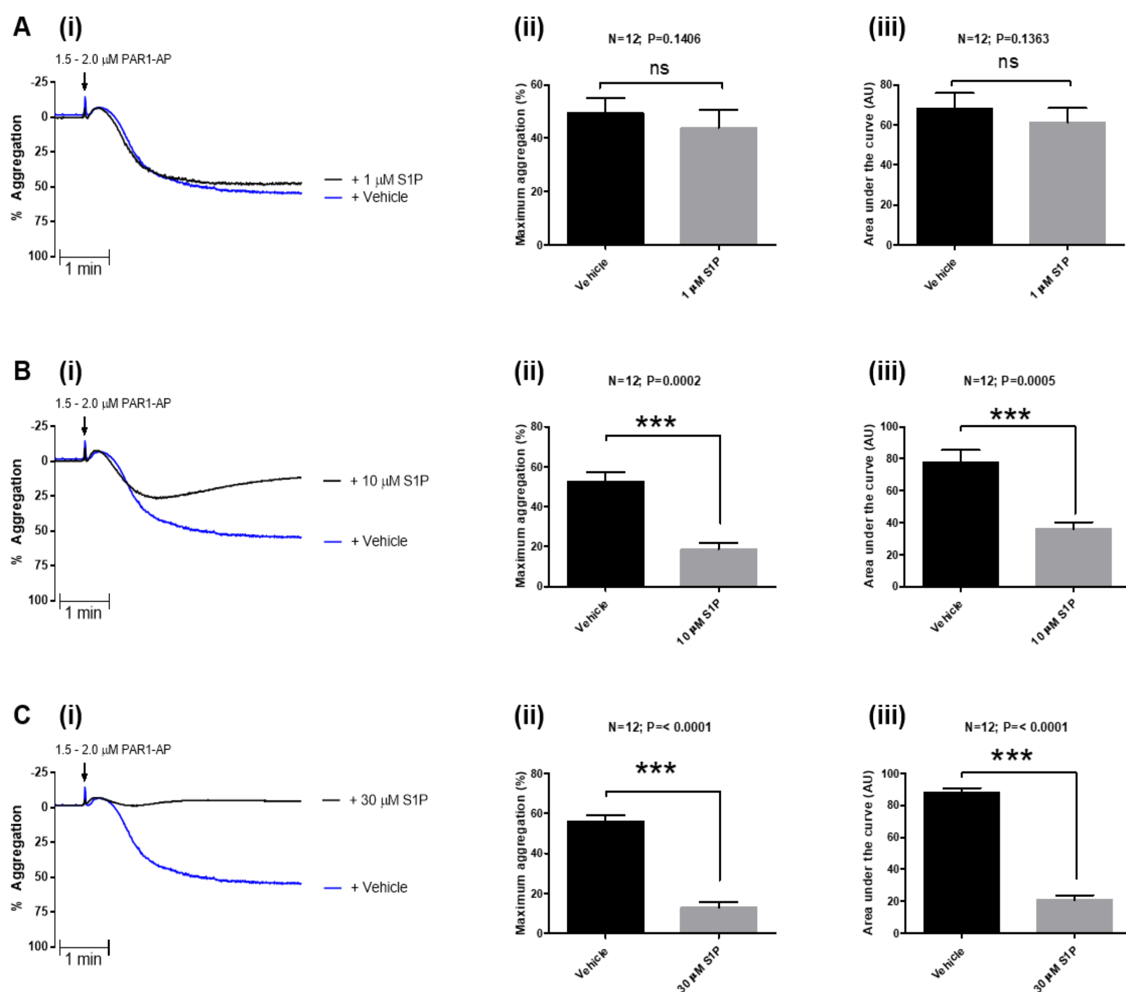


Figure 13. High concentrations of S1P inhibits PAR-induced platelet aggregation. Washed human platelets (2 × 10^8/mL) were pre-incubated with vehicle control (0.2% methanol) or high concentrations of S1P for 5 minutes. Platelet aggregation were induced by maximal concentration (1.5-2.0 µM) of PAR1-AP and recorded for 5 minutes. **(A-C) (i)** Representative traces of maximal aggregation induced by PAR1-AP in the absence or presence of 1, 10, 30 µM S1P. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by high PAR1-AP alone or with the presence of high concentration S1P (1, 10, 30 µM). **(iii)** Histograms graph analysis of AUCs induced by high concentration PAR1-AP alone or with the presence of high concentration S1P (1, 10, 30 µM).

AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. n=12. Statistical analysis: Student's paired t-test. NS, no statistical significance; *** $P < 0.001$.

4.2.3 Activation of S1P receptors by S1PRs pan-agonists results in enhanced platelet aggregation

To investigate whether the S1P priming effect on platelet aggregation was mediated via the activation of S1P receptors, aggregation experiments were carried out by stimulating human platelets with subthreshold PAR1-AP in the presence of the pan-S1PR agonist DHS1P, FTY720 and FTY720-phosphate. The maximal aggregation increased from 19.4 ± 3.83% with PAR1-AP alone to 30.9 ± 4.32% in the presence of 1 μM DHS1P (Fig. 14Aii). There was a 29% increase in the AUC, although this did not reach statistical significance (Fig. 14Aiii). In the presence of 1 μM FTY720, small increases were found in maximal aggregation and AUC, but no significance was found (Fig. 14B). In contrast, maximum aggregation was significantly increased from 18.5 ± 8.26% with PAR1-AP alone to 53 ± 10.9% in the presence of 1 μM phosphorylated FTY720 (Fig. 14Cii). The total aggregation was significantly increased by 89% measured as AUC (Fig. 14Ciii). These data suggest that activation of S1PRs by pan-S1PR agonists can enhance PAR-mediated platelet aggregation. However, DHS1P and FTY720 did not produce a similar degree of priming as 100 nM S1P or FTY720-P did, which might be due to differences in receptor binding affinity or downstream signalling effect between these ligands.

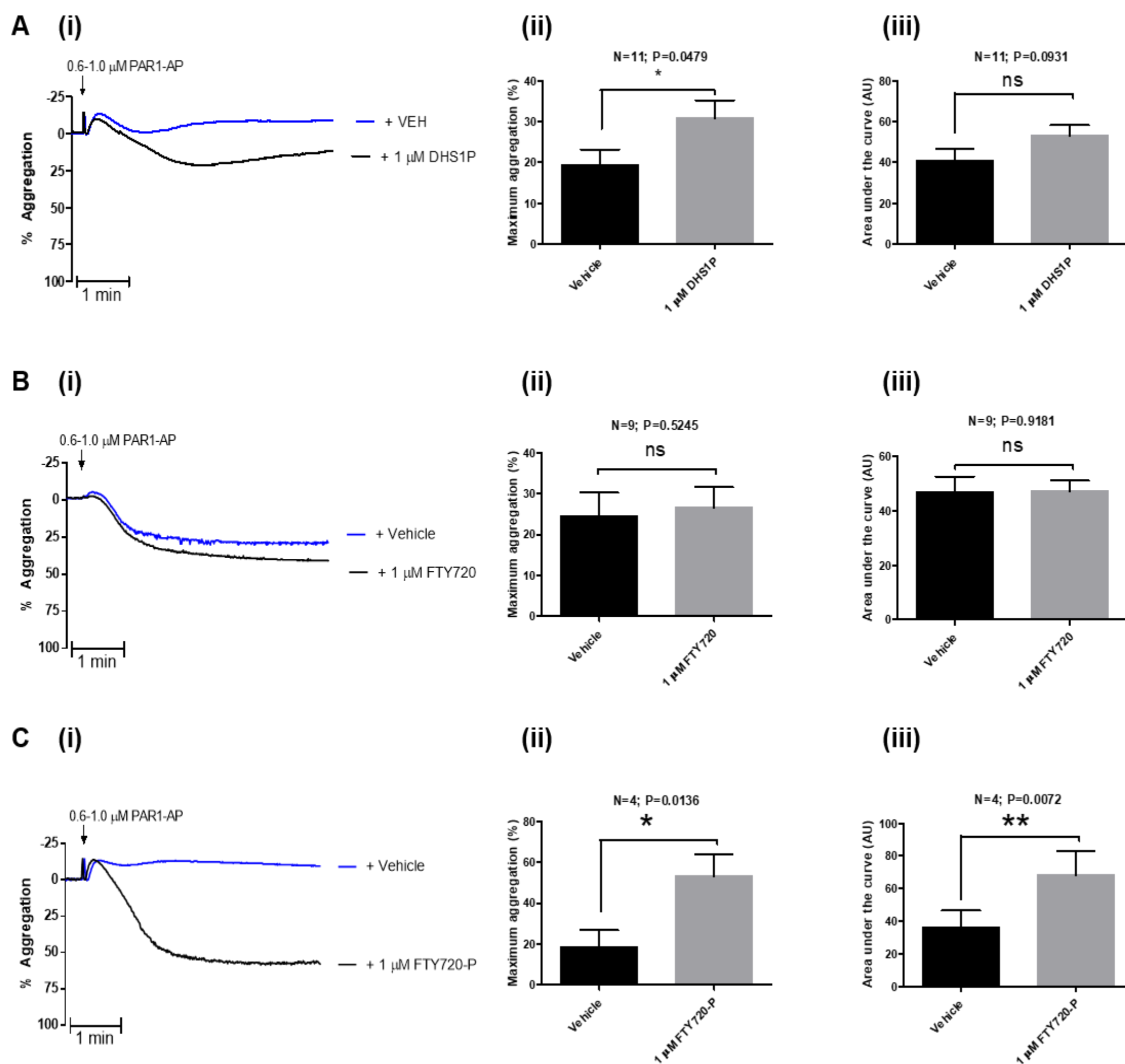


Figure 14. Pan-S1PR agonists enhance PAR-induced platelet aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle control or S1PR agonist for 5 minutes. Platelet aggregation were induced by subthreshold (0.6-1.0 µM) concentration of PAR1-AP and recorded for 5 minutes. **(A-C) (i)** Representative traces of PAR1-AP induced platelet aggregation in the absence or presence of 1.0 µM DHS1P, 1.0 µM FTY-720 or 1.0 µM FTY-720 phosphate .**(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or in presence of pan-S1PR agonists. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or in the presence of S1PRs pan agonists. AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; * $P < 0.05$, ** $P < 0.01$.

4.2.4 High concentration of S1PR agonists inhibit platelet aggregation

To investigate whether the pan-S1PR agonists also exhibit a concentration dependent biphasic effect on platelet aggregation, human platelets were stimulated with a high concentration of PAR1-AP in the absence or presence of 10 μM DHS1P, 10 μM FTY720 or 10 μM FTY720-phosphate. PAR1-AP mediated platelet aggregation was reduced in the presence of high concentrations of S1P receptor pan-agonists (Fig. 15). These findings suggest the inhibitory effects induced by high concentrations of S1P are mediated via S1PRs.

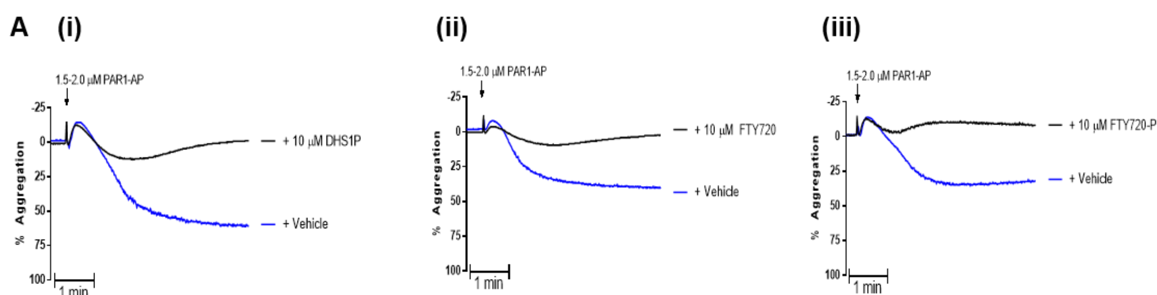


Figure 15. High concentration S1P receptor pan agonists inhibit PAR1-AP induced platelet aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle control or S1PR agonist for 5 minutes. Platelet aggregation was induced by maximal (1.5-2.0 μM) concentrations of PAR1-AP and recorded for 5 minutes. **(A)** Representative traces of PAR1-AP induced platelet aggregation in the absence or presence of 10 μM DHS1P, 10 μM FTY-720 and 10 μM FTY-720 phosphate. N=1.

4.2.5 Activation of $S1PR_1$ enhances PAR1-AP mediated platelet aggregation, whereas activation of $S1PR_4$ and $S1PR_5$ inhibits platelet aggregation.

It is important to identify the S1PR subtypes expressed on human platelets and investigate which S1P receptor subtypes is mediating the effects of S1P on platelet function. Aggregation was induced by either subthreshold or maximal concentrations of PAR1-AP in the presence or absence of S1PR selective agonists. Aggregation experiments confirmed that activating $S1PR_1$ can enhance PAR1-AP mediated platelet aggregation (Fig. 16Ai). In the presence of 1 μM SEW2871, platelet maximal aggregation significantly increased from 7.67 ± 2.91% to 34.5 ± 6.86% and the AUC was increased by 120% (Fig. 16Aii, Aiii).

Aggregation experiments found there was no effect of activating $S1PR_2$ or $S1PR_3$ on PAR1-mediated platelet aggregation (Fig. 16Bi, Ci). Furthermore, no significant effect on maximal aggregation or AUC was observed in platelets pre-treated with 1 μM $S1PR_2$ agonist CYM5520 or 1 μM $S1PR_3$ agonist CYM5541 (Fig. 16B and C). In addition, at 1 μM of concentration $S1PR_1$, $S1PR_2$ or $S1PR_3$ agonists did not show inhibitory effects. However, further increases in the agonist concentration up to 10 μM occasionally resulted in inhibitory effects (data not shown). Activation of $S1PR_4$ or $S1PR_5$ receptors caused a strong inhibition of PAR1-mediated platelet aggregation (Fig. 16Di, Ei). The maximum aggregation induced by PAR1-AP was significantly decreased from 50.8 ± 7.58% to 3.25 ± 2.29% in the presence of 1 μM CYM50260 (Fig. 16Dii) The AUC also decreased significantly as shown in (Fig. 16Diii). In the presence of 1 μM A971432, the maximal aggregation was reduced from 45 ± 6.72% to 4.2 ± 2.54% and there was a 79% decrease in the AUC (Fig. 16Eii, Eiii). The inhibitory effect of CYM50260 and A971432 could be observed with lower concentrations starting at 0.1 μM and maximise at 1 μM. These observations suggest that $S1PR_1$ is expressed on human platelets and responsible for the positive priming effect of submicromolar S1P. No effect was observed with $S1PR_2$ agonist CYM5520 or $S1PR_3$ agonist CYM5541 addition within a concentration range from 500 nM to 10 μM (data not shown), which suggest that $S1PR_2$ and $S1PR_3$ may not be expressed on human platelets. In contrast, my data indicates that $S1PR_4$ and $S1PR_5$ are likely to be expressed on human platelets and responsible for the inhibitory effects induced by high concentrations of S1P.

