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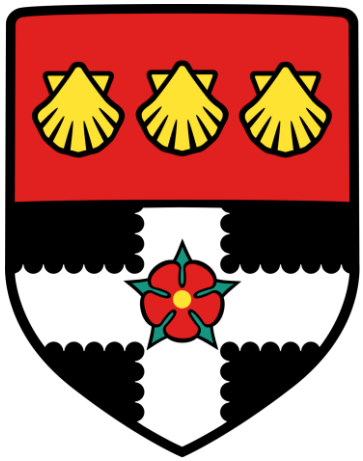

Non-Genomic Effects of the Pregnane X Receptor (PXR) and Retinoid X Receptor (RXR) in Platelets

A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Background: The pregnane X receptor (PXR) is a nuclear receptor (NR) involved in the detoxification of xenobiotic compounds via upregulation of cytochrome P450 enzyme expression. Recently, the presence of PXR was reported in the human vasculature and its ligands were proposed to exhibit anti-atherosclerotic effects. The retinoid X receptor (RXR) is another NR that regulates numerous biological functions. RXR (α and β) is expressed in platelets, and its ligands have been reported to inhibit platelet function mediated by Gq coupled ADP and TXA_2 receptors.

Aims: Platelets play a substantial role towards the initiation of atherosclerosis and express numerous NRs. Given the anti-atherosclerotic effects of PXR ligands, we explored whether PXR is present in human platelets and evaluated the role of its ligands in regulating platelet activation in response to different platelet agonists. Since RXR is known to modulate platelet function in response to ADP and U46619, and is able to function in other cells in collaboration with PXR, we also sought to extend studies of the role of RXR in the modulation of platelet activation stimulated by collagen/CRP-XL or thrombin.

Results: The expression of PXR in human platelets was confirmed using western blot and immunoprecipitation analysis. Platelets treated with PXR ligands (SR12813 or rifampicin) inhibited a range of platelet activities such as aggregation, fibrinogen binding to integrin αIIbβ3, degranulation, intracellular calcium mobilisation, integrin αIIbβ3 outside-in signalling. In the absence of nuclei, the actions of PXR are non-genomic in nature. Human and mouse PXR ligands reduced thrombus formation *in vitro* in human and mouse blood respectively. These effects of human and mouse PXR ligands were observed in a species-specific manner. Anti-thrombotic effects of SR12813 were

observed in humanised PXR knock-in mice using an *in vivo* mouse model of thrombosis. In addition to the reduced tyrosine phosphorylation of multiple GPVI signalling components, caused by PXR ligands, inhibition of phosphorylation of Src family kinases (SFKs) proximal to GPVI, CLEC-2 and integrin αIIbβ3 receptor was also observed. This suggests SFKs as a potential target of PXR function. Furthermore, RXR ligands (9-*cis*-RA or methoprene acid), in a non-genomic manner, down-regulated platelet activity stimulated by collagen/CRP-XL or thrombin. RXR was found to exist in the form of a heterodimer with other NRs such as PXR, liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPARβ and PPARγ). Exposure to 9-*cis*-RA also caused a reduction in thrombus formation *in vitro* and *in vivo*, with impairment of haemostatic response.

Conclusions: This study identifies the ability of PXR and RXR to regulate platelet activation and thrombus formation in a non-genomic manner. The potential anti-atherosclerotic properties of PXR and RXR ligands, together with newly identified anti-thrombotic effects may provide additional cardio-protective benefits.

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Publications

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Presentations

1. **ORAL PRESENTATION: Retinoid x receptor ligands exhibit non-genomic effects to inhibit collagen and thrombin-mediated platelet activation.** UK platelet meeting, Leicester, UK, 2015

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5. **POSTER: Non-genomic effects of the Pregnane x receptor inhibit platelet function.** Joint BSHT, AiP & UK Platelet Group Meeting, Leeds, UK, November 2016

Abbreviations

ACD – Acid Citrate Dextrose

AC – Adenylyl Cyclase

ADP – Adenosine diphosphate

AF – Activation Function

AR – Androgen Receptor

atRA – all-*trans*-retinoic acid

ANOVA – Analysis of variance

ApoE – Apolipoprotein E

ATP – Adenosine triphosphate

AU – Arbitrary Units

BFGF – Basic Fibroblast Growth Factor

BSA – Bovine Serum Albumin

BTK – Bruton's Tyrosine Kinase

Ca^{2+} – Calcium ion

$CaCl_2$ – Calcium chloride

CalDAG-GEFI – Ca^{2+} and DAG-Regulated Guanine Nucleotide Exchange Factor I

cAMP – Cyclic adenosine monophosphate

CD – Cluster of Differentiation

CD62P – P-selectin

Cdc42 – Cell division control protein 42 homolog

CEACAM-1 – Carcinoembryonic Antigen Cell Adhesion Molecule-1

cGMP – Cyclic guanosine monophosphate

CLEC-2 – C-type Lectin-Like Receptor 2

CRP-XL – Cross-Linked Collagen-Related Peptide

Csk – C-terminal Src Kinase

Chk - Csk homologous kinase

CVD – Cardiovascular disease

CYP – Cytochrome p450

Cy5 – Cyanine 5 dye

C57BL/6 – C57 Black 6 mice

DAG – Diacylglycerol

DBD – DNA Binding Domain

DDI – Drug-drug interaction

DioC6 – 3,3'-dihexyloxacarbocyanine iodide

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DTS – Dense Tubular System

ECM – Extracellular Matrix

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

ER – Estrogen Receptors

eNOS – endothelial NOS

FAK – Focal Adhesion Kinase

FcR – Fc Receptor

FITC – Fluorescein isothiocyanate

FSC – Forward scatter

Fura-2 AM – Fura-2-acetoxymethyl ester

FXR – Farnesoid X Receptor

g – g-force

GR – Glucocorticoid receptors

Gads – Grb2 Related Adaptor Protein Downstream of Shc)

GC – Guanylyl Cyclase

GDP – Guanosine diphosphate

GEF – Guanine Nucleotide Exchange Factor

GP – Glycoprotein

GPCR – G-protein Coupled Receptor

GPO – Gly-Pro-Hyp

GPRP – Gly-Pro-Arg-Pro

GTP – Guanosine triphosphate

HDL – High-Density Lipoprotein

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hPXR – Human PXR

HRE – Hormone Response Element

HSP – Heat Shock Protein

Ig – Immunoglobulin

IP receptor – Prostaglandin Receptor

IP3 – Inositol trisphosphate

IP3R – Inositol trisphosphate Receptor

ITAM – Immunoreceptor Tyrosine-Based Activation Motif

ITIM – Immunoreceptor Tyrosine-Based Inhibitory Motif

JAM-A – Junctional Adhesion Molecule A

K_d – Dissociation constant

kDa – kilodalton

KO – Knock-out

LAMP – Lysosomal-associated membrane protein

LAT – Linker for activation of T cells

LBD – Ligand Binding Domain

LDL – Low-Density Lipoprotein

LTA – Light Transmission Aggregometry

LXR – Liver X Receptor

M – Molar

MAPK – Mitogen-Activated Protein Kinase

mg – Milligram

Mg^{2+} – Magnesium ion

$MgCl_2$ – Magnesium chloride

Mins – Minutes

ml – Millilitre

MLC – Myosin Light Chain

mmol/L – Millimolar

MMP – Matrix metalloproteinases

mPXR – Mouse PXR

mRNA – Messenger RNA

μg – Microgram

μl – Microliter

μM – Micromolar

NaCl – Sodium chloride

$NaHCO_3$ – Sodium bicarbonate

Na_2HPO_4 – Disodium phosphate

nm – Nanometre

nM – Nanomolar

NO – Nitric Oxide

NR – Nuclear Receptor

ns – Not significant

OCS – Open Canalicular System

Orai1 – Calcium-Release Activated Calcium Modulator 1

PAR – Protease-Activated Receptor

PBS – Phosphate Buffered Saline

PCC – Pearson correlation coefficient

PCR – Polymerase Chain Reaction

PDE – Phosphodiesterase

PDGF – Platelet Derived Growth Factor

PE – Phycoerythrin

PECAM-1 – Platelet Endothelial Cell Adhesion Molecule-1

PF4 – Platelet Factor 4

pg– picogram

PGI_2 – Prostacyclin

PIP2 – Phosphatidylinositol 4,5-bisphosphate

PIP3 – Phosphatidylinositol 3,4,5-trisphosphate

PI3K – Phosphoinositide 3-kinase

PKA – Protein Kinase A

PKB – Protein Kinase B

PKC – Protein Kinase C

PKG – Protein Kinase G

PLA - Phospholipase A

PLC – Phospholipase C

PPAR – Peroxisome Proliferator-Activated Receptor

PCN – Pregnenolone-16α-carbonitrile

PRP – Platelet Rich Plasma

PVDF – Polyvinylidene difluoride

PXR – Pregnane X Receptor

RAR – Retinoic acid Receptor

RBC – Red Blood Cell

RIAM – Rap1-GTP-Interacting Adaptor Molecule

RIBS – Receptor Induced Binding Site

ROS – Reactive Oxygen Species

RPM – Revolutions Per Minute

RT – Room Temperature

RXR – Retinoic X Receptor

S – Serine

SDF1α – Stromal Cell-Derived Factor α

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

Secs – Seconds

SFK – Src Family Kinase

sGC – Soluble Guanylyl Cyclase

SHP-1 – Src homology 2 domain-containing protein tyrosine phosphatase-1

SHP-2 – Src homology 2 domain-containing protein tyrosine phosphatase-2

SH2 – Src homology 2

SH3 – Src homology 3

SLP76 – SH2 domain containing leukocyte protein of 76 kDa.