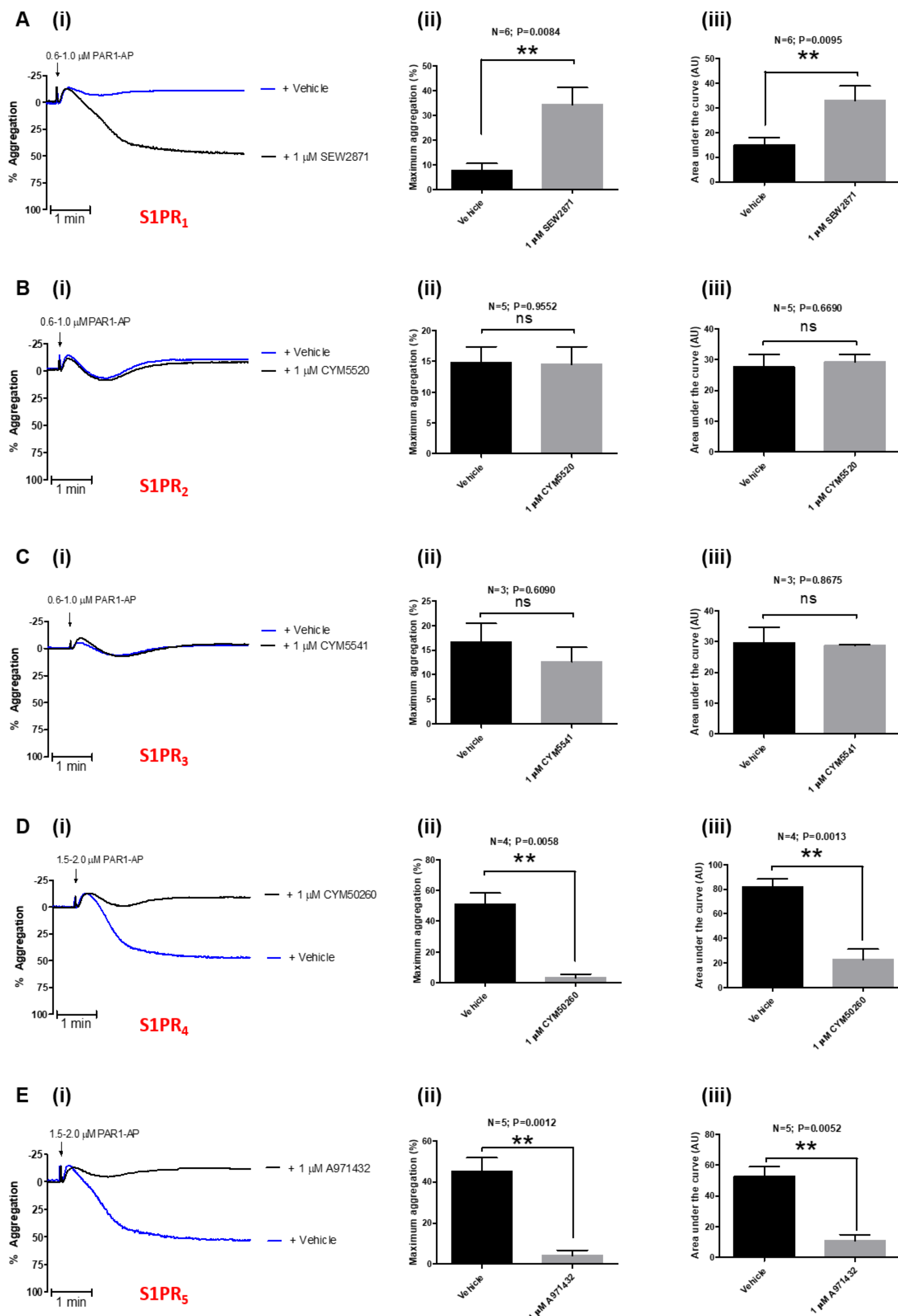


Figure 16. Effect of S1PR selective agonists on PAR-mediated aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle (0.2% DMSO) or S1PR agonists for 5 minutes. Platelet aggregation were induced by subthreshold (0.6-1.0 µM) or maximal (1.5-2.0 µM) concentrations of PAR1-AP and recorded for 5 minutes. **(A-C) (i)** Representative traces of platelet aggregation induced by subthreshold PAR1-AP in the absence or presence of the $S1PR_1$ agonist SEW2871, $S1PR_2$ agonist CYM5520 or $S1PR_3$ agonist CYM5541. **(D, E) (i)** Representative traces of platelet aggregation induced by PAR1-AP in the absence or presence of the $S1PR_4$ agonist CYM50260 or $S1PR_5$ agonist A971432. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or in presence of S1P receptor agonists. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or in the presence of S1P receptor agonists. AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; ** $P < 0.01$.

4.2.6 Antagonism of $S1PR_1$ reduces PAR-mediated aggregation, whereas antagonism of $S1PR_4$ enhances PAR-mediated aggregation.

To investigate the effect of inhibiting S1PR subtypes, platelet aggregation was induced by subthreshold or high concentrations of PAR1-AP in the absence or presence of S1PR selective antagonists. Aggregation experiments determined that inhibition of $S1PR_1$ inhibited PAR1-AP mediated platelet aggregation (Fig. 17Ai). In the presence of 0.1 μM Ex26, high concentration PAR1-AP induced platelet aggregation significantly decreased from 54.5 ± 11.9% to 14.5 ± 2.35% and the AUC was decreased by 61.1% (Fig. 17Aii, Aiii). Aggregation data indicated inhibition of $S1PR_2$ or $S1PR_3$ has no effect on subthreshold concentrations of PAR1-AP mediated platelet aggregation (Fig. 17Bi, Ci). No significant effect on maximal aggregation or AUC was observed in platelets pre-treated with 0.1 μM $S1PR_2$ antagonist JTE-013 or 1 μM $S1PR_3$ antagonist TY52156 (Fig. 17Bii, Cii, Biii and Ciii). $S1PR_2$ or $S1PR_3$ antagonists did not show inhibitory effects on inhibiting platelet aggregation, however, further increasing the concentration of these antagonists up to 10 μM could resulted in some inhibitory effects (data not shown). Interestingly, another $S1PR_1$ receptor antagonist W146 tested earlier in this study induced irreversible “aggregation” resulting in a maximal decrease in the light transmission just by itself (data not shown). We deduce that W146 might exhibit potential platelet lysis or toxic effect, thus it was excluded from the study.

Antagonising $S1PR_4$ using 1 μM CYM50358 hydrochloride enhanced subthreshold PAR1-AP mediated platelet aggregation (Fig. 17Di). The maximum aggregation was significantly increased from 9.4 ± 2.7% with PAR1-AP alone to 43.7 ± 7.68% in the presence of 1 μM CYM50358 hydrochloride (Fig. 17Dii) The total aggregation was significantly increased by 81%, measured as AUC (Fig. 17Diii). This enhancing effect by antagonising $S1PR_4$ is saturated at 1 μM, but it could start to be observed at lower concentration using 0.1 μM CYM50358 hydrochloride.

These data are in line with result from S1P agonist tests, suggesting that $S1PR_1$ is responsible for the positive priming effect of S1P. No effect was observed with addition of JTE-013 or TY52156, which strongly imply that $S1PR_2$ or $S1PR_3$ are not expressed in human platelets. Furthermore, $S1PR_4$ antagonist enhanced PAR1-AP mediated aggregation, suggesting the inhibitory effect of supermicromolar S1P is partly mediated via $S1PR_4$ activation and inhibition of $S1PR_4$ could reverse this effect. However, due to the lack of commercially available $S1PR_5$ antagonists, the effect of inhibiting $S1PR_5$ remains unclear.

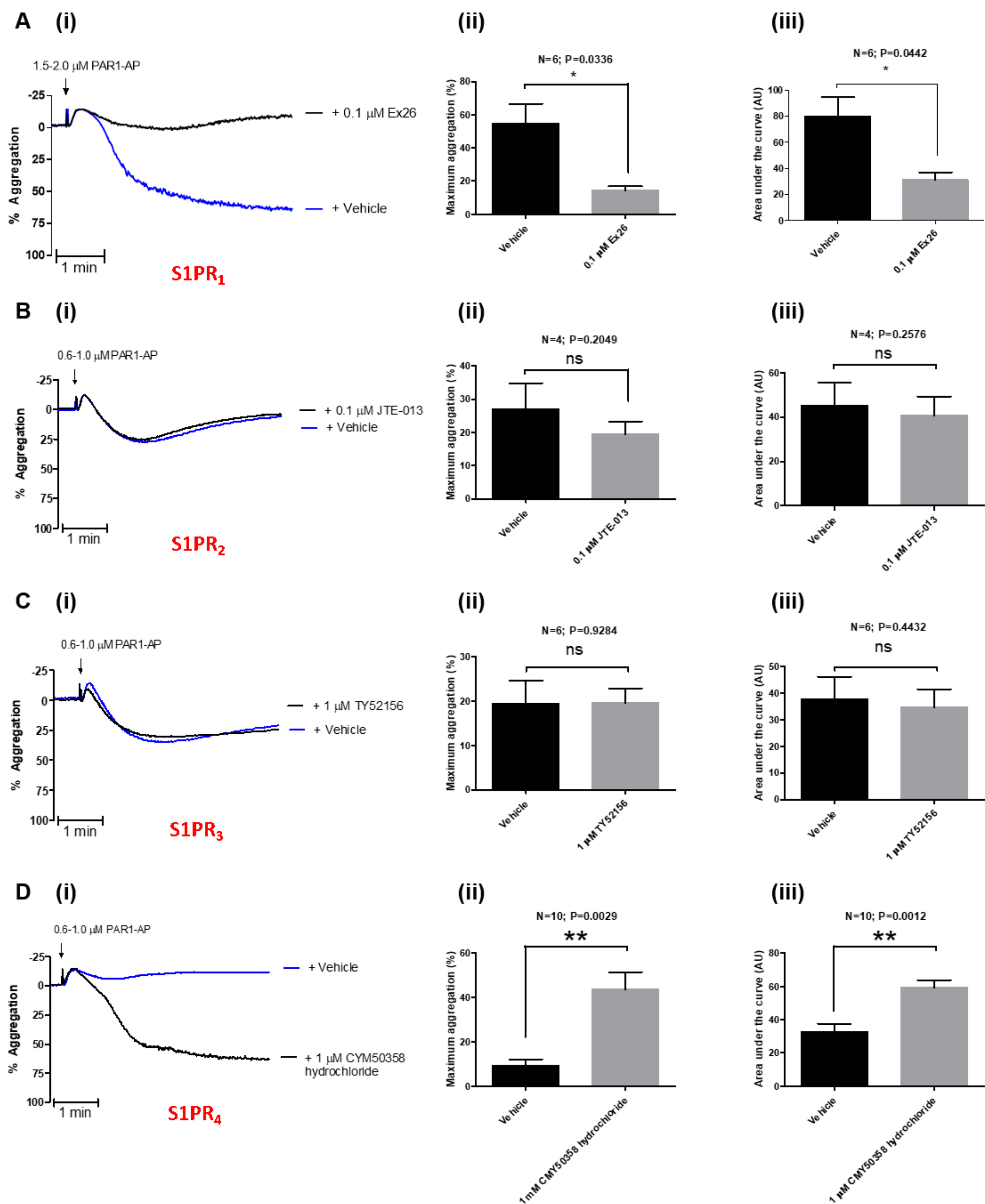


Figure 17. Effect of S1PR antagonists on PAR-mediated platelet aggregation. Washed human platelets (2×10^8/mL) were treated with vehicle (0.2% DMSO) or S1PR antagonists for 5 minutes. Platelet aggregation were induced by subthreshold (0.6-1.0 µM) or maximal (1.5-2.0 µM) concentrations of PAR1-AP and recorded for 5 minutes. **(A-D) (i)** Representative traces of PAR1-AP induced platelet aggregation in the absence or presence of $S1PR_{1-4}$ antagonists: 0.1 µM Ex26 or JTE-013, 1.0 µM TY52156 or CYM50358 hydrochloride. **(ii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or in presence of S1P receptor antagonists. **(iii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or in the presence of S1P receptor antagonists. AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. NS: no statistical significance; ** P < 0.01,

4.2.7 Inhibition of sphingosine kinases reduces PAR1-AP mediated platelet aggregation.

To investigate the effect of inhibiting sphingosine kinases and endogenous S1P synthesis in platelets, aggregation was induced by high concentrations of PAR1-AP in the absence or presence of sphingosine kinase inhibitors. Aggregation experiments indicate that inhibition of sphingosine kinases can reduce PAR1-AP mediated platelet aggregation (Fig. 18Ai, Bi). In the presence of 1 μM SphK1 inhibitor PF543 hydrochloride, aggregation was significantly reduced from 50.3 ± 6.59% to 23.3 ± 8.7% (Fig. 18Aii) and the AUC was significantly reduced (Fig. 18Aiii). In contrast, maximal aggregation induced by high concentrations of PAR1-AP was significantly reduced from 57.5 ± 5.6% to 4.25 ± 1.97% in the presence of 10 μM non-selective SphK1/2 inhibitor DMS (Fig. 18Bii) and total aggregation was decreased by 83.6% (Fig. 18Biii). The inhibitory effect of DMS can be observed at 1 μM concentration (Fig. 18Bi). In contrast, further increasing PF543 hydrochloride concentration up to 10 μM did not produce the same degree of inhibition as 10 μM DMS did. These findings demonstrate that sphingosine kinases play a role in platelet aggregation, possibly via the control of *de novo* S1P synthesis and subsequent autocrine activation on the $S1PR_1$ receptor. Furthermore, the non-selective SphK inhibitor DMS (IC_{50}=500 nM) induced a much stronger inhibitory effect on platelet aggregation than the same concentration of the SphK1 selective inhibitor PF543 hydrochloride (IC_{50}=2 nM). This finding agrees with Urtz et al[54] suggesting that SphK2 might be the major sphingosine kinase controlling S1P synthesis in platelets.

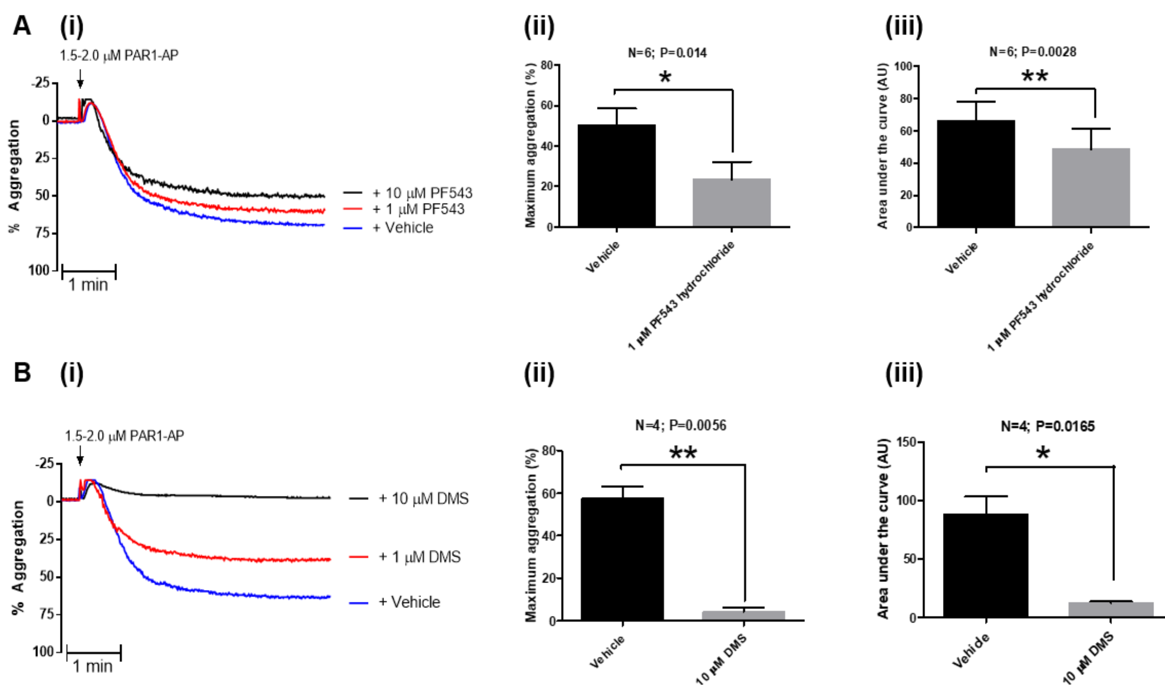


Figure 18. Inhibiting sphingosine kinases reduces PAR1-AP induced platelet aggregation. Washed human platelets (2 × 10^8/mL) were pre-incubated with vehicle control (0.2% DMSO) or sphingosine kinase inhibitors for 5 minutes. Platelet aggregation were induced by maximal (1.5-2.0 μM) concentration of PAR1-AP and recorded for 5 minutes. **(Ai, Bi)** Representative traces of maximal concentration PAR1-AP induced platelet aggregation in the absence or presence of 1.0, 10 μM SPHK1 inhibitor PF543 hydrochloride or 1 μM, 10 μM SPHK1/2 inhibitor DMS (N, N-Dimethylsphingosine) respectively. **(A, Bii)** Histograms graph analysis of quantified percentage maximum aggregation induced by PAR1-AP alone or with the presence of 1 μM PF543 hydrochloride or 10 μM DMS. **(A, Biii)** Histograms graph analysis of AUCs induced by PAR1-AP alone or with the presence of SPHK inhibitors. AU: arbitrary unit. Data are plotted as mean ± standard error of the mean. Statistical analysis: Student's paired t-test. * $P < 0.05$, ** $P < 0.01$.

4.3.1 Dual effect of S1P on integrin activation and P-selectin exposure.

To investigate the underlying mechanism of how S1P affects platelet function in a concentration dependent biphasic manner, flow cytometry was carried out to exam the effects of S1P on PAR1-AP induced platelet integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure (as a measure of α-granule secretion). FACS analysis experiments indicate that PAR1-AP mediated integrin $\alpha_{IIb}\beta_3$ activation was significantly increased in the presence of 100 nM S1P, and the maximal integrin $\alpha_{IIb}\beta_3$ activation was increased from 1522 ± 75.5 to 1768 ± 79.3 (Fig. 19Ai). In addition, the PAR1-AP concentration response curve (blue trace) was shifted to the left in the presence of 100 nM S1P (red trace), with the $LogEC_{50}$ value significantly decreased from -5.59 ± 0.045 to -5.66 ± 0.027 (Fig. 19Aii). PAR1-AP mediated P-selectin exposure was also significantly increased in the presence of 100 nM S1P with maximal P-selectin exposure being increased from 5991 ± 192 to 6374 ± 152 (Fig. 19Bi). In addition, the PAR1-AP concentration response curve was shifted to the left with a reduction in the $LogEC_{50}$ value (Fig. 19Bii).

In contrast, PAR1-mediated integrin $\alpha_{IIb}\beta_3$ activation was significantly reduced in platelets pre-treated with 10 μM S1P (Fig. 19Ci), with maximal integrin $\alpha_{IIb}\beta_3$ activation decreased from 1550 ± 68.5 to 1238 ± 62.6 (Fig. 19Ci). In addition, there was a slight rightward shift in the PAR1-AP concentration response curve, and $LogEC_{50}$ value was increased from -5.57 ± 0.023 to -5.54 ± 0.042 (Fig. 19Cii). PAR1-AP mediated P-selectin exposure was significantly decreased in the presence of 10 μM S1P. Maximal P-selectin exposure reduced from 6387 ± 177 to 5869 ± 242 (Fig. 19Di) and the $LogEC_{50}$ value of the PAR1-AP concentration response curve increased from -5.46 ± 0.019 to -5.44 ± 0.036 (Fig. 19Dii) in the presence of S1P. Therefore, it can be deduced that the biphasic effect of S1P on PAR1-AP mediated platelet function is likely to be the result of affecting both integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure.

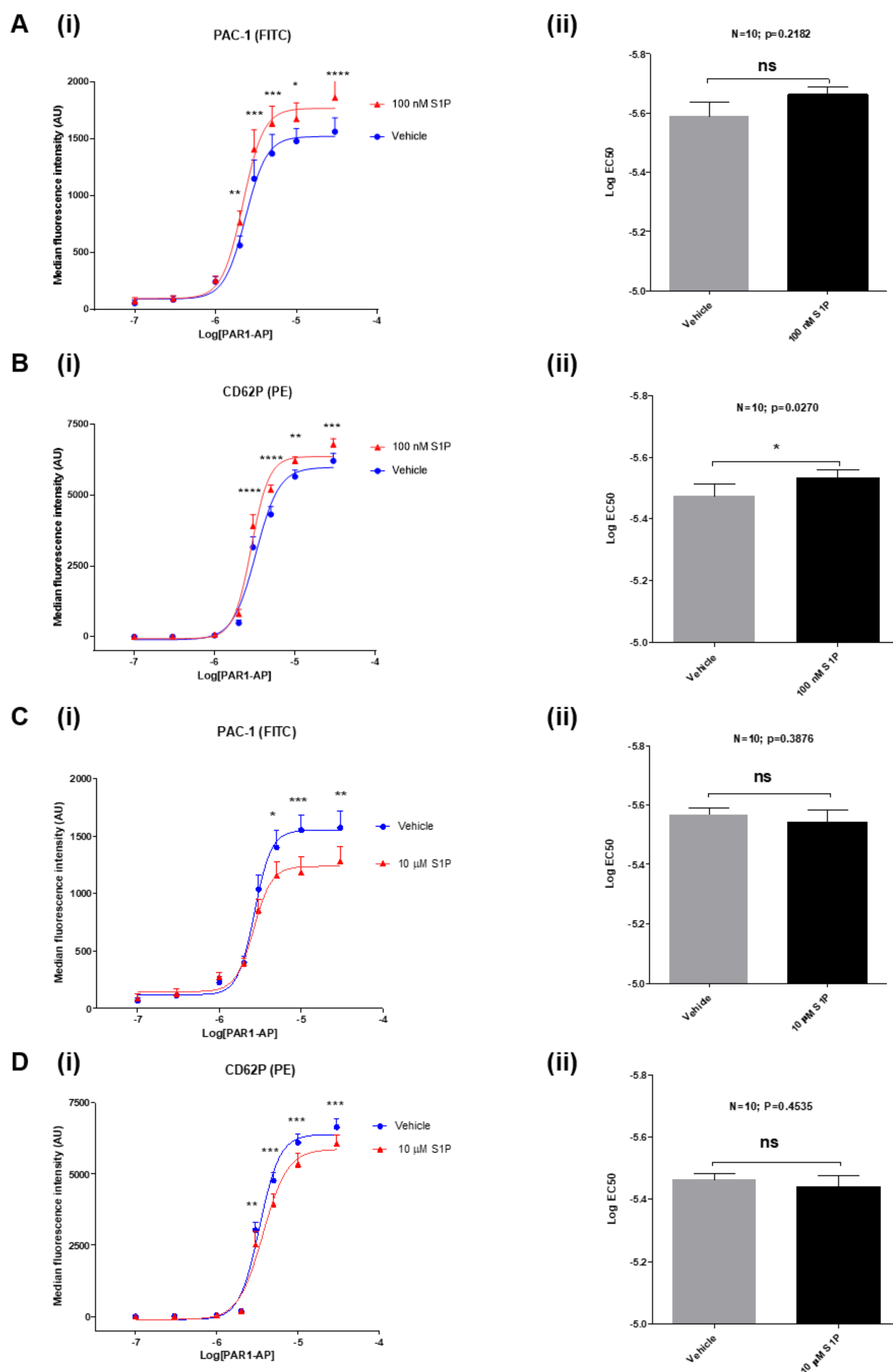


Figure 19. S1P alters PAR1-mediated integrin activation and P-selectin exposure. Washed human platelets (2 × 10^7/mL) were incubated with vehicle (0.2% methanol) or S1P (100 nM, 10 µM, 10 min), before stimulation with PAR1-AP for 10 minutes in the presence of fluorescently conjugated antibodies against the activated form of integrin $\alpha_{IIb}\beta_3$ and P-selectin. Platelet samples were fixed (1 % PFA) before reading on a flow cytometer. **(A and C)** Platelet integrin activation level was measured by binding of PAC-1 antibody. Results are expressed as medium fluorescent intensity. **(B and D)** Platelet P-selectin exposure. Results are expressed as median fluorescent intensity. AU: arbitrary unit. N=10. Statistical analysis: two-way ANOVA with Bonferroni post hoc multiple comparisons. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 **(ii)** Histograms graph analysis of the $LogEC_{50}$ value measured of the PAC-1 and CD62P binding curve. Statistical Analysis: Student's paired t-test. Data are plotted as mean ± standard error of the mean. NS: no statistical significance; * P < 0.05.

4.3.2 Effect of pan-S1PR agonists on integrin activation and P-selectin exposure.

To further investigate effects of activating S1P receptors, FACS analysis was carried out to examine the effect of pan-S1PR agonists on PAR1-AP induced integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure. FACS analysis indicate that PAR1-AP mediated integrin $\alpha_{IIb}\beta_3$ activation level was significantly increased in the presence of 1 μM phosphorylated FTY720 (Fig. 20Ai). Maximal integrin $\alpha_{IIb}\beta_3$ activation increased from 1281 ± 78.6 to 1419 ± 96.4, and the $LogEC_{50}$ value decreased from -5.52 ± 0.047 to -5.58 ± 0.049. However, no significance change in P-selectin exposure was found (Fig. 20Aii). In the presence of 1 μM DHS1P, a slight leftward shift in the PAR1-AP response curve was found, but no significant change in platelet integrin $\alpha_{IIb}\beta_3$ activation or P-selectin exposure level was found (Fig. 20 Bi, Bii). From these data obtained, it can be concluded that both activation of S1PRs and the downstream intracellular effects are required to affect integrin $\alpha_{IIb}\beta_3$ activation.

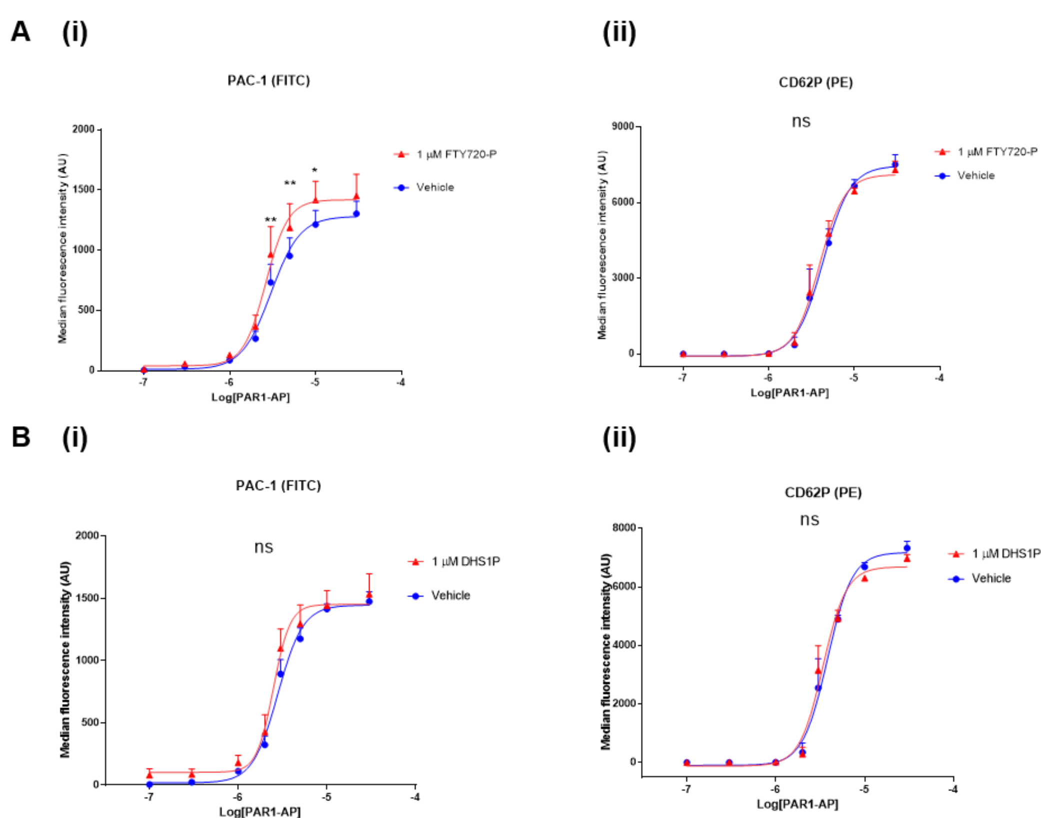


Figure 20. Effect of pan-S1PR agonists on PAR1-mediated integrin activation and P-selectin exposure. Washed human platelets (2 × 10^7/mL) were pre-treated with vehicle or pan-S1PR agonist (10 min), before stimulation with PAR1-AP for 10 minutes in the presence of fluorescently conjugated antibodies against the activated form of integrin $\alpha_{IIb}\beta_3$ and P-selectin. Samples were fixed (1 % PFA) before reading on a flow cytometer. **(Ai, Bi)** Integrin activation. **(Aii, Bii)** P-selectin exposure. Results are expressed as median fluorescent intensity. Data are plotted as mean ± standard error of the mean. N=3. Statistical analysis: two-way ANOVA with Bonferroni post hoc multiple comparisons. NS: no statistical significance. * $P < 0.05$, ** $P < 0.01$.

4.3.3 Activating or inhibiting $S1PR_1$, $S1PR_4$ and $S1PR_5$ affects integrin activation and P-selectin exposure

To demonstrate the mechanism of action of S1PR selective agonists and antagonists on platelet function, FACS analysis was carried out. As shown in Fig. 21A, maximal integrin $\alpha_{IIb}\beta_3$ activation was significantly increased from 1266 ± 56 to 1361 ± 79 in the presence of 1 μM SEW2871, with no significant change in P-selectin exposure. In contrast, the integrin activation level of platelets pre-treated with 1 μM Ex26 remained unchanged (Fig. 21B). But a slight decrease in P-selectin exposure level (6956 ± 392.5 to 6656 ± 358.8) was observed (Fig.21Bii). Surprisingly, the $S1PR_4$ agonist CYM50260, which strongly inhibited platelet aggregation, caused a significant increase (1266 ± 56.1to 1389 ± 84.0) in integrin $\alpha_{IIb}\beta_3$ activation, but not P-selectin exposure (Fig. 21Ci, Cii). In the presence of 1 μM $S1PR_4$ antagonist CYM50358 hydrochloride, the 3 μM PAR1-AP induced integrin activation and P-selectin exposure was significantly increased from 732 ± 149 to 977 ± 224 (asterisk point, Fig. 21Di). In addition, both curves were slightly shifted to the left. In contrast, the $S1PR_5$ agonist A971432, which strongly inhibited platelet aggregation, reduced both integrin activation and P-selectin exposure levels (Fig. 21E). These findings indicate that activation of $S1PR_1$ results in elevated integrin $\alpha_{IIb}\beta_3$ activation and priming of platelet function. Inhibition of $S1PR_4$ receptor increases both integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure. The inhibitory effect of $S1PR_5$ is mediated via inhibition of integrin $\alpha_{IIb}\beta_3$.

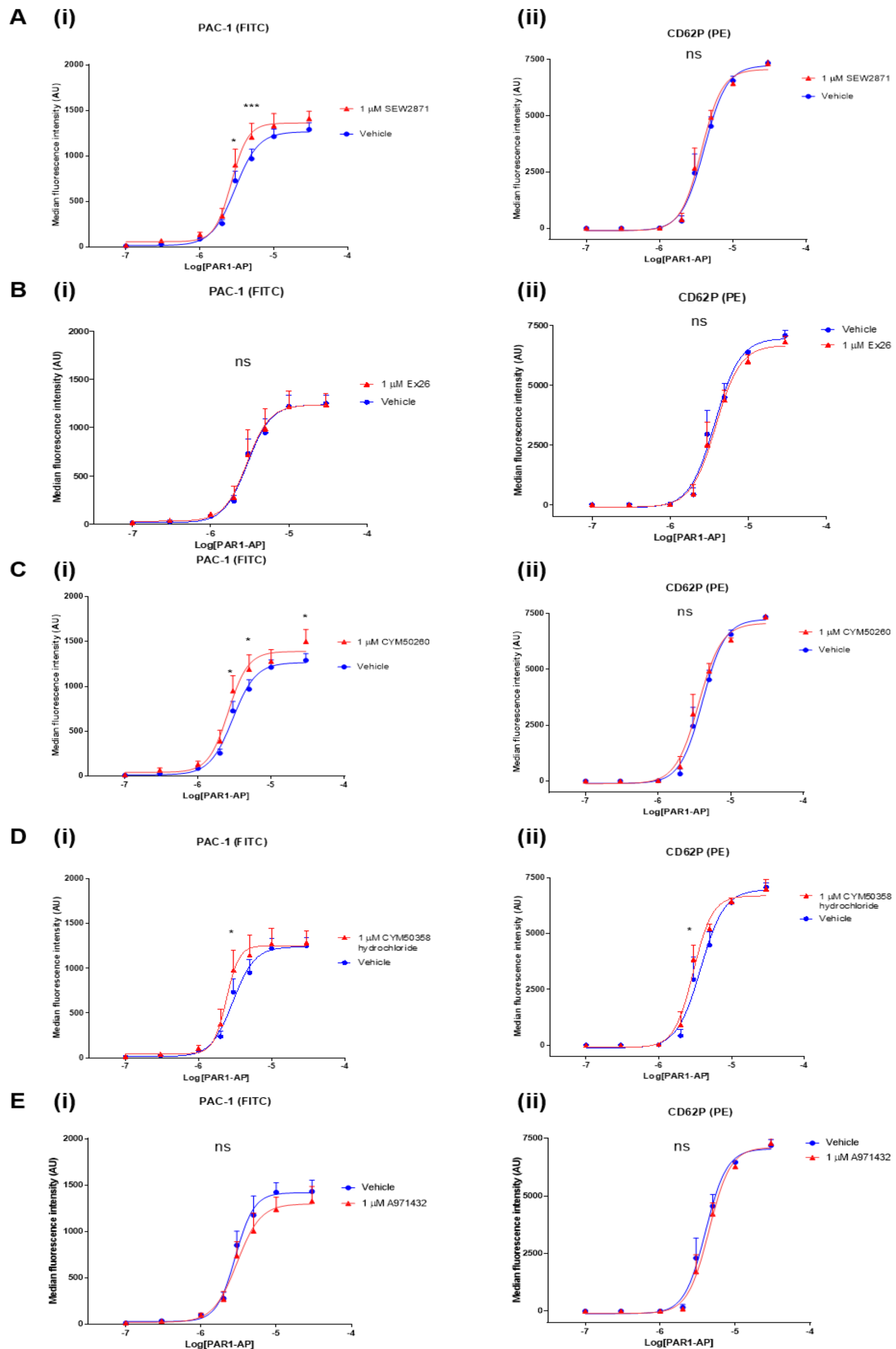


Figure 21. Effect of S1PR selective agonists and antagonists on PAR-mediated integrin activation and P-selectin exposure. Washed human platelets (2 × 10^7/mL) were pre-treated with vehicle or S1PR selective agonist/antagonist (10 min), before stimulation with PAR1-AP for 10 minutes in the presence of fluorescently conjugated antibodies against the activated form of integrin $\alpha_{IIb}\beta_3$ and P-selectin. Samples were fixed (1 % PFA) before reading on a flow cytometer. **(Ai - Ei)** Integrin activation. **(Aii - Eii)** P-selectin exposure. Results are expressed as median fluorescent intensity. Data are plotted as mean ± standard error of the mean. N=3. Statistical analysis: two-way ANOVA with Bonferroni post hoc multiple comparisons. NS: no statistical significance. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