SOCE – Store-Operated Calcium Entry

SSC – Side scatter

STIM1 – Stromal Interaction Molecule 1

Syk – Spleen Tyrosine Kinase

TAE – Tris Acetate EDTA

TBS-T – Tris Buffered Saline with Tween 20

TNFα – Tumor Necrosis Factor alpha

TP – Thromboxane Prostanoid Receptor

TR – Thyroid Hormone Receptor

TRPC6 – Transient Receptor Potential Cation Channel Subfamily C Member 6

TxA_2 – Thromboxane A_2

TxB_2 – Thromboxane B_2

U/ml – Unit/millilitre

U46619 – 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin

V – Volts

VDR – Vitamin D Receptor

VLDL – Very Low-Density Lipoprotein

v/v – Volume/Volume

vWF – von Willebrand Factor

w/v – Weight/Volume

WPL – Whole Platelet Lysate

Y – Tyrosine

9-cis-RA – 9-*cis*-retinoic acid

^{o}C – Degrees Centigrade

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Chapter-1

Introduction

1.1. Platelets: an overview

Platelets (or thrombocytes) were first discovered by Giulio Bizzozero, an Italian professor, in 1882. He categorised these corpuscles as the third morphological element of blood and coined the term '*piastrine*' for them, meaning small plates (Gazzaniga and Ottini, 2001). These particles later became established as 'platelets' and are now regarded as the second most abundant class of circulating blood cells (after red blood cells) (York, 2013). They are small, anucleated and discoid shaped (1-3 μm diameter) arising from cytoplasmic fragmentation of megakaryocytes in the bone marrow. In healthy individuals, approximately 10^{11} platelets are produced per day to maintain a normal platelet concentration of 1.5 – $4x10^8$ cells/ml with an average lifespan of 8-10 days (Ghoshal and Bhattacharyya, 2014). The primary function of platelets is to maintain haemostasis, which is to initiate blood coagulation at the site of injury, resulting in the development of a haemostatic clot and thereby sealing the wound (Clemetson, 2012). Low concentration of circulating platelets, a condition referred to as thrombocytopenia, can lead to bleeding disorders. Under physiological conditions, red blood cells flow in the central core of the blood vessel and marginate platelets towards the vessel wall, enabling them to detect injury to the vessel wall (Mountrakis et al., 2013).

Platelets maintain a quiescent profile while flowing under normal conditions; however, upon encountering any discontinuity in the vessel wall, they undergo a dynamic transformation into an activated state, leading towards the formation of a platelet plug to seal the wound and arrest bleeding. Although, platelet activation is a highly controlled and regulated process, their inappropriate

activation, even in the absence of vessel injury may lead to thrombus formation within a blood vessel (Bergmeier and Hynes, 2012). This condition, referred to as thrombosis can be regarded as a pathological version of haemostasis and may lead to life-threatening tissue ischemia, myocardial infarction or stroke through the occlusion of cerebral blood vessels (Clemetson, 2012; Mackman, 2008). Clinical conditions such as atherosclerosis that involve development of a plaque (consisting of fat, cholesterol, calcium) at the arterial wall can stimulate platelet activation. Rupturing of the fibrous cap (rich in collagen) of the plaque triggers activation of platelets and subsequent thrombosis (Badimon and Vilahur, 2014). Unwanted platelet activation is also associated with obesity, diabetes, cancer and hypertension that may lead to hyperactivity of platelets (Blokhin and Lentz, 2013; Vazzana et al., 2012; Previtali et al., 2011; Elyamany et al., 2014).

There exists a very thin line that demarcates the frontier between physiological haemostasis and pathological thrombosis. Despite many advancements in the field, thrombosis is still one of the leading causes of death globally (Davì and Patrono, 2007). Since, platelets are the major contributors towards this disease, comprehensive understanding of the mechanisms that govern haemostasis and its transition to thrombosis are required. Therefore, paramount emphasis has been given to platelet-research with a clear intention of exploring them as potential therapeutic targets in the field of thrombosis and related cardiovascular diseases (CVDs).

1.2. Platelet biogenesis and clearance

Platelets are produced from megakaryocytes in the bone marrow by a process called thrombopoiesis. Megakaryocytes constitute <0.1% of the nucleated cell population in the bone marrow and are produced from multipotent haematopoietic stem cells (HSCs) via haematopoiesis (Patel et al., 2005). Under the control of hematopoietic cytokine, thrombopoietin; HSCs mature and differentiate to form megakaryocytes, each of which can subsequently produce 10^3-10^4 platelets (Kaushansky, 2006; Kaushansky, 2005).

Thrombopoiesis is initiated with the binding of thrombopoietin to the c-Mpl surface receptor on the megakaryocyte, which stimulates endomitosis (DNA replication without cell division), transforming megakaryocytes into giant cells with a large cytoplasm and polyploid nucleus (Ravid et al., 2002). This process considerably amplifies the levels of DNA (up to 64-fold) which is necessary to produce mRNA, cytoskeletal and platelet-specific proteins, to be packaged into platelets. (Machlus et al., 2014; Patel et al., 2005). This is then followed by the rearrangement of a bulk of megakaryocytic cytoplasm in the form of long projections or pseudopods called proplatelet shafts, while the polyploid nucleus remains embedded inside the megakaryocyte (Italiano and Shivdasani, 2003). These shafts are active structures made up of microtubule bundles with platelet sized swellings at their ends (Thon et al., 2012). Proplatelets are regarded as the assembly lines for platelet production and this process essentially takes place adjacent to the sinusoidal blood vessels in the bone marrow, and into which, proplatelets are released into the circulation (Geddis, 2010; Machlus and Italiano, 2013). Each megakaryocyte can give rise to 10-20 proplatelets, which after their

release undergoes repeated branching and fission in a shear-dependent manner to form discoid shaped platelets (Junt et al., 2007; Richardson et al., 2005).

Apart from the bone marrow, lungs have been a subject of interest for decades as a potential site of thrombopoiesis. Several studies reported the presence of megakaryocytes in lungs (Hansen and Pedersen, 1978; Kaufman et al., 1965; Levine et al., 1993) and noticed that blood leaving the lungs carried fewer megakaryocytes (in comparison with the number that initially entered) and more of platelets (Kallinikos-Maniatis, 1969; Howell and Donahue, 1937). Lefrançais *et al.* (2017) recently provided further evidence that suggest lungs as a major site of platelet biogenesis. Through direct imaging of lung microcirculation in mice, the presence of both mature and immature megakaryocytes (of extrapulmonary origin) and release of platelets from them in extravascular spaces of the lungs was confirmed. It was estimated that platelet production in lungs contributes to around 50% of total platelet biogenesis in mouse (Lefrançais et al., 2017). However, the origin of platelets (bone marrow or lungs) remains a topic of considerable debate.

The pathways underlying clearance of platelets are not well understood. The classical mechanism suggests the role of apoptosis in platelet removal. It explains how dysregulation in the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family (involved in the intrinsic apoptotic pathway) can direct platelets towards apoptosis and eventual clearance from the physiological system (Mason et al., 2007; Grozovsky et al., 2015b). Alternatively, autoantibodies that target platelet integrin αIIbβ3 and/or the GPIb–V–IX complex can also cause platelet removal. Platelets displaying autoantibodies on their

surface attract macrophages, leading to removal through phagocytosis in spleen (Grozovsky et al., 2015c). Immune thrombocytopenia is the most common outcome of this mode of platelet clearance (McMillan, 2007). Recently, studies have reported platelet clearance based on ageing (senescence) induced signals. Loss of sialic acid from the platelet surface marks the senescent platelets for removal, which are cleared via the hepatic Ashwell-Morell receptors (Grozovsky et al., 2015a; Li et al., 2015).

1.3. Platelet ultrastructure

Although platelets possess a deceptively simple appearance, they are quite complex structures (Figure 1.1). The lack of a nucleus is one the feature that distinguishes platelets from other cell types. Their remarkably small size of 2-4 μM provides a mean cell volume of 8-10 femtoliters, whereas, their disc shape aids their flow near to the endothelium in the bloodstream. Important structures of platelets include:

a. Plasma Membrane

The platelet membrane is a typical bilayer of phospholipids and relatively smooth in comparison with other cell types present in circulation. Scanning electron microscopy shows the presence of tiny folds, which are believed to provide additional surface area when platelets spread upon activation (White, 2013). The plasma membrane is structurally supported by a cytoskeleton made up of actin, tubulin, spectrin and filamin. In a resting state, microtubules exist in the form of coils in the submembranous part of the plasma membrane, providing a discoid shape to platelets (Smyth et al., 2010). Actin filaments exist in the cytoplasm and maintain a dynamic equilibrium between a monomeric globular form (G-actin) and a polymeric filamentous form (F-actin). It is this polymerisation that primarily facilitates platelet shape change upon activation (Bearer et al., 2002). Spectrin, on the other hand, laminates the cytoplasmic face of the plasma membrane and is connected to actin filaments (Shin et al., 2017).

Invaginations of the platelet plasma membrane constitute the open canicular system (OCS) that serves a conduit for the release of platelet secretome during

activation along with the movement of substances into the cells (Flaumenhaft and Koseoglu, 2016). The plasma membrane also features a range of surface receptors pivotal for platelet activation and aggregation. These include adhesion receptors, G-protein coupled receptors and integrins, discussed in detail in section 1.4.

b. Dense tubular system

The dense tubular system (DTS) is a derivative of the megakaryocyte smooth endoplasmic reticulum and acts as a storehouse of calcium ions (Ca^{2+}) and several enzymes that are important to control platelet activation (Gremmel et al., 2016). Under resting conditions, the DTS exists as thin elongated membranes, which transform to a round vesicular form upon platelet activation (Ebbeling et al., 1992) along with the release of calcium. This causes a rapid rise in cytosolic calcium-concentration which is extremely important for platelet activation (Jardín et al., 2008). The DTS is also a site where enzymes cyclooxygenase-1 (COX-1) and thromboxane synthetase catabolise arachidonic acid towards the formation of thromboxane A_2 (TxA_2), which provides positive feedback and amplifies platelet activation (Rendu and Brohard-Bohn, 2001).

c. Platelet granules

There are three kinds of granules that have been identified in platelets; α-granules, dense granule and lysosomes (Lam et al., 2015).