4.4.1 S1P induces PKB phosphorylation in a concentration dependent biphasic manner

To investigate the downstream signaling elicited by S1P in platelets and probe the mechanism of S1P priming, the phosphorylation of the PI3K effector PKB (Akt-S^{473}) and MAPK ERK1/2 was determined by immunoblotting. S1P alone induced PKB phosphorylation (Fig. 22A). Both 100 nM and 300 nM S1P evoked significant increases of 321% and 318% in PKB phosphorylation compare to vehicle (Fig. 22Bi). Further increasing the concentration of S1P resulted in less PKB phosphorylation, with the PKB phosphorylation levels reduced back to basal levels in the presence of 30 µM S1P (Fig. 22Bi). In addition, S1P alone did not induce any ERK1/2 phosphorylation (Fig. 22A,) and there was no significance found (Fig. 22Bii). A similar trend was observed in for phospho-PKC substrate phosphorylation levels (data not shown). Together, these findings suggest that S1P can activate PI3K but not ERK1/2 or PKC in platelets.

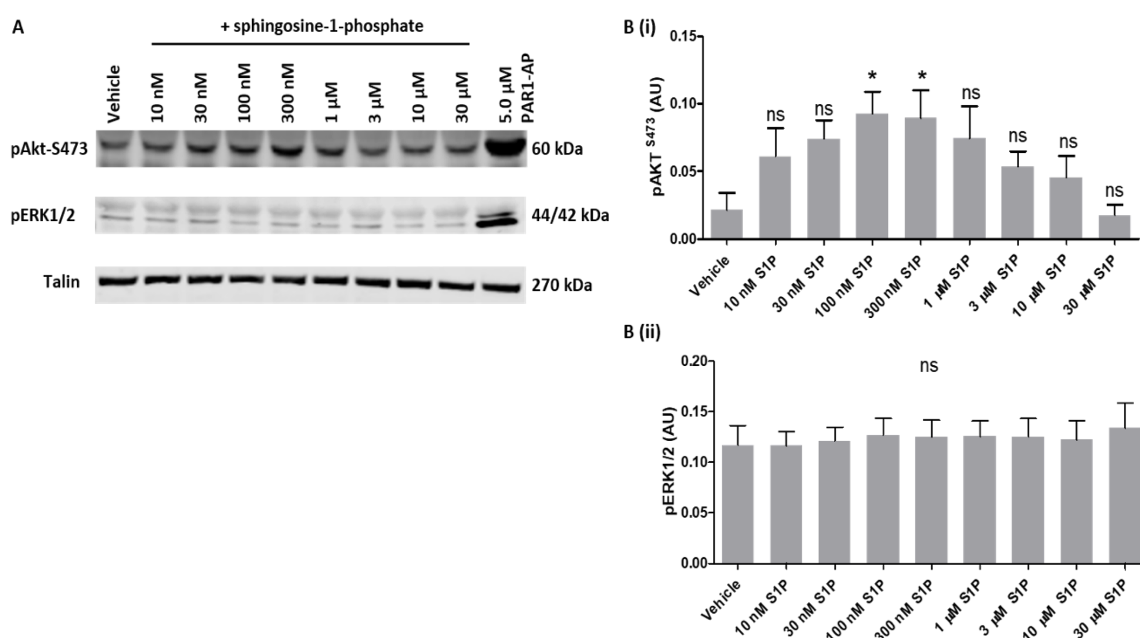


Figure 22. S1P induced phosphorylation of the PI3K effector PKB but not the MAPK ERK1/2. Washed human platelets (4 × 10^8/mL) were treated with vehicle (0.2% methanol) or S1P (10 nM to 30 µM) or 5.0 µM PAR1-AP for 5 minutes at 37 °C. **(A)** Platelet lysates were subjected to SDS-PAGE followed by immunoblotting to measure the phosphorylation of PKB/Akt^{S473} and pERK1/$2^{Thr202/Tyr204}$. Immunoblotting for talin was used as a loading control. **(Bi, ii)** Histograms graph quantification of PKB/Akt^{S473} and ERK1/$2^{Thr202/Tyr204}$ phosphorylation level using LI-COR Image Studio Version 5.2. Results were normalized as fraction to the 5.0 µM PAR1-AP band and expressed in 1 arbitrary unit. N=7. Data are plotted as mean ± SEM. N=8. Statistical analysis: one-way ANOVA with Dunnett's test. NS: no statistical significance. * $p<0.05$

Sphingosine-1-phosphate stimulated a transient increase in PKB phosphorylation (Fig. 23). pAkt^{S473} was observed at 0, 1 and 2-minute incubation. Significant increases in PKB phosphorylation level were observed following a 3 minute or 5 minute incubation with 100 nM S1P (Fig. 23A, B). In contrast, S1P-mediated PKB phosphorylation was reduced after a prolonged (10- or 30-minutes) incubation (Fig.23A, B). Therefore, platelets were incubated with S1P for 5 minutes in all following immunoblotting experiments.

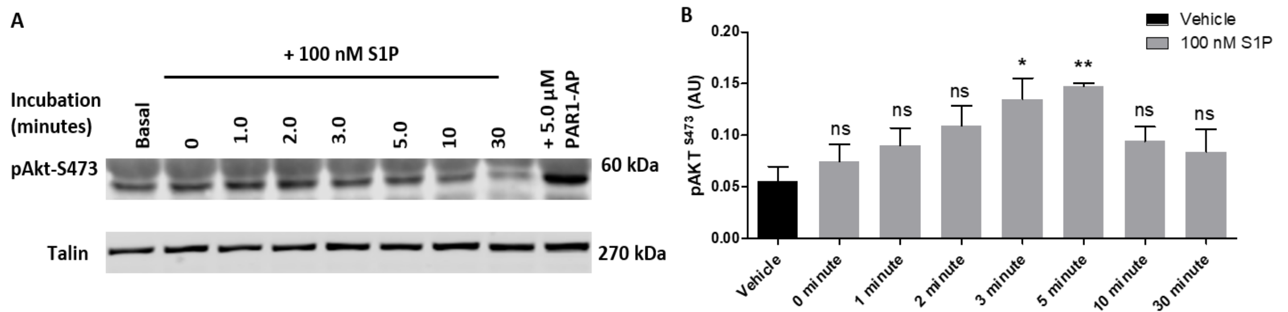


Figure 23. Kinetics of S1P induced PKB phosphorylation. Washed human platelets (4 × 10^8/mL) were treated with vehicle control (0.2 % methanol) or 100 nM S1P (0 - 30 min, 37 °C). **(A)** Platelet lysates were subjected to SDS-PAGE, followed by immunoblotting to measure the phosphorylation of PKB/Akt^{S473}. Immunoblotting for talin was used as a loading control. **(B)** Histogram of quantification of PKB/Akt^{S473} phosphorylation. Quantification was performed using LI-COR Image Studio Version 5.2. Results were normalized to the amount of phosphorylation elicited by PAR1-AP. Data are plotted as mean ± SEM. N=4. Statistical analysis: one-way ANOVA with Dunnett's test. NS: no statistical significance. * $p<0.05$, ** $p<0.01$

4.4.2 Additive effect of S1P on PAR1-induced PKB and ERK1/2 phosphorylation.

To investigate how S1P can prime PAR1-AP mediated platelet function, the phosphorylation levels of PKB and MAPK ERK1/2 were studied. Stimulation with PAR1-AP alone induced an increase in PKB phosphorylation (1417 ± 303) compared to the basal group (404 ± 71.4). In the presence of 10 nM, 30 nM, 100 nM and 300 nM S1P, 2.0 µM PAR1-AP induced PKB phosphorylation was increased from 1417 ± 303 to 1683 ± 409, 2088 ± 528, 1861 ± 400 and 1708 ± 417 respectively (Fig. 24A,B). In the presence of 1 µM S1P, PKB phosphorylation was 1552 ± 364, which was similar with to that induced by 2.0 µM PAR1-AP. In contrast, further increasing in the S1P concentration resulted in a decrease in PAR1-AP mediated PKB phosphorylation (Fig. 24A). In the presence of 3 µM, 10 µM and 30 µM S1P, the PKB phosphorylation level were decreased to 1084 ± 249, 1098 ± 254 and 616 ± 142 respectively (Fig. 24Bi).

Previous experiments indicated that S1P did not induce ERK phosphorylation by itself. Platelets stimulation with 2.0 µM PAR1-AP resulted an increase in ERK1/2 phosphorylation level from basal of 647 ± 108 to 1318 ± 252 (Fig. 24A). This PAR1-AP induced ERK phosphorylation was further enhanced in the presence of 10 nM and 30 nM S1P to 1573 ± 331 and 1856 ± 319, respectively. In the presence of 100 nM S1P, a maximal 61% increase in ERK1/2 phosphorylation (2124 ± 325) was observed in comparison to 2.0 µM PAR1-AP alone (1318). However, at higher S1P concentrations the increase in pERK became less apparent. In the presence of 300 nM, 1 µM, 3 µM and 10 µM S1P, PAR1-AP induced ERK1/2 phosphorylation was 1706 ± 197, 1758 ± 272, 1724 ± 264 and 1422 ± 258 respectively (Fig. 24Bii). In contrast, the highest S1P concentration tested (30 µM) reduced PAR1-AP induced ERK1/2 phosphorylation from 1318 ± 252 to 847.7 ± 112.4 (Fig.24A, Bii).

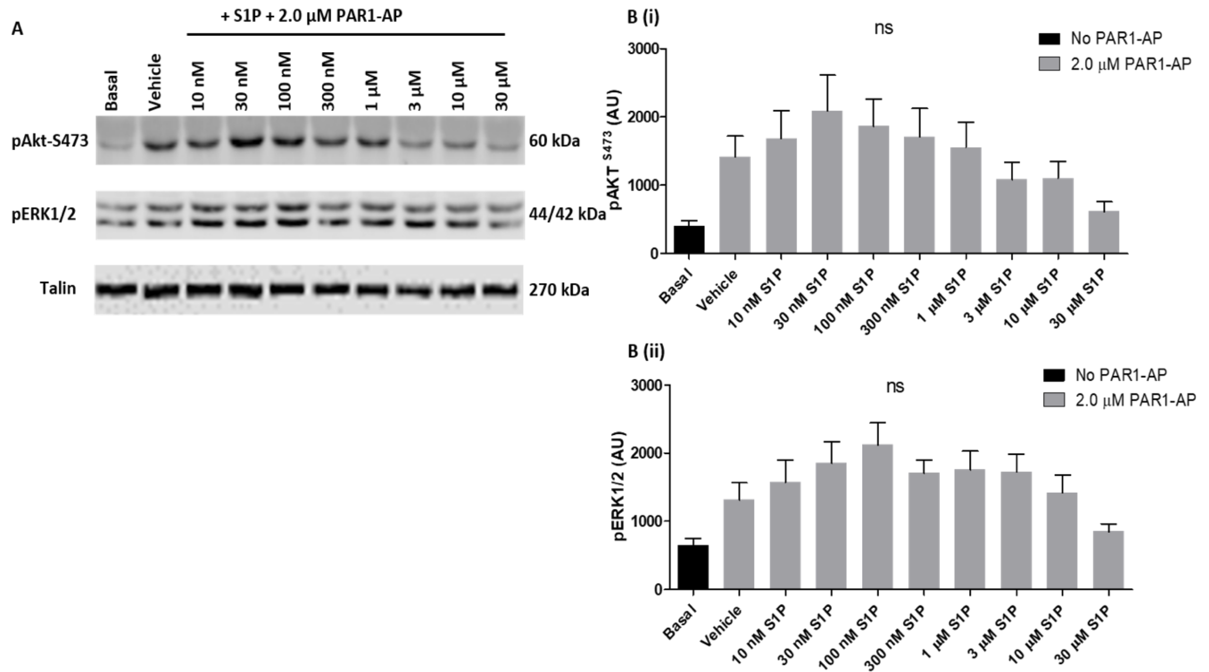


Figure 24. Concentration dependent biphasic effect of S1P on PAR1-AP induced PKB phosphorylation. Washed platelets (4 × 10^8/mL) were pre-treated with vehicle or S1P (10 nM - 30 µM, 3 min) before stimulation with PAR1-AP (2.0 µM, 37 °C). **(A)** Platelet lysates were subjected to SDS-PAGE, followed by immunoblotting to measure the phosphorylation of PKB/Akt^{S473}. Immunoblotting for talin was used as a loading control. **(Bi, ii)** Histograms of quantification of PKB/Akt^{S473} and ERK1/$2^{Thr202/Tyr204}$ phosphorylation. Quantification was performed using LI-COR Odyssey Image Studio Version 5.2. N=7. Data are plotted as mean ± SEM. Statistical analysis: one-way ANOVA with Dunnett's test. NS: no statistical significance.

The immunoblotting results indicate that S1P activates the PI3kinase pathway. To evaluate the role of PI3kinase in S1P mediated priming, we incubated platelets with the pan-PI3K inhibitor wortmannin. As shown in Fig.25, 100 nM S1P increased PKB phosphorylation from a basal level of 434 ± 24.3 to 706 ± 102. In the presence of 100 nM wortmannin, both the basal and S1P-stimulated PKB phosphorylation induced by 100 nM S1P was reduced to 312 ± 31.5 and 436 ± 52.3 respectively (Fig.25Bi).

An additive effect was observed in the presence of both PAR1-AP and S1P, as PKB phosphorylation induced by PAR1-AP alone (1476 ± 294) was increased to 1752 ± 393 in the presence of S1P (Fig.25A, Bi). Wortmannin, reduced PKB phosphorylation induced by PAR1-AP and the combination of both PAR1-AP and S1P to 632 ± 525 to 525 ± 124, respectively (Fig.25A, Bii).

Basal ERK phosphorylation was 426.3 ± 31.4 and changed to 604 ± 118 in the presence of 100 nM S1P. Wortmannin reduced basal and S1P-stimulated ERK phosphorylation to 395 ± 36.5 and 509 ± 40.4 respectively (Fig.25Bi). An additive effect was observed, as the ERK1/2 phosphorylation with by PAR1-AP alone 2007 ± 481 was increased to 2397 ± 683 in the presence of both PAR1-AP and S1P (Fig. 25A, Bii). Wortmannin reduced, ERK1/2 phosphorylation induced by 2.0 µM PAR1-AP and a combination of both PAR1-AP and S1P (Fig. 25A, Bii). The results suggest that the additive effects of S1P on PAR1-AP induced PKB and ERK1/2 phosphorylation was mediated via PI3K pathway.