α-granules: They are spherical or ovoid in shape (diameter of 200–400 nm) and with an approximate number of 50-80 per platelet they outnumber the other two granules (Blair and Flaumenhaft, 2009). α-granules contains the bulk of the platelet secretome and are regarded as the primary secretory granules in platelets. They store several important haemostatic factors such as von Willebrand Factor

(vWF), fibronectin, and fibrinogen along with several transmembrane receptors, for instance, GPIb-V-IX, αIIbβ3, GPVI and Platelet endothelial cell adhesion molecule (PECAM-1). Both haemostatic factors and transmembrane receptors become trafficked to the surface of platelets upon their activation (Golebiewska and Poole, 2015). In addition to these several angiogenic factors (eg, angiogenin, VEGF), anti-angiogenic factors (eg, angiostatin, PF4), growth factors (eg, PDGF, bFGF, SDF1α), proteases (eg, MMP2, MMP9), necrotic factors (eg, TNFα, TNFβ), and cytokines are also present inside α-granules (Whiteheart, 2011).

Dense granules: These granules contain high levels of calcium, magnesium and phosphate ions which make them appear as dense bodies when seen with an electron microscope (Ruiz et al., 2004). While there are only 3-5 dense granules present per platelet, they store sufficient quantities of nucleotides (ATP and ADP) and serotonin that upon release stimulate positive feedback mechanisms in platelets which are important to strengthen activation responses in an autocrine (same platelet) or paracrine (recruiting more platelets) fashion (Flaumenhaft, 2017; Mehta et al., 2012). Both α-granules and dense granules secrete their contents into the OCS and then pass into the blood.

Lysosomes: A platelet typically contains 1–3 lysosomes that are rich in acid hydrolases such as cathepsins, hexosaminidase, β-galactosidase, arylsulfatase, β-glucuronidase and acid phosphatase (Heijnen and van der Sluijs, 2015; Lam et al., 2015). The lysosomal-associated membrane proteins (LAMP); LAMP-1, LAMP-2, and LAMP-3 (CD63) present on the lysosome surface are highly glycosylated and provide protection to the lysosomal membrane from the acidic luminal (pH <5) and proteolytic enzymes present within the lysosome (autodigestion) (Fukuda,

1991; Schwake et al., 2013). The functions of platelet lysosomes are not well understood. Apart from the hydrolysis of phagocytic and cytosolic components, it is believed that lysosomes participate in digestion and resolution of thrombi (Rendu and Brohard-Bohn, 2001).

d. Mitochondria

Platelet mitochondria, like in nucleated cells, are primarily involved in the production of ATP using oxidative phosphorylation to meet energy requirement of the quiescent cell (Zharikov and Shiva, 2013). Recent observations have suggested that mitochondria influence non-ATP mediated thrombotic signalling in platelets through the regulation of redox balance and apoptosis (Matarrese et al., 2009; Garcia-Souza and Oliveira, 2014).

e. Glycosomes

Platelet cytoplasm stores numerous glycosomes or glycogen particles that are important cargos of energy (White, 1999). Since platelets contain all the enzymes required for the conversion of glycogen to glucose (via glycolysis), they are regarded as important sites of energy for platelets (Rocha et al., 2014).

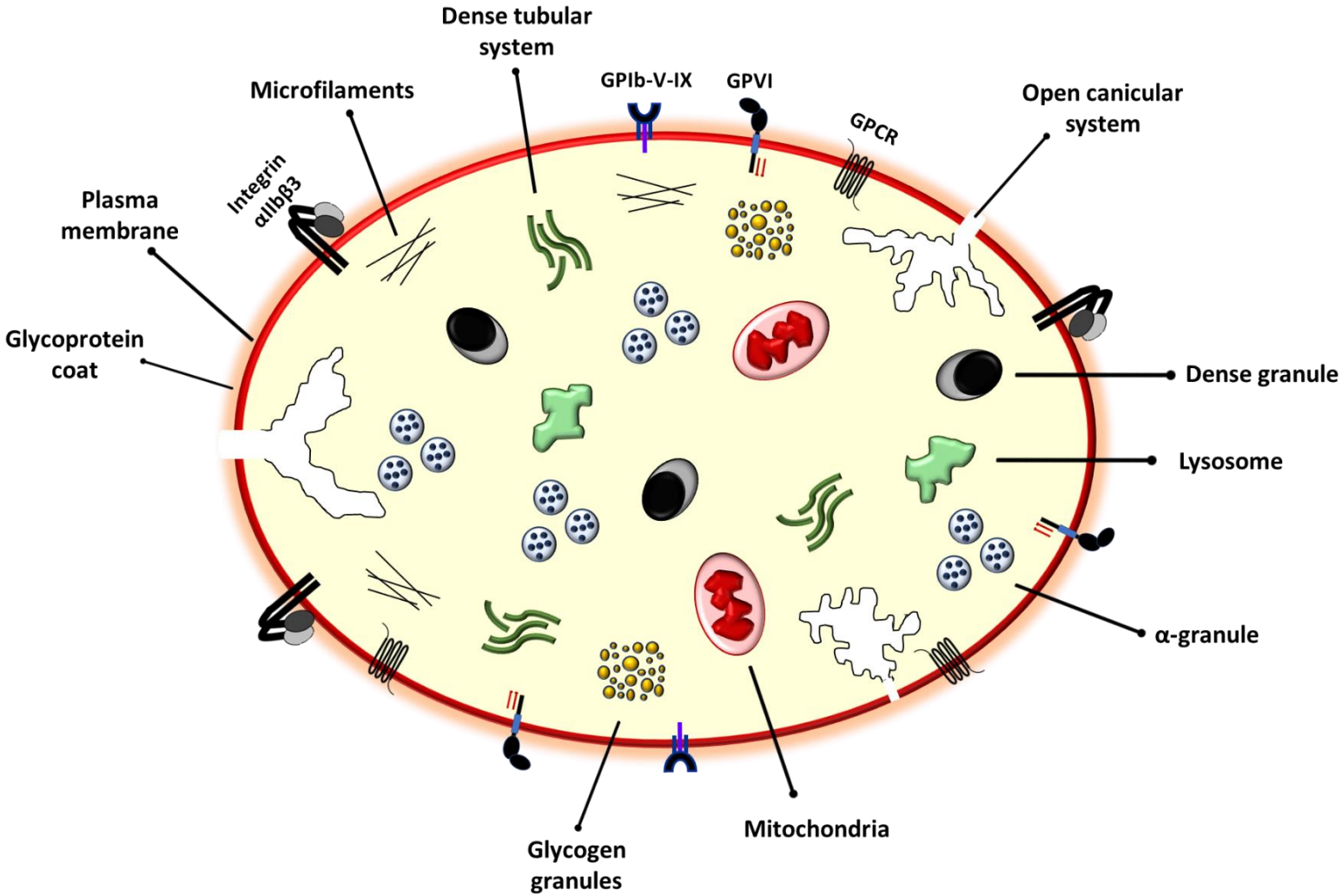


Figure 1.1: A diagrammatic representation of the platelet ultrastructure. The plasma membrane of a platelet is surrounded by an external glycoprotein coat. The submembrane region is rich in actin, microtubules and microfilaments that maintain the discoid shape of resting platelets and assist in shape change upon activation. The cytosol comprises of many organelles such as alpha and dense granules, mitochondria, lysosomes and glycogen granules. Two distinct membrane systems are present in platelets; the open canalicular systems (OCS), which provides a route for the trafficking of molecules in and out of the platelets, while, the dense tubular system (DTS) acts as a store house of calcium.

1.4. Role of platelets in haemostasis

Haemostasis is a normal physiological response of the human body to stop bleeding and prevent loss of blood following an injury to a blood vessel. A series of coordinated cellular and biochemical events are involved in a haemostatic response, which ultimately results in the formation of a blood clot at the site of injury, thereby sealing the wound (Wang et al., 2014b). Vascular spasm or vasoconstriction is the first response of a blood vessel following injury. It involves contraction of the muscular wall that results in narrowing of the blood vessel to reduce the volume of blood flowing near the injured area and thus limits the blood loss (Velnar et al., 2009). Formation of a *platelet plug* at the injured site marks the second stage of haemostasis. The dynamics of platelet plug formation can be categorised into 3 phases; initiation or tethering phase, extension phase and stabilisation phase (Figure 1.2), which will now be discussed:

a. Initiation or tethering phase (adhesion of platelets)

Upon vascular injury, immobilised adhesive proteins such as collagen and vWF present in underlying subendothelial matrix becomes exposed, which triggers the adhesion of platelets (Nuyttens et al., 2011). Initially, the GPIbα subunit of the platelet glycoprotein Ib–V–IX receptor (GPIb–V–IX) forms a reversible complex with its ligand vWF, causing platelets to slow down and roll on the endothelium (Hou et al., 2015; Lopez and Dong, 1997). This binding enables a stable interaction of collagen with platelet receptors glycoprotein VI (GPVI) and integrin α2β1, resulting in the formation of a platelet monolayer at the injured site and transmission of activation signals across the platelets. Although, both the collagen

receptors are necessary for the complete activation of platelet, their role in adhesion of platelets is controversial. Nieswandt *et al.* (2001a) using β1 and GPVI null platelets suggested that the role of α2β1 is superfluous for platelet adhesion and thrombus growth on fibrillar collagen under static and flow conditions, whereas, these processes are eliminated in the absence of GPVI. On the other hand, Pugh *et al*. (2010) reported that α2β1 is predominantly involved with adhesion of platelets, while, GPVI function as the primary signalling receptor to activate platelets. Substrates other than collagen found in the endothelial matrix have also been shown to play a fundamental role in platelet adhesion. For instance; fibronectin binds to integrin α5β1 and αIIbβ3, laminin attaches with GPVI and integrin α6β1, whereas, thrombospondin-1 interacts with GPIb–V–IX complex in a vWF independent manner (Jurk et al., 2003; Ruggeri and Mendolicchio, 2007; Inoue et al., 2006).

b. Extension (activation and secretion)

Interaction with collagen results in the activation of platelets via stimulation of tyrosine kinase signalling downstream of the GPVI receptor, which can be regarded as the major collagen receptor on platelets (Clemetson et al., 1999; Gibbins et al., 1996; Gibbins et al., 1997). Degranulation is one the outcomes of this signalling event and involves the release of a myriad of pro-thrombotic agents, such as adenosine diphosphate (ADP) and TxA_2 (produced by sequential oxygenation of arachidonic acid by cyclooxygenase-1 and thromboxane A_2 synthase). Thrombin precursor prothrombin is also secreted by α-granules upon platelet activation, which is a potent platelet agonist (Golebiewska and Poole, 2015; Gibbins, 2004). These platelet agonists act via their specific platelet surface-

receptors, which belong to the family of G-protein coupled receptors (GPCRs). This activates platelets circulating near the injury site, resulting in an amplification of primary signal initiated by collagen. Irrespective of the receptor through which these platelet agonists act, they ultimately facilitate transformation of integrin αIIbβ3 (GPIIb-IIIa) receptor from a low affinity to high-affinity state. In high-affinity conformation, integrin αIIbβ3 can bind to its bivalent ligand fibrinogen (or other adhesive ligands such as vWF, fibronectin and thrombospondin-1) present in plasma. This results in the formation of crosslinked bridges between platelets and allows them to form aggregates, leading to the formation of a platelet plug (Ma et al., 2007b; Bennett, 2005).

c. Perpetuation (stabilisation)

This stage refers to the events that are involved in the consolidation of the platelet plug to prevent premature disaggregation of platelets. Following the formation of a platelet plug, contact-dependent signalling occurs in platelets that are attached with one another to stabilise the thrombus (Prevost et al., 2003). One of the classic examples of this is outside-in signalling, which is initiated by the attachment of fibrinogen to integrin αIIbβ3. This process is characterised by cytoskeletal reorganisation and clot retraction (discussed in detail in section 1.5.5), which contributes towards the stability of haemostatic plug (Payrastre et al., 2000; Phillips et al., 2001). Apart from integrin αIIbβ3, contact-dependent signalling is also stimulated by the family of Eph receptor tyrosine kinases that are activated by their ligand ephrin (Vaiyapuri et al., 2015; Prevost et al., 2002). Recently, the presence of several forms of connexins were reported in platelets. They were found capable of regulating the stability of thrombus through the

formation of hemichannels (isolated platelets) and gap junctions (within thrombus) that regulate intercellular trafficking of molecules between platelets (Vaiyapuri et al., 2013; Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012).

Simultaneously, in coordination with platelet plug formation, coagulation cascade also becomes activated, which involves the conversion of blood from a liquid state to a gel-like structure to prevent blood loss. Coagulation is a complicated process that involves at least 30 different kinds of proteins (known as coagulation factors) acting via two different pathways (extrinsic and intrinsic) in a sequential manner to generate activated factor X (Spronk et al., 2003; Walsh, 2004). The production of activated factor X results in the cleavage of prothrombin (factor II) to thrombin (factor IIa), which acts as a catalyst for the conversion of soluble plasma-protein fibrinogen (factor I) into long, sticky threads of insoluble fibrin (factor Ia) (Chu, 2011). Fibrin, through the action of factor XIII becomes cross-linked and forms a mesh-like network on aggregated platelets that provide structural stability to the growing thrombus to form a stable clot (Monroe and Hoffman, 2006; Hoffman, 2003; Bagoly et al., 2012).

Lastly, haemostasis is marked by fibrinolysis, which is a highly regulated enzymatic process that prevents unnecessary accumulation of intravascular fibrin and enables the removal of thrombi (Chapin and Hajjar, 2015). This limits the growth of thrombus, which can be fatal and may lead to stroke, tissue ischaemia or myocardial infarction. The thrombus is lysed by the action of plasmin which is generated from plasminogen on the surface of fibrin clot through the action of tissue plasminogen activator (Chapin and Hajjar, 2015). The degradation products

from fibrinolysis possess anticoagulant properties and are cleared by the monocyte-macrophage system (Jennewein et al., 2011).

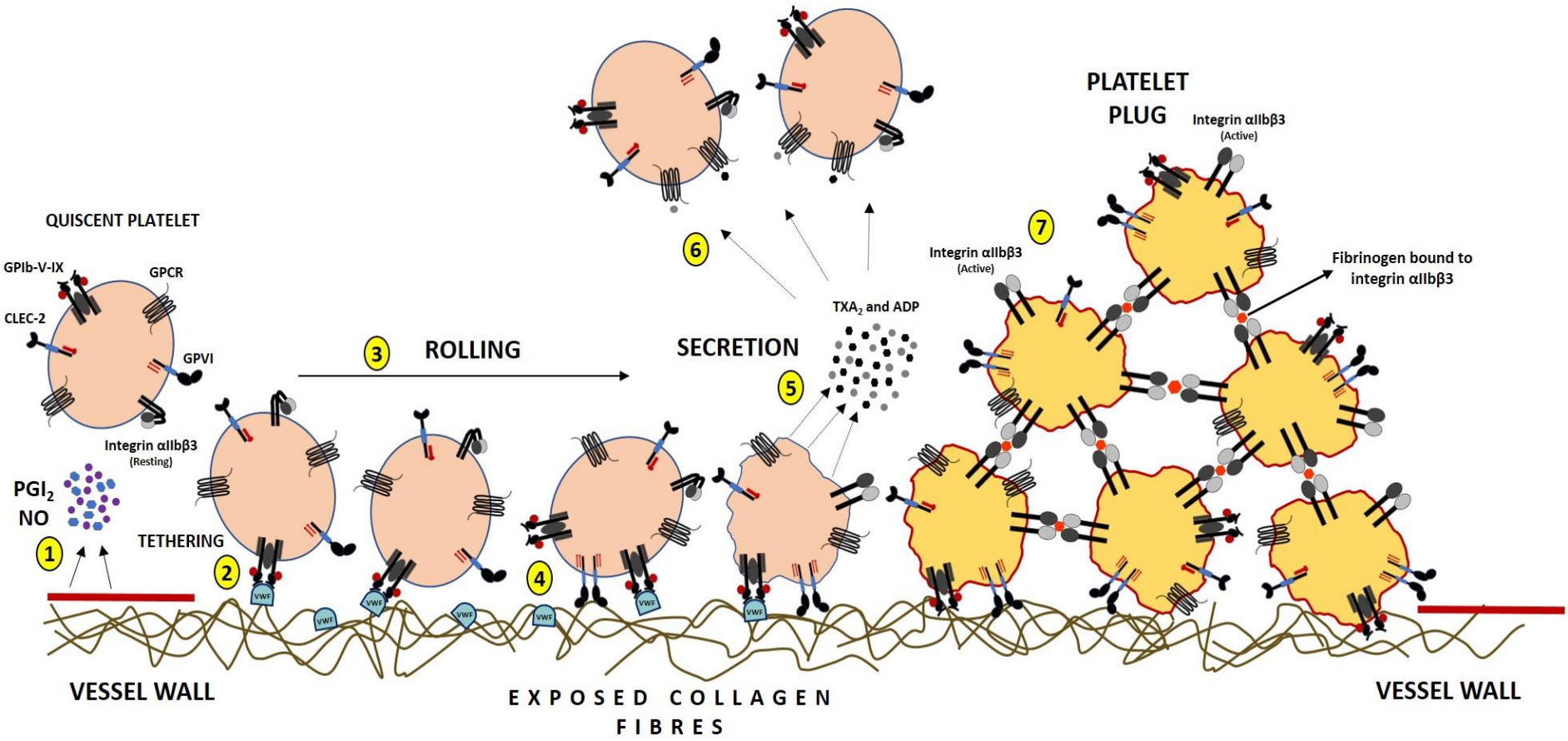


Fig 1.2: Representation of haemostasis in response to vascular injury. (1) Platelets in circulation are maintained in a quiescent state by the action of NO and PGI_2 released from intact endothelial cells. (2) Injury to blood vessel exposes VWF and collagen present in the sub-endothelial matrix. At high shear rates, circulating platelets tether to the VWF through their GPIb-V-IX complex, which slows-down their movement. (3) This tethering facilitates platelet rolling along the sub-endothelium. (4) This allows collagen to bind with platelet surface receptor GPVI, which stimulate a signalling event that causes platelet shape change, affinity upregulation of integrin αIIbβ3 and (5) secretion from α and dense granules. (6) Release of TXA_2, ADP and activate more platelets in the vicinity. (7) Activated integrin αIIbβ3 (with high-affinity state) present on the platelet surface binds to its ligands, vWF and fibrinogen, which results in formation of bridges between platelets, favouring the formation of a platelet plug

1.5. Platelet receptors and signalling

Activation of platelets is a highly dynamic and rapid process. A large number of proteins act in a concerted manner via different pathways to regulate this complex process. Briefly, platelet activation is initiated by the binding of an agonist to its surface receptor on platelets. This provides a stimulus that begins a signal transduction cascade via tyrosine kinase-dependent phosphorylation of numerous proteins acting in a sequence, ultimately allowing platelets to stick together to form aggregates. Platelet receptors can be divided into three broad categories: a) receptors that bind to immobilised matrix proteins such as collagen or vWF and include GPIb-V-IX complex, integrin α2β1 and GPVI. b) G-protein coupled receptors that are activated by soluble mediators found within the plasma or secreted from platelets (thrombin, ADP, TxA_2 or epinephrine). c) Receptors that function to stabilise a thrombus once platelets are in contact with each other, including integrin αIIbβ3 and Eph receptors. Signalling pathways initiated by these receptors will now be discussed in detail.