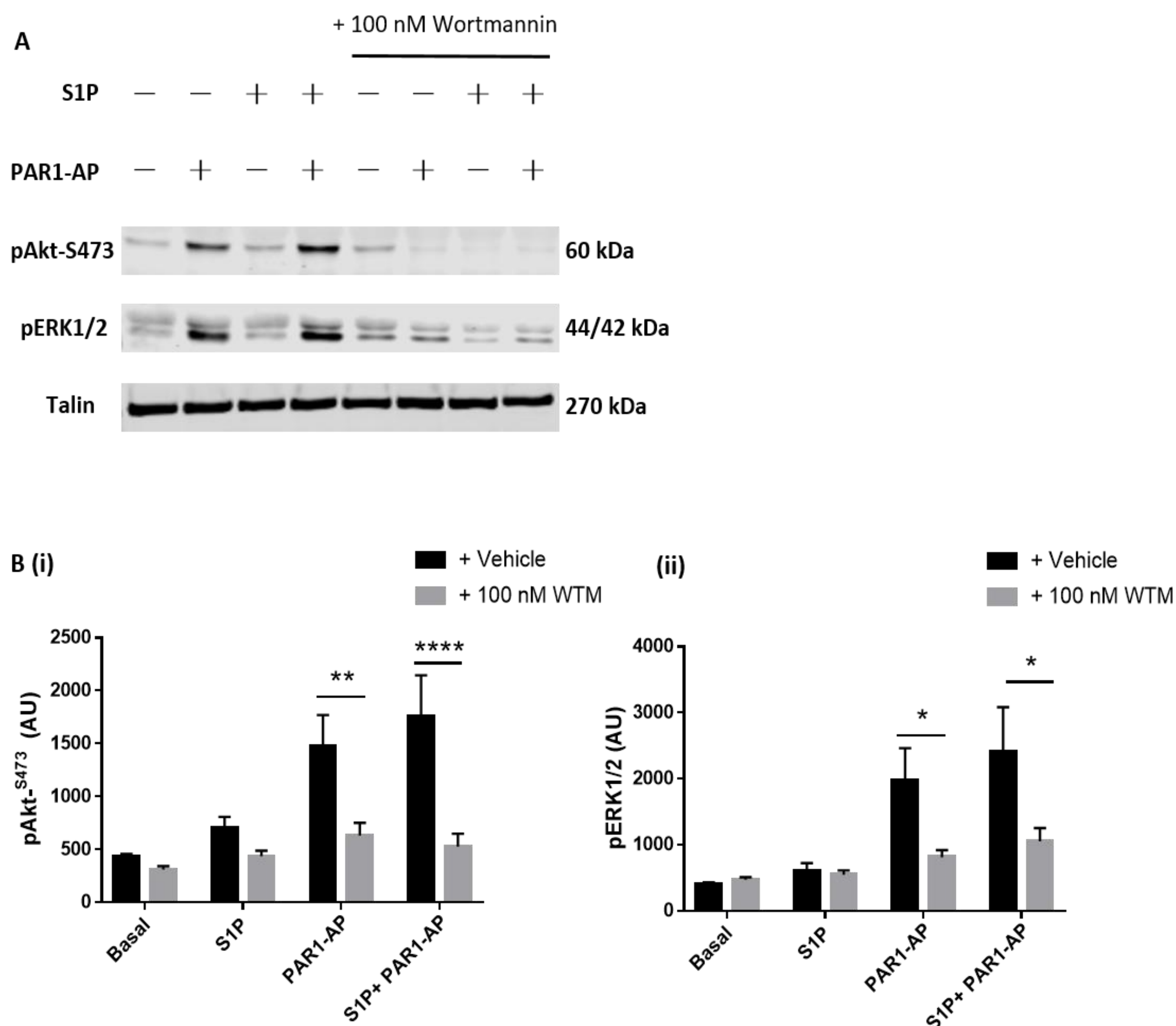


Figure 25. Role of PI3K in S1P and PAR1-AP mediated PKB phosphorylation. Washed platelets (4×10^8/mL) were pre-treated with vehicle or wortmannin (100 nM), before incubation with vehicle or S1P (100 nM) prior to stimulation with PAR1-AP (2.0 µM, 37 °C). **(A)** Platelet lysates were subjected to SDS-PAGE, followed by immunoblotting to measure the phosphorylation of PKB/Akt^{S473} and ERK1/2, Immunoblotting for talin was used as a loading control **(Bi, ii)** Histograms of quantification of PKB/Akt^{S473} and ERK1/2$^{Thr202/Tyr204}$ phosphorylation. Quantification was performed using LI-COR Odyssey Image Studio Version 5.2. Data are plotted as mean ± SEM. N=6. Statistical analysis: two-way ANOVA with Bonferroni post hoc multiple comparisons. NS: no statistical significance. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4.4.3 S1P supresses PGE1-mediated VASP phosphorylation

To investigate the downstream signalling cascade of S1P receptors and probe the receptors coupling in platelets, the phosphorylation level of the vasodilator-stimulated phosphoprotein (VASP) were studied. VASP is a downstream effector of the cAMP-dependent pathway that abundantly expressed in platelet. Activation of adenylyl cyclase (AC) will result in cyclic adenosine monophosphate (c-AMP) production, c-AMP then activate downstream protein kinase A (PKA) which then phosphorylates VASP on the $serine^{157}$ or $serine^{239}$ residue. For instance, activation of $G_{\alpha s}$ coupled prostacyclin receptor by PGE_1 activate AC and c-AMP pathway, which cause downstream VASP phosphorylation. In contrast, activating the $G_{\alpha i/o}$ coupled $P2Y_{12}$ receptor by ADP reduce VASP phosphorylation. Therefore, an increase in VASP phosphorylation level indicates activation of $G_{\alpha s}$ coupled receptor and Vice Versa.

In order to investigate whether S1P receptors have as $G_{\alpha s}$ coupled signalling component, platelets were treated with increasing concentrations of S1P and 100 nM PGE_1 was used as a positive control, the phosphorylation level of VASP-$serine^{239}$ was examined by immunoblotting. 100 nM PGE_1 was used as a positive control. In the presence of 100 nM PGE_1, the VASP $serine^{239}$ phosphorylation level increased from 964 ± 205 to 14670 ± 3472 (Fig.26Ai, Aii). In contrast, increasing concentrations of S1P did not induce VASP phosphorylation compared to the vehicle control group (Fig.26Aii). Therefore, there is no evidence that supports Gαs coupling of the receptors. 10 µM and 30 µM S1P did not induce VASP phosphorylation, although platelet aggregation was strongly inhibited in the presence of these concentration of S1P. Hence, high concentration S1P inhibiting platelet function is not mediated via activation of $G_{\alpha s}$ coupled GPCR.

In contrast, a decrease in VASP phosphorylation level was found when platelets were co-stimulated treated with 20 µM ADP and 100 nM PGE_1 co-stimulation (2310 ± 525), in comparison to the vehicle group (13940 ± 2884) stimulated by 100 nM PGE_1 alone (Fig. 26B). This indicates that activation of $G_{\alpha i/o}$ coupled $P2Y_{12}$ receptor by ADP can reduce PGE_1 mediated VASP phosphorylation. Similarly, decreases in VASP phosphorylation were level observed in platelets treated with PGE_1 and S1P. PGE_1-stimulated pVASP-S^{239} was decreased from 13940 ± 2884 to 10110 ± 4933 in the presence of 100 nM S1P (Fig. 26B). A similar in pVASP-S^{239} was also found in the presence of 10 nM, 30 nM, 300 nM and 1 µM S1P. Interestingly, the pVASP-S^{239} level was reduced to 11156 ± 3565 and 7913 ± 3164 in the presence of 10 µM and 30 µM S1P respectively, although at these concentrations S1P strongly inhibited platelet aggregation. These data indicate that S1P can signal via $G_{\alpha i}$ coupled receptors resulting in inhibition of cAMP production.

The effect of S1PR selective agonists on $VASP^{S239}$ was also examined by immunoblotting. S1P receptor selective agonists (10 nM - 10 µM) did not induce VASP phosphorylation (N=1, data not shown). This suggests that S1P receptor subtypes do not couple to $G_{\alpha s}$ in platelets

and support the finding that the inhibitory effect of high concentrations of S1P on platelet aggregation are not mediated via activation of Gs coupled receptors.

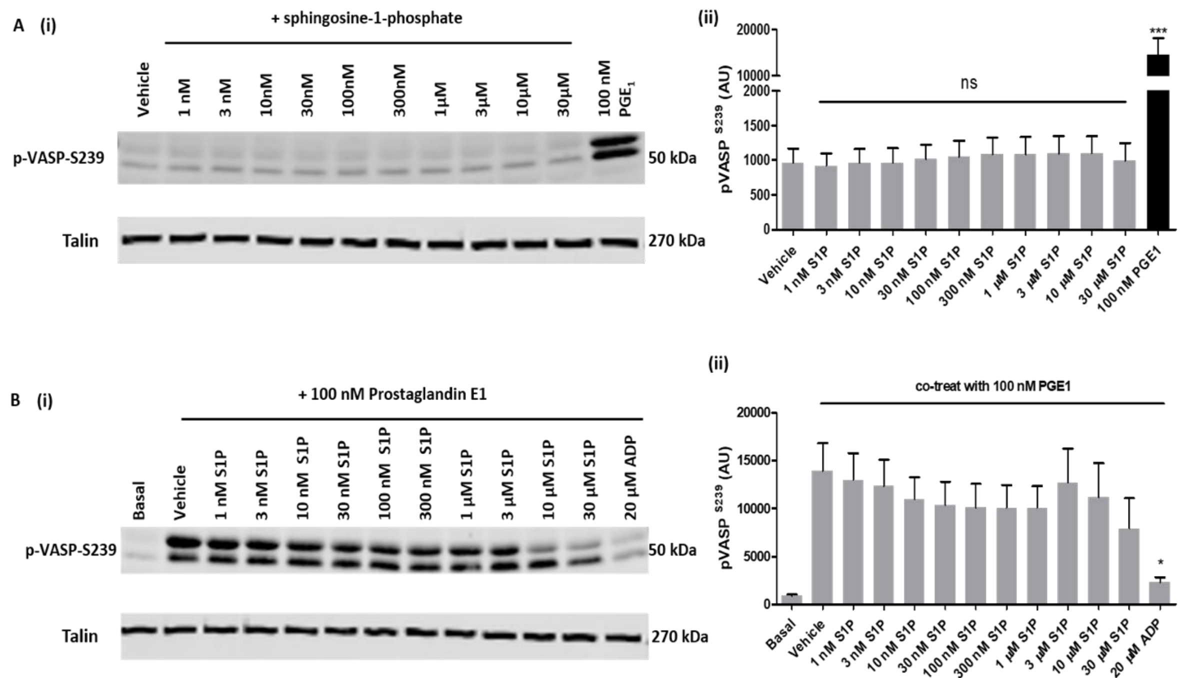


Figure 26. Stimulation of platelets with S1P suppresses PGE_1-mediated VASP phosphorylation. (A) Washed platelets (4×10^8/mL) were treated with vehicle (0.2% methanol, 5 min), S1P (1 nM - 30 µM, 5 min) or PGE_1 (100 nM, 3 minutes). Platelet lysates was subjected to SDS-PAGE, followed by immunoblotting to measure the phosphorylation of VASPS239. Immunoblotting for talin was used as a loading control. **(ii)** Histograms of quantification of $VASP^{S239}$. **(B)** Washed platelets (4×10^8/mL) were stimulated with PGE1 (100 nM) in the presence of S1P (1 nM – 30 µM) or ADP (20 µM). Platelet lysates were subjected to SDS-PAGE, followed by immunoblotting to measure the phosphorylation of $VASP^{S239}$. Immunoblotting for talin was used as a loading control. **(ii)** Histograms of quantification of $VASP^{S239}$. Quantification was performed using LI-COR Odyssey Image Studio Version 5.2. N=4. Statistical analysis: one-way ANOVA with Dunnett's test. NS: no statistical significance. * $p<0.05$, *** $p < 0.001$.

4.5.1 PAR1-AP mediated platelet calcium mobilization is not altered by S1P

Several studies have indicated that S1P can mobilize intracellular calcium release by activating S1P receptors, or act as a intracellular secondary messenger to directly cause calcium flux[132]. To explore whether S1P can modulate platelet calcium mobilization function, Ca^{2+} mobilization assays were carried out to exam the effect of S1P on PAR-induced platelet calcium flux. Platelets loaded with the cell permeant calcium indicator, Fura-2 AM were pre-treated with S1P before stimulation with PAR1-AP. The level of calcium mobilization was measured as the total area under the relative fluorescence units (RFU) curve. High concentration of PAR1-AP induced a large RFU curve result in a bigger AUC (Fig. 27Ai). Interestingly, S1P alone did not induce calcium release in human platelets with a concentration range from 10 nM to 10 μM tested (Fig. 27Aii).

In the presence of 100 nM S1P, a slight increase in the total calcium mobilization level was observed (Fig. 27B). In contrast, platelet calcium mobilization was reduced in the presence of 10 μM S1P, the maximal calcium flux was decreased from 35.9 ± 3.03 to 34 ± 3.12. A rightward shift on the curves was observed, which the $LogEC_{50}$ increased from 6.102 ± 0.17 to -6.009 ± 0.16. Since S1P did not directly induce calcium release or facilitate PAR1-AP mediate calcium mobilization, it can be deduced that there is no $G_{\alpha q}$ coupled S1P receptor signalling in platelets, thus further confirming there is no $G_{\alpha q}$ coupled $S1PR_2$ or $S1PR_3$ expression. Moreover, these findings contradict the studies suggesting that S1P can act as an intracellular secondary messenger to mobilize calcium flux in platelets.

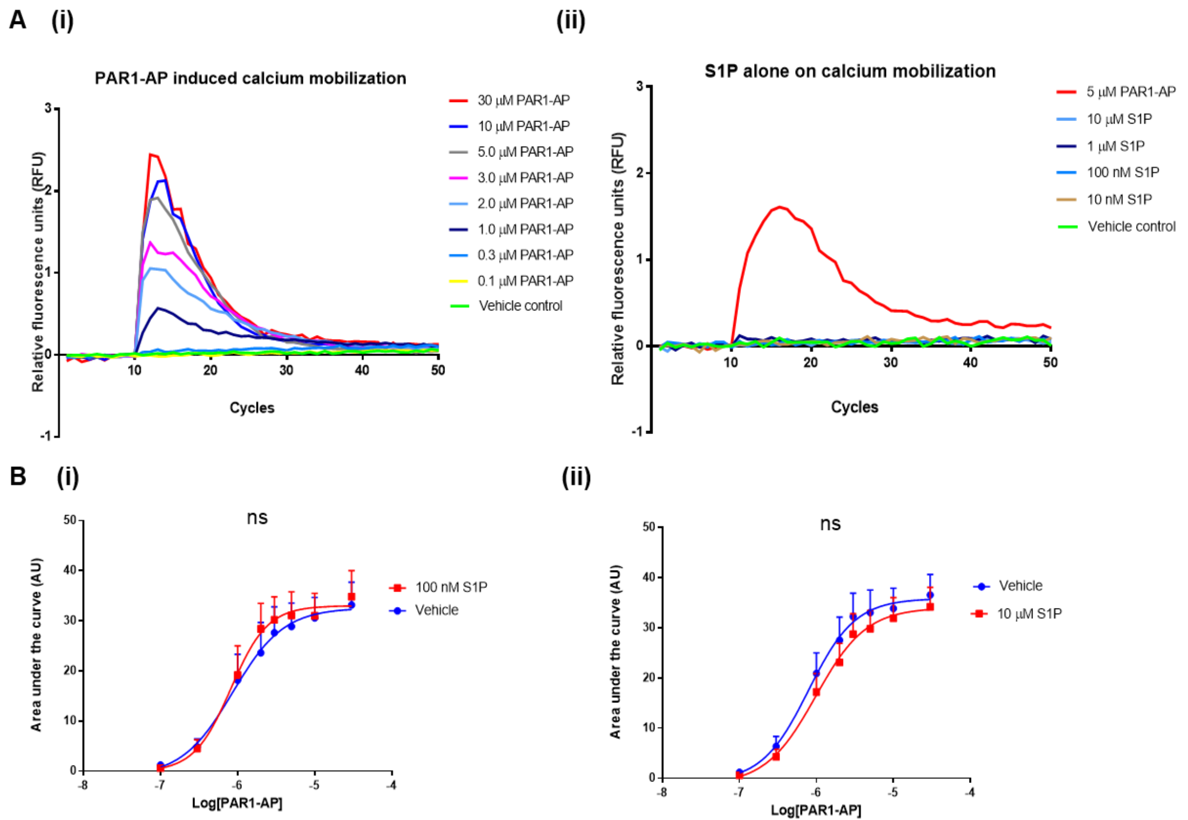


Figure 27. Effect of S1P on PAR-mediated calcium mobilization. (A) Washed platelets (2 × 10^8/mL) loaded with Fura2-AM in the presence of 1 mM $CaCl_2$ were stimulated with PAR1-AP or S1P. **(i)** Representative traces of PAR1-AP mediated calcium mobilization, measured as change in 340/380 nm excitation ratio. **(ii)** Representative traces of S1P mediated calcium mobilization. **(B)** Washed platelets (2 × 10^8/mL) loaded with Fura2-AM in the presence of 1 mM $CaCl_2$ were pre-incubated with either vehicle (0.2% methanol) or S1P (5 min) before stimulation with PAR1-AP. **(i)** PAR1-AP concentration response in the absence and presence of 100 nM S1P. **(ii)** PAR1-AP concentration response in the absence and presence of 10 µM S1P. Results are expressed as either R.F.U or A.U.C. Data are plotted as mean ± standard error of the mean. N=3. Statistical analysis: two-way ANOVA with Bonferroni post hoc multiple comparisons. NS: no statistical significance.