1.5.1. Collagen mediated platelets signalling

Collagens make up approximately 40% of the total proteins constituting the extracellular matrix of the blood vessel wall. They exist in the form of insoluble scaffolds, that is characterised by the presence of a triple helical structure composed of three separate polypeptide chains (α-chains) (Smethurst et al., 2007). Collagen not only provides mechanical and structural strength to the vessel wall but they also act as a substrate for the adhesion of platelets and their subsequent activation (Farndale et al., 2004; Roberts et al., 2004). There are 25

different kinds of collagens that have been identified, of which, 10 exist in human blood vessel, but only the type I, III, V, and VI (fibrillar collagen) and type IV and VIII (nonfibrillar collagen) are thrombogenic in nature (Barnes and Farndale, 1999; Kauskot and Hoylaerts, 2012).

As discussed previously, collagen-evoked platelet activation begins with the interaction of the platelet GPIb–V–IX receptor complex with vWF bound to exposed collagen (at the site of the ruptured vessel). This slows down the movement of platelets and enables their stable interaction with collagen receptors GPVI and integrin α2β1, leading to their firm adhesion. This is followed with intracellular tyrosine kinase signalling, resulting in platelet calcium mobilisation, granule secretion, integrin αIIbβ3 activation and the formation of platelet aggregate (Gibbins, 2004; Li et al., 2010).

1.5.1.1. GPIb-V-IX complex

The GPIb-V-IX complex is a receptor originating in the megakaryocytes and found exclusively in platelets, with the exception of a weak expression in certain endothelial cells (Li and Emsley, 2013). It is composed of four distinct transmembrane proteins; GPIbα, GPIbβ, GPIX, and GPV, each of which belong to leucine-rich repeat superfamily. Attachment of one GPIbα subunit with two GPIbβ subunits via disulphide bonds forms GPIb, whereas GPIX and GPV are non-covalently associated with GPIb (Nuyttens et al., 2011). All 4 subunits are present in high copy numbers, and deficiency in either one of them can affect the surface expression of the entire complex. For instance, defects in the expression of GPIb causes Bernard-Soulier syndrome (BSS), which is a hereditary bleeding disorder

(Lanza, 2006). While vWF is the primary ligand for GPIb-V-IX, it also binds to coagulation factors (thrombin, factor XI, factor XII), thrombospondin-1 and membrane glycoprotein (P-selectin, Mac-1).

As described earlier, the association between GPIb-V-IX complex and the plasma protein vWF (attached to collagen) acts as the first mode of interaction between platelets and the damaged endothelial matrix. This interaction can occur in the venous system where low shear rates are observed (20-200 s^{-1}), however, it is of much more significance in arteries, where high shear rates (300-800 s^{-1}) exist (Dopheide et al., 2002; Andre et al., 2000). vWF-GPIb-V-IX interaction is characterised by a rapid on-and-off rate, still, it provides sufficient opportunity for the collagen receptors GPVI and α2β1 to elicit stable adhesion and activation of platelets (Pugh et al., 2010).

Although principally associated with the adhesion of platelets, there is accumulating evidence that suggests the role of GPIb-V-IX in stimulating tyrosine kinase signalling in platelets. It is believed that the presence of the GPIb–V–IX complex in the lipid raft fraction of the cell membrane provides an ideal location for transmitting activation signals (Ozaki et al., 2013). Lipid rafts are rich in numerous cytoplasmic signalling proteins that include Src family kinases (SFKs), phosphoinositide 3-kinase (PI3K), the adapter molecule 14-3-3ζ, and the actin cytoskeleton-associated protein filamin, which are now known to be active interactive partners of GPIb-V-IX complex (Mangin et al., 2004; Wu et al., 2003; Andrews and Fox, 1991; Munday et al., 2000). Liu *et al.* (2005) reported the role of SFKs as important mediators of vWF-GPIb-IX-V signalling to facilitate TxA_2 production. In a similar manner, the Bruton tyrosine kinase (Btk) has also been

found to regulate TxA_2 production through its participation in signalling initiated by GPIb-V-IX (Liu et al., 2006). PI3K has also been implicated in signalling via GPIb-V-IX, leading to calcium mobilisation (Yap et al., 2002). These findings were supported by Kasirer-Friede *et al*. (2004), where GPIb-V-IX mediated signalling was reported to activate integrin αIIbβ3, through the actions of SFKs and PI3K. Wu et al. (2001) identified the importance of FcRγ-chain in signal transduction downstream of GPIb-V-IX. They reported that in comparison to wild-type platelets, FcRγ chain-deficient platelets stimulated with vWF plus botrocetin (snake venom protein that enhances the affinity of vWF for the platelet GPIbα) formed smaller platelet aggregates suggesting the potential role of FcRγ chain in the activation of platelets mediated by GPIb.

1.5.1.2. Integrin α2β1

Integrin α2β1 (or GPIa/IIa) was the first collagen receptor to be identified in platelets (Santoro, 1986). The number of α2β1 receptors present on platelet surface varies between 2000-4000 (Clemetson and Clemetson, 2013). Like all integrin receptors, α2β1 is a heterodimeric transmembrane receptor containing single α2 and β1 chains being non-covalently associated with each other. α2 subunits possess an I-domain containing Mg^{2+} ions that act as a site for collagen binding (Saboor et al., 2013). Also, as a characteristic feature of integrins, α2β1 maintains a low-affinity state in resting platelets and undergoes an activation-dependent conformation change in the I-domain to efficiently attach to collagen (Cosemans et al., 2008). Binding of α2β1 with collagen types I, III, IV, V,

VI through interaction with GFOGER sequence has been reported (Surin et al., 2008).

The manner in which integrin α2β1 functions in platelets has been a question of debate. According to the two-step, two-site model; platelets first attach to collagen via integrin α2β1 but become activated only through the signalling generated by a second receptor (GPVI in general) (Santoro et al., 1991). However, the most recent model suggests that the initial contact of platelets with collagen, under high shear stress, is mediated by GPVI (Nieswandt et al., 2001a). This connection, however, does not ensure a firm adhesion and allows platelets to tether. Nonetheless, the interaction of GPVI with collagen generates intracellular signals leading to affinity upregulation of α2β1, which increases its ability to adhere to collagen (Massberg et al., 2003; Nieswandt et al., 2009). The activation of integrin α2β1 was also observed to be mediated independently of GPVI signalling through a range of agonists that includes ADP, TxA_2 and vWF/GPIb-specific stimuli (Jung and Moroi, 2000; Jung and Moroi, 1998; Moroi et al., 2000; Cruz et al., 2005).

The importance of integrin α2β1 in platelets has also been an area of conflict. There are studies that support the role of integrin α2β1 in facilitating firm adhesion on collagen and subsequent activation of platelets (Verkleij et al., 1998)., Atkinson et al. (2003) reported that α2β1 plays a vital role in enhancing the avidity of GPVI receptor towards collagen along with increased platelet activation by GPVI. In another study, integrin-deficient ($\alpha2\beta1^{-/-}$) mice exhibited impaired adhesion to collagen substrates under arterial flow conditions coupled with reduced thrombus formation (He et al., 2003). On the contrary, Gruner *et al*.

(2003), using intravital fluorescence microscopy showed that platelet adhesion and thrombus growth in α2β1 deficient mice was largely unaffected. It has also been shown that β1-null platelets can adhere to collagen under low as well as high shear stress, where the number and size of platelet aggregates were not found to differ significantly between normal and β1-null platelets. Furthermore, no adhesion was observed in the absence of GPVI receptor (Nieswandt et al., 2001a). The basis of these discrepancies are not known.

It is now known that the role of integrin α2β1 is not just confined to adhesion and extends to the stimulation of tyrosine kinase signalling that regulates platelet function. Upon activation by integrin-specific triple helical peptide sequence from collagen (GFOGER), integrin α2β1 (in the absence of the GPVI–FcRγ-chain complex) could stimulate tyrosine phosphorylation of many of the proteins that participate in the GPVI–FcRγ-chain cascade, including Src, Syk, SLP-76, and PLCγ2 as well as plasma membrane calcium ATPase and focal adhesion kinase (FAK). These signalling events were reported to control integrin α2β1 dependent spreading in platelets (Inoue et al., 2003). Inoue and group also presented evidence, which suggests the potential ability of α2β1 to generate activation signals that involve Rac, Cdc42, and PAK (Suzuki-Inoue et al., 2001b). In support of these findings, activation of PLCγ2 was also reported downstream of α2β1 via a Src kinase-dependent pathway and a Rac GTPase pathway (Guidetti et al., 2009).

1.5.1.3. GPVI receptor

GPVI receptor (64 kDa) is a type I transmembrane glycoprotein of the immunoglobulin (Ig) superfamily and is found exclusively in platelets (around 4000 copies per platelet) (Andrews et al., 2014; Clemetson et al., 1999). It is regarded as the major collagen receptor on platelets and consists of two extracellular Ig-like domains, an extracellular mucin-like domain, followed by a transmembrane domain, and a cytoplasmic tail that participates in the transmission of collagen-mediated signals. The transmembrane GPVI domain via its positively charged arginine residue forms a non-covalent (salt bridge) association with the aspartic acid residue of the Fc receptor γ-chain (FcRγ). The FcRγ chain exists in the form of a homodimer and each chain is characterised by copy of an immunoreceptor tyrosine-based activation motif (ITAM) containing two YxxL sequences separated by six to eight amino acids [YxxL-$(X)_{6–8}$-YxxL) (Gibbins et al., 1997; Miura et al., 2002; Rabie et al., 2007). GPVI binds to Gly-Pro-Hyp (GPO) rich sequences of collagen (Jarvis et al., 2008) and other than collagen, GPVI receptor can be activated by the snake toxin, convulxin and synthetic ligand; cross-linked collagen-related peptides (CRP-XL), which contain repeating GPO motifs (Yip et al., 2005).