4.5.2 S1P has no effect on PAR1-AP mediated TxA2 production

As previously described, TxA2 plays an important role in platelet function and thrombus formation, with its production being the target of the antiplatelet therapy, aspirin. Ulrych et al[107] have suggested that S1P can signaling through ERK1/2 pathway and that there are close interactions between S1P production and TxA2 synthesis. To gain insight of how S1P can enhance platelet function we explored the possibility that S1P could enhance PAR1-AP mediated TxA2 production. TxB2 ELISA assays were carried out to evaluate the PAR1-AP induced TxA2 production in the absence or presence of 100 nM S1P at non-stirring condition. A logarithmic standard curve was generated from TxB2 standard samples (Fig.28Ai).

In the absence of PAR1-AP, no significant difference was found between the vehicle group (2.31 ± 0.355 ng/mL) and 100 nM S1P treated (2.93 ± 0.469 ng/mL) platelets. In contrast, there was an 11% increase in 1 µM PAR1-AP induced TxA2 production between vehicle control (83. 8 ± 13.1 ng/mL) and 100 nM S1P treated (93.4 ± 14.1 ng/mL) group, although this did not reach significance. These data demonstrate that S1P did not directly induce TxA2 synthesis, which is in line with the S1P induced pERK phosphorylation data. However, it is still possible that S1P can potentiate PAR1-mediated platelet function via increases in TxA2 synthesis. Additional experiments are required.

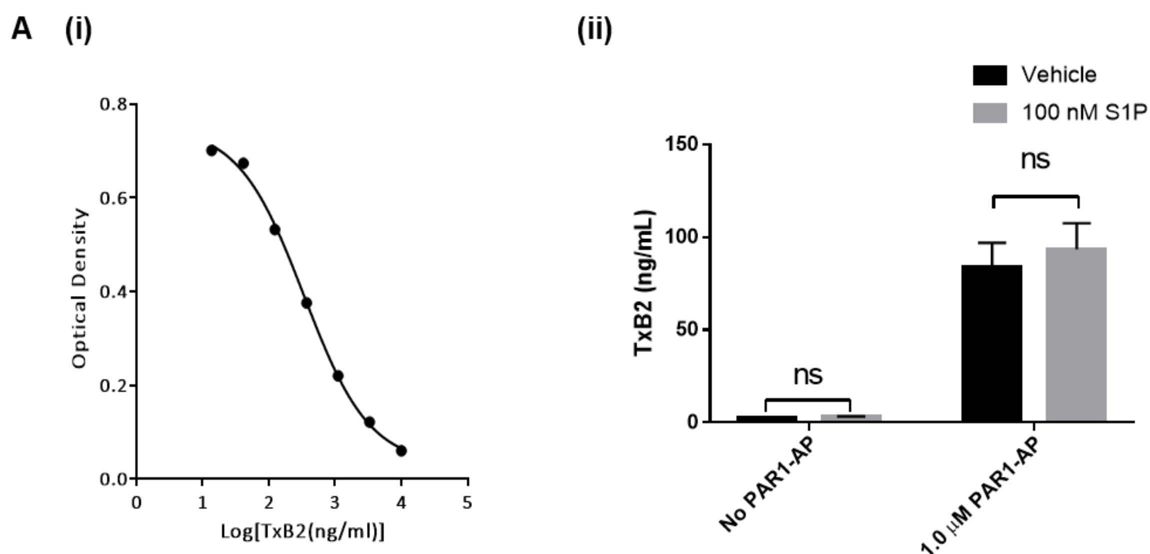


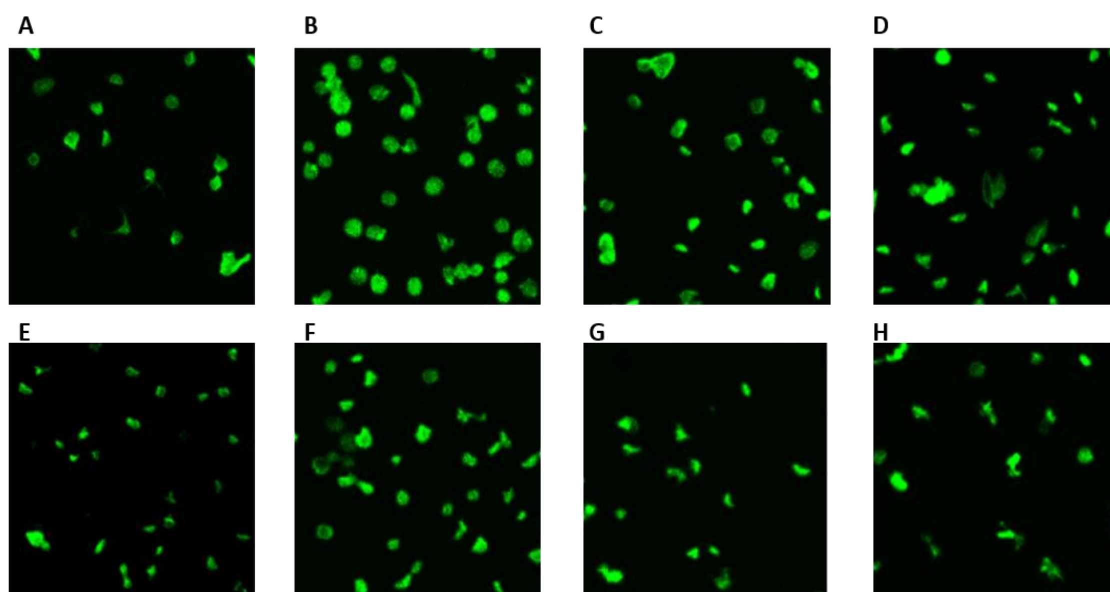
Figure 28. Effect of S1P on PAR1-AP mediated platelet TxA2 production. Washed platelets (4 × 10^8/mL) were incubated with 100 nM S1P (5 min) in prior to stimulation with 1 µM PAR1-AP (5 min) under non-stirring condition. The reaction was stopped by addition of 5 mM EDTA and 100 µM indomethacin. **(i)** A standard curve was used to determine concentrations of TxB2. **(ii)** Histogram of the concentration of TxB2 generated by S1P, PAR1-AP and S1P + PAR1-AP. Data are plotted as mean ± standard error of the mean. N=7. Statistical analysis used was two-way ANOVA with Bonferroni post hoc multiple comparisons. NS: no statistical significance.

4.5.3 Activation of $S1PR_4$ or $S1PR_5$ and inhibition of sphingosine kinase reduces platelet spreading on fibrinogen.

Recently, Gazit et al[133] using sphingosine kinase knock-out mice demonstrated that S1P is required for both platelet aggregation and spreading. To investigate the role of S1P in platelet spreading, platelets in the presence of S1PR agonists, antagonists or a sphingosine kinase inhibitor were spread onto glass wells coated with 100 µg/mL fibrinogen and labelled by F-actin specific fluorescent dye. The platelet average cell area was 11.8 ± 0.791 without fibrinogen stimulation (Fig. 29A). With stimulation by 100 µg/mL fibrinogen, platelet spread, and the average platelet cell area was increased to 15.9 ± 0.314 μm^2 (Fig.29B). In addition, more platelets were found to be adhered to the 100 µg/mL fibrinogen coated wells than the no fibrinogen wells.

In the presence of 1 µM FTY720-P, the average cell area remained unchanged (Fig. 29C). The average platelet cell size increased slightly to 16.58 ± 0.27 μm^2 in the presence of 1 µM SEW2871 (Fig. 29D). In contrast, significant decreases in the average cell area were found in platelets treated with 1 µM $S1PR_4$ agonist CYM50260, 1 µM $S1PR_5$ agonist A971432 and 1 µM SphK inhibitor DMS (Fig.29E, G, H and I). with the average cell area decreased from 15.9 ± 0.314 μm^2 to 9.23 ± 0.21 μm^2, 11.8 ± 0.306 μm^2, and 11.2 ± 0.246 μm^2 respectively (Fig. 29I). Platelets treated with CYM50260, A971432 or DMS small and changed to an elongated/triangular shape (Fig. 29E, G, and H). In addition, there were fewer platelets found per field of view. Surprisingly, there was no increase, but a slight decreased of the platelet average cell area (15.3 ± 0.32 μm^2) in the presence the $S1PR_4$ antagonist CYM50358 hydrochloride (Fig.29F), which was the ligand that significantly enhanced platelet aggregation.

These data suggest that the inhibitory effects of S1P are mediated via activation of the $G_{\alpha12/13}$ coupled $S1PR_4$ and $S1PR_5$ receptors, negatively regulating platelet morphological change. Studies have previously demonstrated that $S1PR_2$, $S1PR_4$ and $S1PR_5$ receptors are the unique $G_{\alpha12/13}$ coupled GPCRs that negatively regulate Rac activity[134,135,136]. Furthermore, the non-selective SphK inhibitor DMS also induced negative effects on platelet shape change, which suggest the intrinsically synthesized S1P play a role in regulating platelet function via controlling membrane polarity or cell morphology. This is concurrent with SphK KO mouse study demonstrated by Gazit et al[133], and study on exogenous S1P increase membrane ruffling and cell motility in CHO cells migration experiments[137].



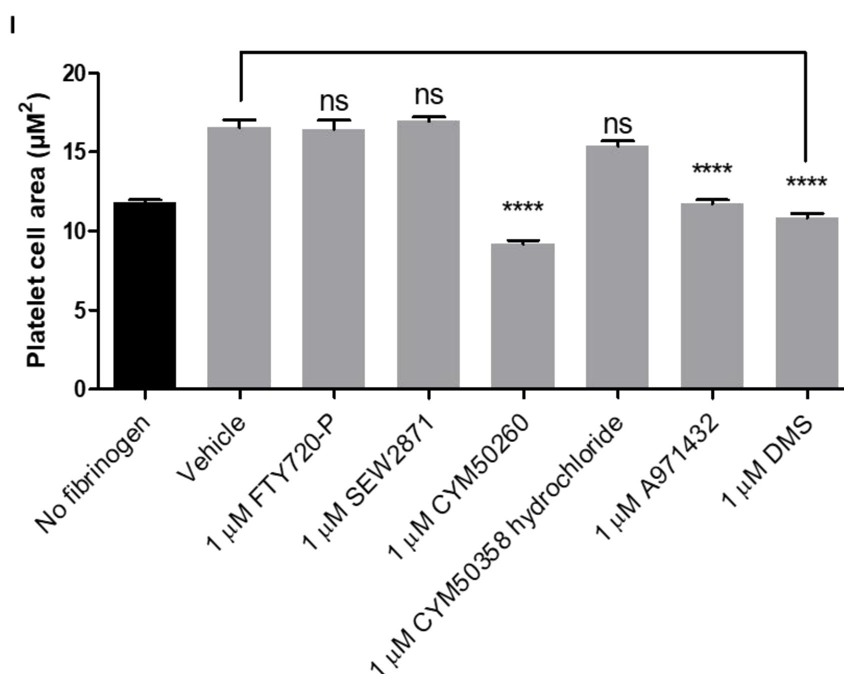


Figure 29. Platelet spreading on fibrinogen is inhibited by $S1PR_4$, $S1PR_5$ agonists and a SphK inhibitor. Washed platelets (7 × 10^7/mL) were incubated with vehicle (0.2% DMSO) or S1PR agonists/antagonists or the SphK inhibitor DMS (10 min). Platelets were then left to adhere onto a 96 well glass bottom plate coated with 100 μg/mL fibrinogen (60 min, 37°C). Samples were fixed (1 % PFA) before permeabilisation (0.1% triton) and staining with ActinGreen 488 ReadyProbes reagent. **(A)** Resting/basal 3% BSA **(B)** vehicle-treated **(C)** 1 μM FTY720 phosphate **(D)** 1 μM SEW2871 **(E)** 1 μM CYM50260 **(F)** 1 μM CYM50358 hydrochloride **(G)** 1 μM A971432 **(H)** 1 μM DMS. **(I)** Histogram displaying the average platelet area (μm²). Three images were taken per well. Average cell area was measured from 50 cells, data are plotted as mean±SEM. N=3. Statistical analysis: One-way ANOVA with Dunnett's test. **** $P < 0.0001$.

4.5.4 Human platelet spreading is reduced by high concentrations of S1P.

To investigate whether S1P affect fibrinogen induced spreading, human platelets treated with S1P were spread onto glass wells coated with 100 µg/mL fibrinogen and labelled by F-actin specific fluorescent dye. The platelet average cell area was 11.8 ± 0.791 µm^2 without fibrinogen (Fig. 29A). Upon in contact with 100 µg/mL fibrinogen, the average platelet cell area was increased to 17.7 ± 0.256 µm^2 (Vehicle control, 0.2% methanol). In the presence of 100 nM S1P, the average cell area was slightly increased to 18.2 ± 0.287 µm^2 (Fig. 30E). In contrast, pre-treatment of platelets with either 1 µM or 10 µM S1P decreased the average platelet cell size to 15.56 ± 0.19 µm^2 and 9.43 ± 0.29 µm^2 (Fig. 30E).