The proline-rich juxtamembrane tail of the GPVI associates with the active form of SFKs (Fyn and Lyn) via their SH3 domains, keeping them in close proximity of the FcRγ chain. The C-terminal Src kinase (Csk) or its family member CSK-homologous kinase (Chk) phosphorylate the C-terminal inhibitory tyrosine residues on SFKs and inhibit their activity (Okada, 2012). Whereas, the activated state of SFKs is maintained by the receptor-like PTP CD148, which

dephosphorylates their C-terminal inhibitory tyrosine residues. Binding of collagen to GPVI causes clustering of the receptor, resulting in the autophosphorylation of the SFKs activation loop tyrosine residue, leading to their maximal activation. SFKs, thereafter, phosphorylate the tandem tyrosine residues in ITAMs within FcRγ chain (Ezumi et al., 1998; Quek et al., 2000; Rabie et al., 2007; Senis et al., 2014). This is followed by the binding of FcRγ chain with the Src homology 2 (SH2) domain of the spleen tyrosine kinases (Syk), which is subsequently autophosphorylated and also phosphorylated by SFKs. The phosphorylated form of Syk further regulates tyrosine phosphorylation of its downstream targets such as linker for activated T cells (LAT), Src homology 2 domain-containing leukocyte phosphoprotein of 76-kDa (SLP-76), Tec family kinases, the Vav family of guanine nucleotide exchange factors (GEF) and grb2-related adapter protein (Gads), resulting in the formation of a LAT-signalosome (Gibbins et al., 1998; Pasquet et al., 2002; Stegner and Nieswandt, 2011). The formation of this signalosome is critical for the recruitment of phospholipase Cγ2 (PLCγ2) and phosphatidylinositol-3 kinase (PI3K).

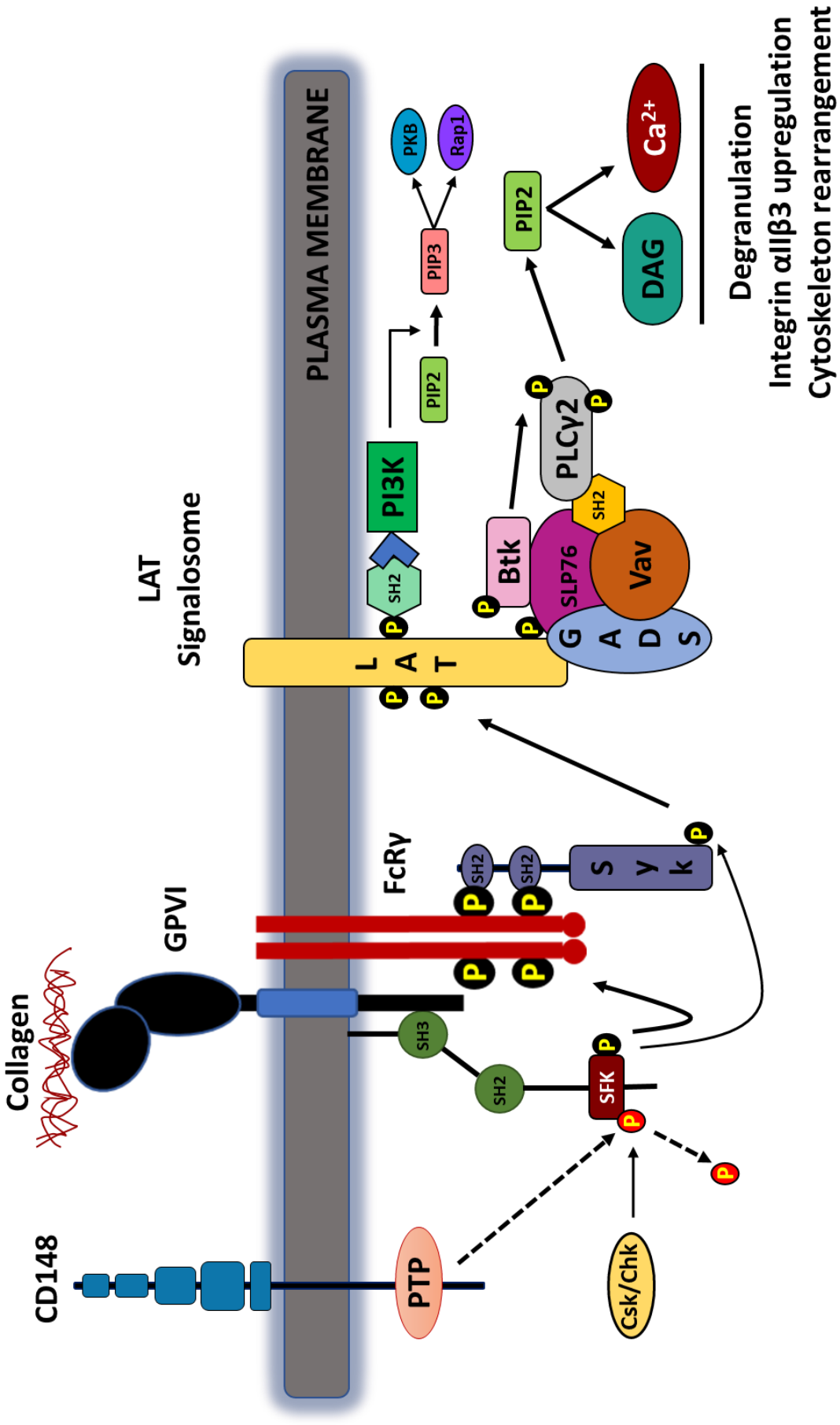
PI3K evokes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3), which then enables PLCγ2 and Bruton's tyrosine kinase (Btk) to colocalise at the plasma membrane (Pasquet et al., 1999a; Pasquet et al., 1999b). Btk thereafter stimulates the phosphorylation and activation of PLCγ2 (Oda et al., 2000) that generates secondary messengers, inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG) through the cleavage of PIP2. Binding of IP3 to its receptor on the dense tubular system promotes mobilisation of calcium from intracellular stores, while

DAG induces the activation of protein kinase C (PKC) (Li et al., 2010; Moraes et al., 2010a; Varga-Szabo et al., 2009). These events finally lead to alpha and dense granule secretion coupled with the transformation of integrin receptors (both α2β1 and αIIbβ3) from a low affinity to high-affinity state, crucial for platelet aggregation (Dunster et al., 2015; Andre, 2012; Jackson et al., 2003) (Figure 1.3).

While GPVI plays a major role in platelet adhesion on collagen and subsequent activation, only a few cases of bleeding disorders in patients suffering from GPVI mutation, deficiency or presence of autoantibodies against GPVI have been reported (Hermans et al., 2009; Dumont et al., 2009; Matsumoto et al., 2007). This might be attributed to the compensatory effects in platelet activation by multiple platelet receptors (Hermans et al., 2009; Dumont et al., 2009; Matsumoto et al., 2007). When perfused over collagen, GPVI knock-out platelets displayed reduced formation of platelet aggregates. However the level of adhesion was found to be normal and no increase in tail bleeding time was observed (Kato et al., 2003). Similarly, administration of an anti-GPVI antibody attenuated collagen-induced platelet aggregation (Nieswandt et al., 2001b). These findings were further supported in a study where deletion of GPVI was related with reduced platelet aggregation and adhesion to collagen, however, no prolongation in bleeding time was observed (Lockyer et al., 2006).

Figure 1.3: The GPVI signalling pathway. The SFKs (Lyn and Fyn) are associated with GPVI via their SH3 domains. The Csk or its family member Chk phosphorylate the C-terminal inhibitory tyrosine residues on SFKs and inhibit their activity. The receptor-like PTP CD148 maintains SFKs in an activated state by dephosphorylating their C-terminal inhibitory tyrosine residues. Collagen-induced clustering of the GPVI receptor results in the autophosphorylation of SFKs, which subsequently phosphorylate tyrosine residues in the ITAM-containing FcRγ-chain. The phosphorylated sites of FcRγ chain provides a docking site for the SH2 domain–containing protein-tyrosine kinase Syk, which is also phosphorylated by the SFKs. Syk further regulates tyrosine phosphorylation of LAT, resulting in the formation of the LAT-signalosome consisting of SLP-76, Tec, Vav and Gads. The signalosome recruits PI3K, responsible for the the conversion of PIP2 into PIP3, which further activated PKB and Rap1. The interaction of Btk and Tec kinases with PIP3 facilitates the phosphorylation and activation of PLCγ2, responsible for the generation of IP3 and DAG. IP3 promotes mobilisation of calcium while DAG activates PKC, which leads to degranulation and affinity upregulation of integrin αIIbβ3, resulting in platelet aggregation.