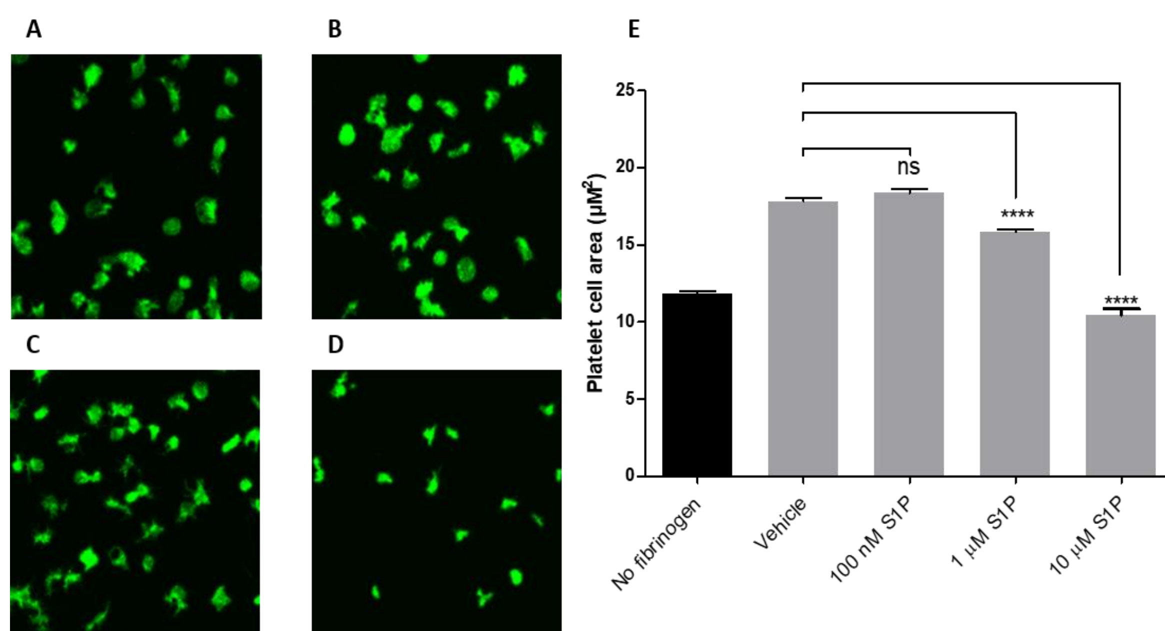


Figure 30. Platelet spreading on fibrinogen is inhibited by 1 µM and 10 µM S1P. Washed platelets (7 x 10^7/mL) were incubated with vehicle (0.2% methanol) or S1P (100 nM, 10 µM) for 10 min. Platelets were then left to adhere onto a 96 well glass bottom plate coated with 100 µg/mL fibrinogen (60 min, 37°C). Samples were fixed (1 % PFA) before permeabilisation (0.1% triton) and staining with ActinGreen 488 ReadyProbes reagent. **(A)** Vehicle control **(B)** 100 nM S1P **(C)** 1 µM S1P **(D)** 10 µM S1P **(E)** Histogram displaying the average platelet area (µm^2). Data obtained from one experiment with three individual platelet donors. Three images were taken per well. Average cell area was measured from 50 cells, data are plotted as mean±SEM. N = 3. Statistical analysis: One-way ANOVA with Dunnett's test. **** $P < 0.0001$

In 10 μM S1P treated group, platelets shrunk and lost the spherical shape, with no cell protrusion and much less platelet-platelet interactions observed (Fig.30D). In addition, there were much smaller number of platelets found on the field of view. Interestingly, more platelet-platelet interacts in the 100 nM S1P or 1 μM S1P treated platelets and more cell protrusions were observed (Fig.30B, C). These data suggest high concentration S1P inhibit platelet function via negatively regulating the platelet spreading and adhesion process, possibly through activation of $S1PR_4$ and $S1PR_5$ receptors.

4.6 Discussion

This study has demonstrated for the first time that exogenous S1P can concentration-dependently alter platelet function, with low concentrations of S1P priming platelet function and high concentrations of S1P inhibiting platelet responses. S1P enhanced PAR1-AP mediated platelet aggregation, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure. S1P did not affect PAR1-AP mediated TxA2 production nor calcium release in platelets.

Based on the data obtained from aggregation experiments and FACS assays using S1P receptor agonist and antagonists, I suggest $S1PR_1$, $S1PR_4$ and $S1PR_5$ receptor subtypes are expressed on human platelets, while I found no evidence to support $S1PR_2$ or $S1PR_3$ expression. This study demonstrates activation of $S1PR_1$ or inhibition of $S1PR_4$ can enhance platelet function, while inhibition of $S1PR_1$, activation of $S1PR_4$ and $S1PR_5$ reduce platelet responses, which suggest the concentration dependent biphasic effect of S1P is mediated via differential signalling through different S1P receptor subtypes. The positive priming effects of S1P might be mediated via activation of $G_{\alpha i/o}$ coupled $S1PR_1$ receptor and downstream PI3K signalling pathway (Fig. 1). In contrast, the inhibitory effects of S1P may be mediated via activation of $G_{\alpha 12/13}$ coupled $S1PR_4$ and $S1PR_5$, resulting in negative regulation of cytoskeletal dynamics and potential inhibition of ERK. Furthermore, we found inhibition of sphingosine kinases reduced PAR1-AP mediated platelet aggregation and platelet spreading, suggesting *de novo* synthesized S1P plays an important role in controlling platelet activation and morphology change.

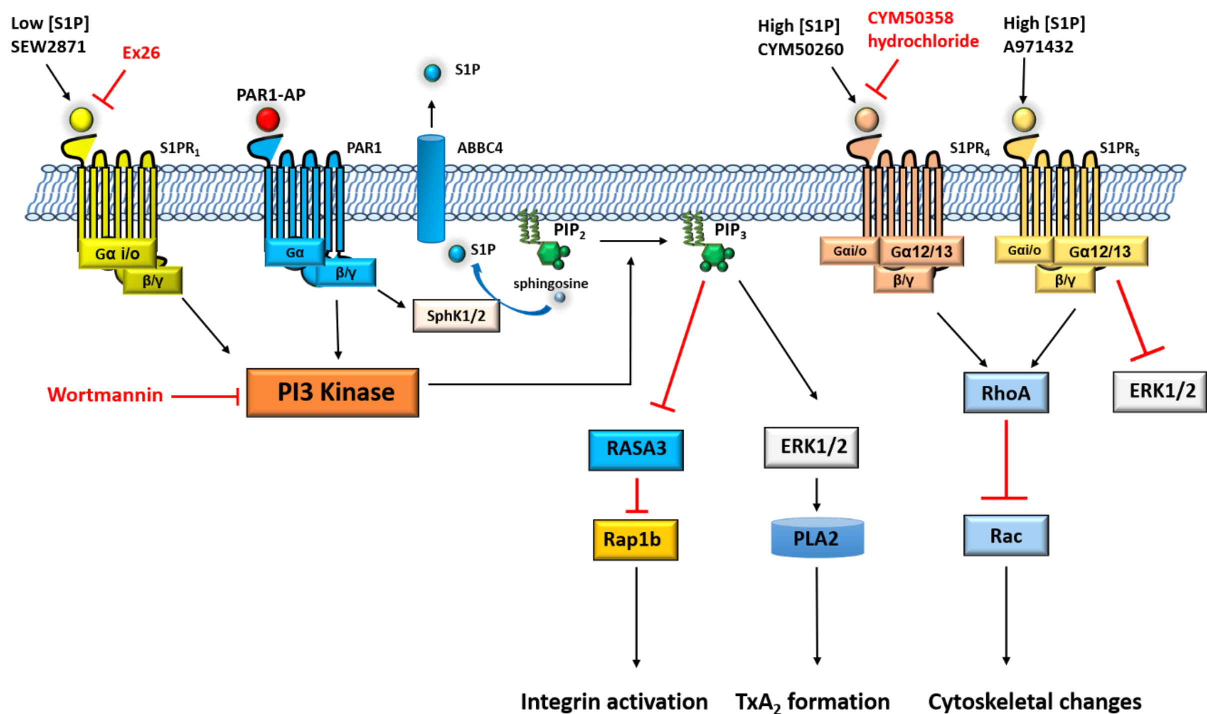


Figure 31. Schematic diagram showing the hypothesized mechanism of S1P induced concentration dependent biphasic effects on PAR1-AP mediated platelet function. S1PR: S1P receptor; PAR1: protease-activated receptor 1; Rho: Rho family of GTPases; Rac: Ras-related C3 botulinum toxin substrate; PIP_2: phosphatidylinositol 4, 5-biphosphate; PIP_3: phosphatidylinositol (3, 4, 5)-triphosphate; PKB: protein kinase B. PLA2: phospholipase A2; Rap1b: Ras-related protein 1b.

4.6.1 Activation of S1P receptors affects PAR1-AP mediated platelet function in a concentration dependent biphasic manner.

S1P exerted a characteristic, concentration dependent biphasic effect on PAR-mediated platelet function. Platelet aggregation, integrin $\alpha_{IIb}\beta_3$ activation, P-selectin exposure, phosphorylation of AKT and ERK were enhanced in the presence of 100 nM S1P but inhibited in the presence of supermicromolar S1P. This is the first time the S1P biphasic effect has been observed in a platelet study. Similar S1P concentration dependent biphasic effects have previously been observed in other cell types. A study by Wang et al[138] found the 0.5 - 2 μM of S1P stimulated *in vivo* ovarian cancer cell growth and migration, and this was inhibited by 5 μM S1P and ablated by 20 μM S1P. Another study based on three different osteoblastic cell lines found significant increases in cell growth with 100 nM - 1 μM S1P but decreases in the presence of 5 μM[139]. Furthermore, S1P enhanced T cell migration at a concentration range from 1 - 100 nM but exerted inhibitory effects from 100 - 500 nM. Machida et al[140] reported that S1P induced a biphasic effect on calcium release in vascular smooth muscle cell starting at 100 nM. As many studies reported, this biphasic effect may be due to differential signalling via S1P receptor subtypes, and the turning point concentration is dependent on the incubation time and/or cellular response measured.

Similar biphasic effects were also observed in platelets pre-incubated with the pan-S1PR agonist dihydro-S1P, FTY-720 and phosphorylated FTY-720. Dihydrosphingosine-1-phosphate (DHS1P) is a synthetic analogue of S1P, a study by Bu et al[141] indicate that DHS1P can activate the ERK1/2 pathway in fibroblasts. My data demonstrates that DHS1P (1 μM) induces a milder enhancement in platelet aggregation compared to S1P, this may be due to DHS1P having a lower binding affinity for the S1PRs and/or that it cannot evoke intracellular effects such as calcium mobilization[142]. No obvious effect of DHS1P on platelet integrin activation or P-selectin exposure was detected. Further increasing the DHS1P concentration resulted in platelet inhibition.

FTY720 (Fingolimod) is a pro-drug used for treating multiple sclerosis, which is a synthetic analogue of sphingosine. FTY720 can be phosphorylated by sphingosine kinases to form FTY720-P which is able to activate $S1PR_{1, 3, 4, 5}$ receptors. Aggregation data demonstrated that the FTY-720 only induced a mild increase in platelet aggregation. This agrees with previous findings by Ocwieja et al [143], who showed that oral treatment of Fingolimod could somewhat enhance ADP mediated platelet aggregation. In contrast, phosphorylated FTY720 significantly promoted platelet aggregation. In addition, FTY720-P significantly potentiated platelet integrin $\alpha_{IIb}\beta_3$ activation. This is supported by a study demonstrating that FTY720-P enhanced chemokine induced calcium release and cell migration in hematopoietic stem cells[144]. Study by Anada et al[145] has demonstrated that FTY720 can be phosphorylated in platelets to FTY720-P and transported out of the cell without platelet activation. However, this process is relatively slow as only 47 % of FTY720 was phosphorylated during a 1-hour incubation with 3×10^7 human platelets[145]. This may explain why FTY720 evoked a lesser effect than FTY720-P on platelet aggregation. Furthermore, it was reported that Fingolimod treatment can activate $S1PR_1$ on mouse megakaryocytes resulted in increased platelet production[127].

4.6.2 S1P receptor subtypes expression on human platelets

It is important to identify the S1PR subtypes expressed on platelets in future studies examining the role of S1P in regulating platelet function. It was reported that $S1PR_1$, $S1PR_2$ and $S1PR_3$ are widely expressed[146], while $S1PR_4$ is predominantly expressed in lymphoid tissue[147] and $S1PR_5$ expressed in spleen and the central nervous system[148]. Previously it was suggested $S1PR_1$, $S1PR_2$, $S1PR_4$ and $S1PR_5$ might be expressed in human platelets[125, 126]. Using the commercially available pharmacological tools listed in Table 1, platelet aggregations were carried out in the absence or presence of S1PR agonists and antagonists to probe the expression of S1P receptors on human platelets. Selective agonists and antagonists for the $S1PR_2$ and $S1PR_3$ were tested using a concentration range from 100 nM to 10 μM. However, no effect on platelet aggregation was observed with any of these ligands. This suggests that $S1PR_2$ and $S1PR_3$ are not expressed on human platelets, which is in line

with transcriptome expression data demonstrating that no mRNA transcript for $S1PR_2$ or $S1PR_3$ is in human platelets. In addition, calcium mobilization assays demonstrated that S1P alone or in combination with PAR1-AP does not modulate platelet calcium signalling. Suggesting that S1P is not binding to any S1PRs which couple to $G_{\alpha q}$ such as $S1PR_2$ and $S1PR_3$[149]. However, the $S1PR_2$ agonist CYM5520 and $S1PR_3$ agonist CYM5541 used in this study are allosteric agonists for their respective receptors and are the only ligands for these receptors commercially available. As shown in Table 1, these two compounds have a much lower binding affinity than the other S1PR agonists or the orthosteric ligand S1P. Studies by Satsu et al[150] and Jo et al[151] using receptor binding site mutation technique and ligand competitive assay have indicated these two compounds bind to different binding sites on the $S1PR_2$ or $S1PR_3$ receptor than the orthosteric ligand S1P. This binding affinity and binding site difference might result in signalling differences or functional bias. Therefore, to confirm the S1P receptor expression, immunoblotting experiments using $S1PR_2$ and $S1PR_3$ receptor specific antibody might need to be carried out in the future.

In contrast, $S1PR_1$ agonists enhanced platelet activation whereas the $S1PR_1$ antagonist inhibited platelet activation. Therefore, it is likely that the $S1PR_1$ is expressed on human platelets and that the positive priming effect of S1P is mediated through the $S1PR_1$ receptor (Fig. 31). Studies indicate activation of the $G_{\alpha i/o}$ coupled $S1PR_1$ results in that activation of PI3K-Akt, ERK and Rac in various cell types such as ovarian cancer cells[152], endothelial cells[153] and smooth muscle cells[154], and can induce cell migration, secretion and proliferation. However, further studies on $S1PR_1$ signal transduction in platelets need to be conducted.

Pre-treatment of platelets with the $S1PR_4$ agonist significantly inhibited PAR1-AP mediated aggregation, this finding agrees with Onuma et al[85] which found that high concentrations of S1P or addition of the $S1PR_4$ agonist strongly inhibited CRP-mediated platelet function. Furthermore, aggregation data indicated that inhibition of the $S1PR_4$ significantly enhanced platelet aggregation. These findings suggest $S1PR_4$ is expressed on platelets and might play a role in negatively regulating platelet function. Intriguingly, CYM50260 did not inhibit but slightly increased platelet integrin $\alpha_{IIb}\beta_3$ activation. In addition, aggregation data showed for the first time, that pre-treatment of platelets with the $S1PR_5$ agonist A971432 can significantly reduce platelet responses. It was reported A971432 strongly inhibit forskolin cAMP production in $S1PR_5$ overexpressing CHO cells[155]. In addition, activation of $S1PR_5$ can enhance blood brain barrier function and reverse cognitive decline in animal models[156]. However, the lack of a commercially available $S1PR_5$ antagonist means we were unable to determine the effect of inhibiting this receptor in regulating platelet. From these data we can deduce that both $S1PR_4$ and $S1PR_5$ are likely to be expressed on platelets, which is in agreement with platelet transcriptome data.

Activation of $S1PR_4$ and $S1PR_5$ inhibited platelet spreading, suggesting that $S1PR_4$ and $S1PR_5$ negatively regulate platelet cytoskeletal dynamics. Several authors have indicated that $S1PR_4$ and $S1PR_5$ primarily couple to $G_{\alpha 12/13}$ subunit over $G_{\alpha i/o}$, however, activation of the $S1PR_4$ and $S1PR_5$ uniquely results in inhibition of Rho family small GTPase Rac1[157, 158]. It was demonstrated Rac1 plays an important role in regulating platelet lamellipodia formation and contributes to aggregate stability[16]. Recently, Okamoto et al[137] have demonstrated the inhibitory effects of S1P on CHO cells migration was mediated via activation of a Rac GAP (GTPase-activating protein). Activation of Rac-GAP converts Rac to a GDP bound low affinity state, which limits plasma membrane ruffling, cell migration and cytoskeletal rearrangement. High concentrations of S1P were also found to inhibit PDGF and IGF1 induced Rac activation in airway smooth muscle cell[159]. Therefore, this study suggests that the inhibitory effect of high concentrations of S1P are mediated through the activation of $S1PR_4$ and $S1PR_5$ resulting in Rac1 inhibition (Fig. 31). In addition, $S1PR_5$ was reported to inhibit ERK signalling pathway, which might result in less TxA2 being synthesized and released from platelets[160]. However, the activity of Rac1 and Rac-GAP and signal transduction downstream of these two receptors in platelets needs further examination.

4.6.3 The positive priming effect of S1P on PAR1-induced platelet function is mediated via activation of S1PR and PI3K.

As this project is focused on studying the positive effects of primers, it was important to examine the underlying mechanism by which S1P could enhance platelet activation. Aggregation data confirmed that S1P alone did not induce platelet aggregation but synergized with low concentrations of PAR1-AP to enhance platelet function. The inability of S1P to induce aggregation by itself is in line with findings by Ulrych et al[107] but contradicts the findings of Igarashi et al[161]. Platelet aggregation induced by subthreshold concentrations of PAR1-AP was significantly increased in the presence of 100 nM S1P, this finding is in line with work by Urtz et al[54]. Similar observations were also found by Ruan et al[162] where S1P was found to significantly potentiate ADP and adrenaline induced platelet aggregation. In contrast, S1P only caused small increases when platelets were stimulated with higher concentrations (1.5 - 2.0 μM) of PAR1-AP, which might be due to the platelets having reached maximal aggregation. Furthermore, PAR1-AP mediated integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure was significantly enhanced with pre-incubation of 100 nM S1P. This is consistent with studies on sphingosine kinase 2 knock out mice, where a significant decrease in PAR4-AP mediated integrin $\alpha_{IIb}\beta_3$ activation was rescued by applying exogenous S1P[133].

Immunoblotting experiments were carried out to investigate the downstream signalling of S1P in priming platelet function, with the phosphorylation of Akt^{S473}, ERK1/2 and $VASP^{S239}$ investigated. Immunoblot results demonstrated that S1P alone induced PKB/Akt phosphorylation in human platelets. Similar observation were also found in hippocampus

neurons[163] and intestinal epithelial cells[164] treated with sub-micromolar exogenous S1P. 100 nM and 300 nM S1P induced the greatest amount of $pAkt^{S473}$ with higher concentrations not inducing phosphorylation. This might be the result of different S1P receptors being activated, the S1P binding affinity on $S1PR_1$ and $S1PR_5$ are 8 nM and 64 nM respectively. A possible explanation is high concentrations of S1P start to activate the low affinity $G_{\alpha12/13}$ coupled $S1PR_4$ receptors resulting in less PKB phosphorylation and platelet inhibition. No obvious ERK phosphorylation was induced by S1P alone in human platelets, in contrast, studies found significant increases in ERK phosphorylation in osteoblasts cell treated with S1P and $S1PR_1$ overexpressed HeLa cells[139, 165]. This might because ERK phosphorylation mediated via $S1PR_1$ is weak or counteracted by effect of S1P at other S1PRs. In addition, there was a time-dependent effect of S1P on $pAkt^{S473}$, S1P induced peak phosphorylation within 3 - 5 minutes with less phosphorylation of Akt observed at 10 - 30 minutes, suggesting possible receptor internalization occurring. It was reported S1P receptors can be rapidly internalized upon stimulation with S1P and recycled back to cell membrane after 30 minutes[166].

Additive effects between S1P and PAR1-AP were observed on platelet aggregation, integrin activation and signal transduction. PAR1-AP mediated Akt^{S473} and ERK1/2 phosphorylation were enhanced in the presence of 100 nM S1P. Akt^{S473} phosphorylation was ablated by the addition of the pan-PI3K inhibitor wortmannin, as expected. Similarly, Takeya et al[167] found that S1P enhanced thrombin induced tissue factor expression and sustain thrombin induced activation of ERK. Since S1P enhances PAR1-mediated ERK signalling, theoretically PAR1-AP mediated TxA2 production could be enhanced in the presence of S1P. Furthermore, Cheng et al[168] found S1P induce COX-2 expression and upregulate PGE_2 release in human granulosa cells. However, measurements of TxB2 production found only a small non-significant increase in PAR1-AP mediated TxA2 synthesis induced by S1P. In this study, the TxB2 samples were prepared under non-stirring condition, therefore the effect of S1P under stirring conditions should be investigated in future studies. Furthermore, as sphingolipids are extremely hydrophobic, under non-stirring condition S1P may form a monolayer on the aqueous surface instead of interacting with platelets[130].

Lastly, immunoblotting experiment indicate S1P alone did not induce phosphorylation of $VASP^{S239}$ but did reduce PGE_1 mediated $VASP^{S239}$ phosphorylation. It was reported S1P pre-treatment reduced forskolin induced cAMP production and resulted in a rapid phosphorylation of Akt in mouse embryonic fibroblasts, these observations were abolished by pertussis toxin (PTX)[169], suggest $G_{\alpha i}$ coupling of the S1P receptors. Another study also found PTX treatment completely abolished S1P or dihydro-S1P induced cell proliferation in mesangial cells. My data is in consistent with these studies suggest S1P signalling via $G_{\alpha i/o}$ coupled receptors.

4.6.4 Sphingosine kinases play a role in regulating platelet function

Platelets lack SPP and SPL but exhibit constitutive activity of sphingosine kinases (Sphk). Inhibition of sphingosine kinase significantly reduced PAR1-AP mediate platelet aggregation. The competitive sphingosine kinase-1/2 inhibitor DMS significantly inhibited PAR1-AP induced platelet aggregation in a concentration dependent manner. This is consistent with experiments showing DMS inhibited platelet aggregation[170], and concentration dependently inhibited *de novo* S1P synthesis in human platelet. Similarly, pre-treatment with the selective SphK1 inhibitor PF543 hydrochloride (IC_{50} = 2nM, K_i = 3.6 nM) reduced platelet aggregation, but the inhibitory effect induced by PF543 hydrochloride was much less than with the same concentration of DMS. Although the DMS has a much higher IC_{50} of 500 nM and Ki of 3 μM. These results suggest SphK2 is the major sphingosine kinase in regulate platelet function, which is in agreement with SphK2 knock out mice studies showing deficiency in platelet aggregation and thrombus formation resulted prolonged tail bleeding time[54]. However, human platelet transcriptome data indicate that SphK1 has a 48-fold higher expression level than SphK2. Due to there being no commercially available selective SphK2 inhibitors when this study was conducted, the effect of selectively inhibiting SphK2 in human platelets remains unclear. Recently, a novel selective SphK2 inhibitor SLM 6031434 hydrochloride (Ki = 0.4 μM) has become available on the market, which could be used to test the effect of inhibiting platelet SphK2 in future experiments.

Inhibition of sphingosine kinases also reduced platelet spreading, the concentration of DMS used (1 μM) was not capable of inhibiting PKC[171]. The average platelet area was reduced, and fewer platelets were adhered. Suggesting sphingosine kinases are involved in regulating platelet morphology change. A recent study suggests the *de novo* synthesized S1P is transported through the phospholipid bilayer membrane, rapidly activate the S1P receptors expressed on the cell surface[110]. In line with aggregation data and platelet spreading results, we suggest sphingosine kinases can regulate platelet function, possibly via control the *de novo* synthesized S1P and subsequent autocrine signalling via the S1P receptors expressed.

Interestingly, a much less selective SphK2 inhibitor ABC294640 (Ki = 9.8 μM) is in phase I/II clinical trial under the brand name Yeliva® (RedHill Biopharma) for the treatment of cholangiocarcinoma, multiple myeloma and solid tumor[172,173]. Ongoing clinical trials data suggest the plasma S1P levels can be used as a novel biomarker for monitor cancer treatment. Orally administrated ABC294640 resulted in a rapid decrease of plasma S1P levels in patients with progressing cancer and mouse model study[174,175].

Chapter 5. General discussion

5.1 Summary of the study

Platelets play a central role in maintaining haemostasis but also contribute to the development of thrombotic disorders and atherosclerosis. Cardiovascular disease is the current leading cause of death worldwide, diseases such as diabetes, obesity and chronic inflammation contribute to the pathogenesis of thrombotic events[176]. Studies have found that elevated levels of circulating platelet primers can enhance platelet reactivity increasing the risk of thrombosis. The underlying mechanism of existing platelet primers such as IGF-1, TPO and PGE_2 has been well established, however, there is overwhelming evidence to suggest that other primers of platelet function exist.

In Chapter 3 of this thesis, a list of potential platelet primers was selected based on the corresponding receptor signalling mechanism, receptor transcriptome expression level and literature studies. Aggregation data confirmed that IGF-1, TPO and sulprostone enhance platelet function, some novel platelet primers including CCL17, CCL22, S1P, IL-34, and PDGF-AA were identified. After thoroughly evaluating the priming effect and comparing the novelty of these primers in the literature, S1P was selected as the “novel platelet primer” for further study. In Chapter 4 of this thesis, the effect of S1P on platelet function was examined in detail. Data indicate that S1P exhibit a concentration dependent biphasic effect on human platelet function; the positive priming effect might be mediated via $S1PR_1$ activation resulting in the activation of PI3K and the negative priming effect mediated via $S1PR_{4,\ 5}$ activation resulting in the inhibition of Rac small GTPase. Furthermore, this study also demonstrates that sphingosine kinases play a role in controlling platelet function.

5.2 Clinical implications and the bigger picture of S1P

As previously described, elevated S1P levels have been found to be associated with many diseases including ovarian cancer, atherosclerosis and diabetes. It was reported that S1P can synergize with thrombin to promote the blood clotting process and play an important role in regulating endothelial cell response[128]. This study found S1P can both enhance and inhibit PAR1-AP platelet function. A proposed mechanism of *in vivo* S1P priming platelet function is shown in Fig. 32. Diseases such as diabetes result in increased level of oxidation and glycation on ApoM/ApoA1 associated HDL, which cause defected HDL function. This disease induced HDL modification impairs the HDL-S1P interaction, resulting in more S1P being released into the circulation[177]. Elevated bioactive S1P can bind to the S1P receptors expressed on platelets or endothelial cells to amplify downstream signalling through S1P receptors, resulting in increased platelet function and elevated endothelial responses. It was reported that exogenous S1P can induce $S1PR_1$ receptor expression, which also contributes to priming of cellular responses [174,178].

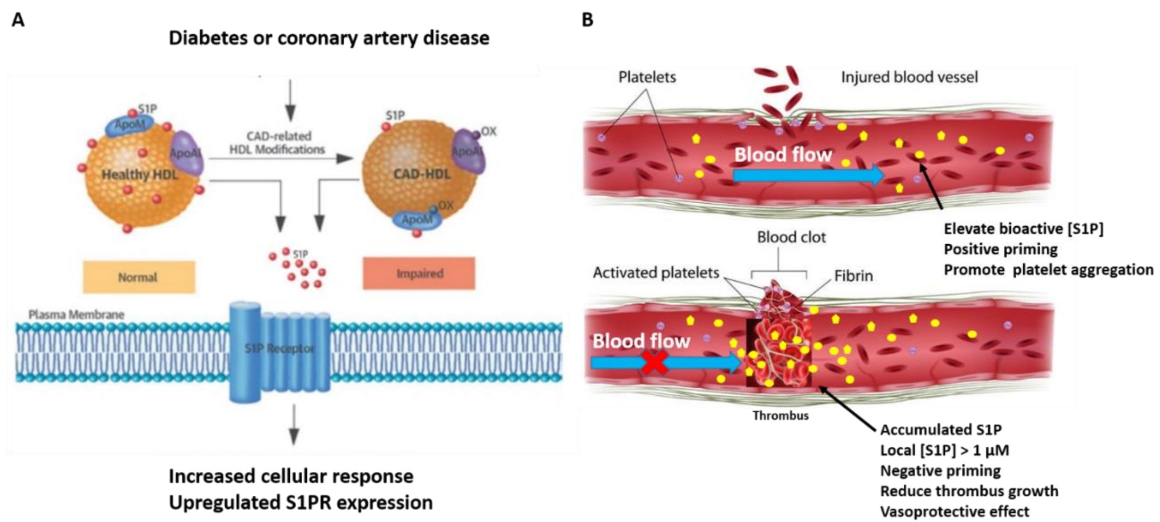


Figure 32. Schematic diagram showing (A) Proposed mechanisms of disease induced HDL dysfunction resulted S1P priming of platelet and endothelial cell response. **(B)** A potential vasoprotective effect mediated by the concentration dependent biphasic effects of S1P. Figures adapted from Sattler et al[179] and Alila Medical Media[180]

Interestingly, many studies on endothelial cells and cardiomyocytes also found S1P exhibits both vasoprotective and cardioprotective effects. Exogenous S1P application was found to enhance the viability of cardiomyocytes under hypoxia condition, this protective effect is primarily mediated via activation of $S1PR_{1,2,3}$ receptors expressed on the cells[181]. A similar study on endothelial cells found low level thrombin induced vasoprotective effects were abolished in the presence of $S1PR_1$ siRNA or pertussis toxin (PTX), which imply there is a synergistic protective effect between PAR1 and $S1PR_1$ receptors[182]. Downstream signalling of S1P receptors on these cells results in increased cell proliferation, promotes PI3K-Akt mediated survival signalling and increases PKC mediated nitric oxide synthase activation[183]. A proposed mechanism of S1P vasoprotective effect is shown in Fig. 32B. Injury to blood vessels causes endothelial cells and red blood cells to release S1P. Initial increases of bioactive S1P primes the circulating platelet function, promoting blood clotting and platelet aggregation at the site of injury. Subsequently, activated platelets release S1P contained in their granules and initiate *de novo* S1P synthesis, resulting in an increase in the local concentration of S1P. High local concentrations of S1P will induce inhibitory effects on platelet function, limiting thrombus growth. Furthermore, local S1P also activates S1P receptors expressed on endothelial cells to promote barrier integrity, initiate cell survival and proliferation process. By limiting the size and rate of thrombus growth and promote cell survive prose, S1P can induce protective effects on cardiomyocytes in a similar way. It was reported that low dosage sphingosine kinase inhibitor DMS can provide cardioprotective effects[184]. Although this model is hypothesized based on literature reports and data obtained, future studies on S1P protective effect might be conducted and examined in detail.

In the S1P research field, several drugs have been developed or in the clinic trails. Targeting $S1PR_1$ on lymphocytes exhibit immunomodulating effects. Fingolimod is the first S1P receptor

modulator developed for treating multiple sclerosis, it can act as a functional antagonist on $S1PR_1$ cause receptor degradation and inhibit lymphocytes migration[185]. Novel S1P related immunomodulators can cat as dual agonist on both $S1PR_1$ and $S1PR_5$, which Siponimod and Ozanimod are in phase III clinical trials for treating multiple sclerosis and Crohn's disease[186]. Furthermore, $S1PR_1$ agonist SEW2871 have been shown to reduce ischemic injury and exhibit cardioprotective effects in myocardial ischemia mice model[187]. Moreover, it was reported that antagonizing $S1PR_2$ using JTE-013 reduce vascular permeability and promote endothelial function in mouse stroke model study. The biphasic effect of S1P on platelets might lead to identification of novel antiplatelet targets, for instance, targeting the sphingosine kinase or $S1PR_4$ and $S1PR_5$ receptors[188]. Recently, it was reported that Fingolimod treatment reduce leukocytes recruitment and inhibit endothelial activation, which might reduce local platelet-leukocyte or platelet-endothelial cell interactions[189].

5.3 Future work and conclusion

There are genetic variations between individual platelet donors which might affect the results obtained, since some experiments only have been conducted for limited times, more repeat experiments need to be performed. FACS assay using annexin V conjugates can be conducted to determine whether the inhibitory effects of high concentrations S1P were cytotoxic effects. The S1P receptor subtypes expression profile on human platelets might need to be confirmed on immunoblotting with receptor specific antibodies. Moreover, studies have shown the synthesis and release of S1P from activated platelets is also regulated by PKC and TxA2. Therefore, future investigation is needed to examine the effect of aspirin or indomethacin treatment on S1P priming. Future experiments are needed to quantify PAR1-AP induced *de novo* S1P synthesis in human platelets using S1P ELISA kit, and the S1P release from platelets also could examined by using ABBC4 inhibitor. Furthermore, S1P receptor gene knockout mice model could be developed, in particular, $S1PR_4$ knock out mice might be designed to study the regulatory effect of this receptor on platelet function. Moreover, epidemiological studies could be carried out to exam correlation between S1P level and cardiovascular events in patients with diabetes, obesity or chronic inflammation.

There has been limited research conducted on the effect of S1P on human platelet function, while many controversies exist in the literatures. In conclusion, this study has identified S1P as a novel platelet primer and demonstrated the underlying mechanism by which S1P affect PAR1 mediated platelet function in a concentration dependent biphasic manner. These findings may contribute to identify other primers and understand their mechanisms in thrombotic disorders. The biphasic effect of S1P might have clinical importance in developing novel drugs to diversely modulate platelet function. Since targeting S1P receptors also have shown immunomodulating effects, disrupt the platelet-leukocyte interactions via S1P receptors might be a novel approach in treatment of thrombosis and chronic inflammation.

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