(Abbreviations- Csk: C-terminal Src kinase, Chk: CSK-homologous kinase, SFKs: Src family kinases, ITAM: Immunoreceptor tyrosine-based activation motif, FcRγ: Fc receptor γ-chain, LAT: Linker for activated T cells, SLP-76: Src homology 2 domain-containing leukocyte phosphoprotein of 76-kDa, PIP2: Phosphatidylinositol-4,5-bisphosphate, PIP3: Phosphatidylinositol-3,4,5-trisphosphate, PKB: Protein kinase B, Btk: Bruton's tyrosine kinase, PLCγ2: Phospholipase Cγ2, DAG: Diacylglycerol, IP3: Inositol (1,4,5)-trisphosphate


Collagen
GPVI
CD148
PTP
FcRγ
SH2
SH2
SH3
SH2
SFK
P
Csk/Chk
LAT
Signalosome
PLASMA MEMBRANE
PI3K
SH2
PIP2
PIP3
PKB
Rap1
Btk
SLP76
Vav
SH2
PLCγ2
PIP2
DAG
Ca2+
Degranulation
Integrin αIIβ3 upregulation
Cytoskeleton rearrangement

1.5.1.4. C-type lectin receptor-2 (CLEC-2)

Originally found in immune cells, CLEC-2 is a transmembrane receptor that has been identified in human and mouse platelets (Suzuki-Inoue et al., 2006). Its endogenous ligands include podoplanin, while it can also be activated potently by an exogenous ligand, rhodocytin, which is a protein isolated from the venom of Malayan pit viper (*Calloselasma rhodostoma*) (Suzuki-Inoue et al., 2006; Suzuki-Inoue et al., 2007).

CLEC-2 shares quite a remarkable structural and functional similarity with the GPVI receptor. The cytosolic tail of CLEC-2 is characterised by a motif, which resembles the ITAM of GPVI. However, unlike the classic ITAM with tandem YxxLs, CLEC-2 features a single YxxL (hemITAM) in its cytoplasmic domain, which undergoes tyrosine phosphorylation by SFKs upon activation (Ozaki et al., 2009). Treatment with SFKs inhibitors or presence of a mutation in the hemITAM can completely block signalling via CLEC-2 (Suzuki-Inoue et al., 2006). Binding of Syk occurs at hemITAM via its SH2 domain, resulting in its phosphorylation and activation. However, unlike the GPVI receptor which requires dually phosphorylated ITAM on FcRγ chain for the recruitment of Syk, phosphorylation of single YxxL is sufficient to facilitate attachment of Syk in CLEC-2 mediated signalling (Hughes et al., 2013; Séverin et al., 2011). This event is followed further by signalling downstream leading to the tyrosine phosphorylation of LAT, and formation of the LAT signalosome, which, similar to GPVI, is composed of an adapter SLP-76, Tec family tyrosine kinases, Vav GTP exchange factors and PLCγ2 (Fuller et al., 2007; Ozaki et al., 2013). While the absence of SLP-76 can completely block the signalling downstream of Syk in case of GPVI receptor, weak activation

of platelets in SLP-76 deficient platelets, using high concentrations of rhodocytin has been observed (Suzuki-Inoue et al., 2006; Fuller et al., 2007). This suggests that signalling by CLEC-2 is only partially dependent on the recruitment of SLP-76 and CLEC-2 might recruit alternative adapter proteins that can replace SLP-76 and thus facilitate signalling.

Platelets from mice treated with a monoclonal antibody that specifically targets and functionally inactivate CLEC-2 showed normal adhesion to collagen under flow but thrombus formation *in vitro* and *in vivo* was substantially impaired, suggesting a fundamental role of CLEC-2 in haemostasis and thrombosis (May et al., 2009). CLEC-2 deficient mice are embryonically lethal. However, generation of an irradiated chimeric mouse lacking CLEC-2 has been reported (Suzuki-Inoue et al., 2010). Platelets from CLEC-2$^{-/-}$ mice didn't aggregate upon stimulation by rhodocytin while retaining a usual aggregation profile to collagen, thrombin or ADP. They were also able to adhere and normally spread on collagen or fibrinogen. Interestingly, thrombus formation *in vitro* and *in vivo* was inhibited in CLEC-2 chimaeras (Suzuki-Inoue et al., 2010). In contrast to these finding, *in vitro* thrombus formation in CLEC-2 chimaeras generated by Hughes *et al.* (2010), was not inhibited and no significant increase in tail bleeding time was observed. The reason for this discrepancy is unclear and was reasoned to be due to the difference of genetic background of the mice used for generating chimaeras.

1.5.2. G-protein coupled receptors (GPCR) mediated signalling

After the formation of a platelet monolayer on the site of injury, the development of a platelet plug requires the involvement of platelets circulating near the injured site. This is achieved by thrombogenic mediators such as thrombin, ADP and TxA_2 that are released by platelets once they adhere to the damaged extracellular matrix. All these mediators commonly activate platelets through autocrine and paracrine signalling via different kinds of GPCRs present on the platelet surface. Thus, it can be said that role of GPCRs is linked with the second phase of platelet aggregation, which aims to provide positive feedback to amplify platelet aggregation by many folds.

GPCRs constitute the largest superfamily of proteins encoded by animal genomes and are activated by a diverse range of ligands. All the GPCRs share a common structure of seven transmembrane helices spanning the plasma membrane with an extracellular N-terminus and intracellular C-terminus. Three loops present on the N-terminus region provide the ligand binding site, while three loops found at the cytoplasmic front presents binding sites for intracellular signalling proteins (Kobilka, 2007; Rosenbaum et al., 2009). GPCRs derive their name from their direct association with heterotrimeric G-proteins, comprising three subunits, referred to as Gα, Gβ and Gγ. The α and γ subunits of G-proteins are connected to the plasma membrane via lipid chains. The Gα subunit is bound to guanine nucleotide GDP in a resting state, while upon activation of the receptor by its ligand, there occurs a replacement of GDP with GTP, resulting in a conformational change in Gα. This further leads to the dissociation of the trimeric complex into Gα and Gβγ (Tuteja, 2009; Oldham and Hamm, 2008). Both the

subunits are capable of instigating signalling pathways independently of each other in platelets. G-proteins are classified based on the sequence and function of their Gα subunits, which are categorised into four families; $G\alpha_q$, $G\alpha_s$, $G\alpha_i$ and $G\alpha_{12/13}$. $G\alpha_q$ family regulates signalling through the activation of Phospholipase Cβ (PLCβ). $G\alpha_s$ is coupled with adenylyl cyclase and activates it, whereas, $G\alpha_i$ function by inhibiting adenylyl cyclase, thereby modulating cyclic adenosine monophosphate (cAMP) levels within the cell. $G\alpha_{12/13}$ involves signalling via activation of RhoGTPase nucleotide exchange factors (RhoGEFs) (Siehler, 2009; Ellis, 2004; Harden et al., 2011; Liebmann and Bohmer, 2000).

1.5.2.1. Thrombin

Thrombin belongs to the family of serine proteases and is a highly potent platelet agonist. Two independent pathways contribute to its production. Firstly, the coagulation pathway (intrinsic and extrinsic), where multiple enzymatic reactions culminate in the generation of Factor Xa, which converts zymogen prothrombin to thrombin (Offermanns, 2006). The second route is referred to as contact-activation pathway that begins with the interaction of injured vessel and Factor XII (Vogler and Siedlecki, 2009). Upon activation of platelets, α-granules release prothrombin from platelet granules, which further enhances the thrombin levels at the site of injury. As mentioned previously, thrombin also plays an important role in the formation of a stable clot by facilitating the conversion of fibrinogen to fibrin monomers that binds the aggregated platelets together (Monroe et al., 2002).

Activation of platelets by thrombin is mediated by protease-activated receptors (PAR), which exist in two isoforms, PAR-1 and PAR-4, both present on the surface of human platelets (mouse platelets display PAR-3 and PAR-4). PAR-1 is reported to have high affinity towards thrombin, whereas, a higher concentration of thrombin is required to activate signalling via PAR-4 (Angiolillo et al., 2010; Li et al., 2010). Both PAR-1 and PAR-4 are coupled to $G\alpha_q$ and $G\alpha_{12/13,}$ and exist in the form of heterodimers, which enhance thrombin mediated platelet activation. Upon exposure to thrombin, the N-terminal domain of the PAR receptor becomes cleaved, which reveals a new sequence at the N-terminus that acts as a tethered ligand and stimulates receptor signalling via $G\alpha_q$ and $G\alpha_{12/13}$ (Coughlin, 2000; Barrett et al., 2008).

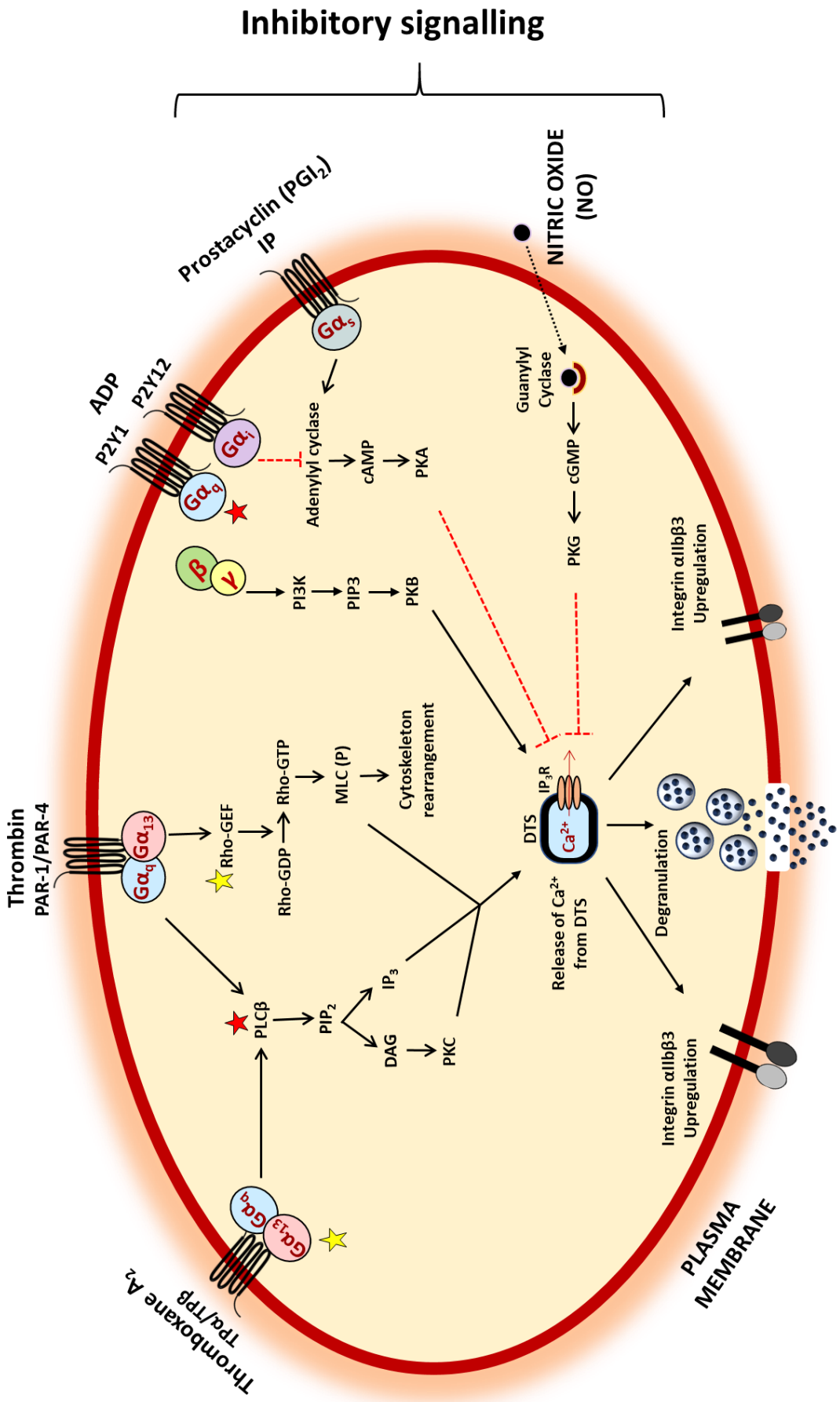
$G\alpha_q$ stimulated events are initiated by the activation of PLCβ, which cleaves PIP2 to generate secondary messengers, DAG and IP3. This triggers granule secretion, increase in cytosolic calcium concentration and ultimately increasing the affinity of αIIbβ3 towards fibrinogen (Stalker et al., 2012; Zhang et al., 2013; Joo, 2012). $G\alpha_{13}$ induced pathway involves RhoGEF mediated activation of Rho kinase, which phosphorylates myosin light chain (MLC), resulting in degranulation and cytoskeleton rearrangement (shape change) of platelets (Rivera et al., 2009; Klages et al., 1999; Moers et al., 2003). PAR-1 has also been reported to function via G_i-coupled signalling, which reduces cAMP levels. The cAMP has an inhibitory effect on platelet function, and its suppression promotes platelet activation (Figure 1.4).

Sambrano *et al.* (2001), reported the significance of PAR-4 in thrombin-mediated platelet activation by generating PAR-4 knock-out mice. PAR-$4^{-/-}$

platelets did not aggregate to thrombin and displayed markedly prolonged bleeding times. Similar results were seen in another independent study on PAR-4 deficient mice (Hamilton et al., 2004). This study identifies that mouse PAR-3 is incapable of stimulating transmembrane signalling by itself and it only acts as a cofactor for thrombin cleavage and activation of mouse PAR4.

Figure 1.4: G-protein–coupled receptor-mediated signalling pathways in platelets. Both inhibitory and activatory responses can be stimulated by GPCRs present on platelets. Inhibitory signalling is induced in platelets by binding of PGI_2 to IP receptors coupled with $G\alpha_s$. This stimulates adenylyl cyclase to generate cAMP, which further activates PKA. This suppresses calcium mobilisation, Rap1b activation, platelet adhesion and spreading, which ultimately down-regulates platelet activation. Besides PGI_2, NO inhibits platelet activation by binding to guanylyl cyclase. This causes the formation of cGMP and subsequent activation of PKG. Several soluble agonists are released from platelets during activation, which stimulates activatory signalling in platelets via GPCRs. Thromboxane A_2 receptors (TP), thrombin receptors (PAR1/4) and ADP receptor (P_2Y_1) are coupled to $G\alpha_q$ that signals via PLCβ leading to the cleavage of PIP2 to generate DAG and IP3. This triggers granule secretion, increase in cytosolic calcium concentration and ultimately increase in the affinity of integrin αIIbβ3 towards fibrinogen. TP and PAR1/4 also signal via $G\alpha_{13}$. This includes Rho kinase-mediated phosphorylation of MLC, resulting in degranulation and cytoskeleton rearrangement (shape change) of platelets. ADP receptors P_2Y_{12} are coupled with $G\alpha_{i,}$ which reduces adenylyl cyclase evoked synthesis of cAMP. This attenuates PKA activation and thus promotes platelet activation. Gβ/γ subunits also participate in signalling via the activation of PI3K, that subsequently activates PKB and Rap1b involved in degranulation and integrin αIIbβ3 activation.

(Abbreviations- PGI_2: prostacyclin, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, NO: nitric oxide, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G, MLC: myosin light chain, PKA: protein kinase A, PKB: protein kinase B, PIP2: phosphatidylinositol-4,5-bisphosphate, DAG: diacylglycerol, IP3: inositol (1,4,5)-trisphosphate, PI3K: phosphoinositide 3-kinase)


Inhibitory signalling
Prostacyclin (PGI_2)
IP
NITRIC OXIDE
(NO)
ADP
P2Y12
P2Y1
$G\alpha_s$
$G\alpha_i$
$G\alpha_q$
Adenylyl cyclase
cAMP
PKA
Guanylyl
Cyclase
cGMP
PKG
β
γ
PI3K
PIP3
PKB
Integrin αIIbβ3
Upregulation
Thrombin
PAR-1/PAR-4
$G\alpha_q$
$G\alpha_{13}$
Rho-GEF
Rho-GTP
Rho-GDP
MLC (P)
Cytoskeleton
rearrangement
DTS
IP_3R
Ca^{2+}
Release of Ca^{2+}
from DTS
Degranulation
PLCβ
PIP_2
IP_3
DAG
PKC
Integrin αIIbβ3
Upregulation
Thromboxane A_2
TPα/TPβ
$G\alpha_q$
$G\alpha_{13}$
PLASMA
MEMBRANE

1.5.2.2. Adenosine diphosphate (ADP)

Adenosine diphosphate (ADP) is another prothrombotic secondary mediator released from dense granules upon platelet activation. It acts in an autocrine and paracrine fashion to amplify the platelet activation process. Although ADP was one of the earliest known platelet agonists, it is only recently that its receptors have been defined clearly (Whiteheart, 2011; Born, 1962; Hollopeter et al., 2001). ADP can act through two different purinergic receptors, P2Y1 and P2Y12. While P2Y1 is coupled with $G\alpha_q$, P2Y12 is known to act through $G\alpha_i$ (Murugappa and Kunapuli, 2006; Gachet, 2006).

Approximately 150 P2Y1 binding sites are expressed on the platelet surface, which is quite low in comparison with other adhesion and GPCR receptors present on platelets. P2Y1 being coupled with $G\alpha_q$ instigates signalling through the activation of PLCβ as described previously for PAR receptors. This results in the mobilisation of calcium from internal stores, platelet shape change and a transient rapid, reversible aggregation evoked by ADP (Savi et al., 1998; Daniel et al., 1998; Hechler et al., 1998b). Two selectively potent P2Y1 antagonists, MRS2179 and MRS2500 have been developed, which have been helpful in identifying the functions of this receptor (Leon et al., 2001; Hechler et al., 2006; Cattaneo et al., 2004). Mice lacking P2Y1 displayed reduced platelet aggregation to ADP along with an increase in bleeding time and resistance to thromboembolism (Fabre et al., 1999). In general, P2Y1 elicits a weak response to ADP in comparison to P2Y12 (Gachet, 2006). Nonetheless, it plays a vital role in haemostasis and thrombosis.

On the other hand, P2Y12 is coupled to $G\alpha_i$, which acts by blocking the production of cAMP from the enzyme, adenylyl cyclase. The cAMP is responsible

for activating protein kinase A (PKA), which negatively regulate calcium release and degranulation, causing platelet inactivation (Hardy et al., 2005; Woulfe et al., 2001). P2Y12 also signals via $G_{\beta\gamma}$ subunit, which increases PI3K activity and subsequently regulates PIP3 production. PI3K has two important targets; Akt or protein kinase B (PKB), which is a serine/threonine kinase, and GTPase Rap1B that contribute to the activation of integrin αIIbβ3 and granule secretion (Kim et al., 2004; Dorsam and Kunapuli, 2004; Lova et al., 2002) (Figure 1.4). P2Y12 knock-out mouse platelets show impaired aggregation when stimulated by ADP (Foster et al., 2001). Treatment with a P2Y12 antagonist has also been reported to associate with a reduction in the formation of emboli, without affecting the stability of the initial thrombus (van Gestel et al., 2003).

Coactivation of P2Y1 and P2Y12 receptors is required to attain a normal sustained platelet aggregation, especially in response to stimulation by collagen. Selective inhibition of either of the ADP receptor by their antagonists have been shown to reduce platelet aggregation (Hechler et al., 1998a; Gachet, 2006). The importance of ADP receptors can be assessed from their potential involvement in the development of therapeutic targets (especially P2Y12) for the treatment of thrombosis. Clopidogrel, cangrelor, prasugrel and ticagrelor possess antithrombotic properties through irreversible inhibition of P2Y12 (Wijeyeratne and Heptinstall, 2011; Hollopeter et al., 2001).

1.5.2.3. Thromboxane A_2 (TXA_2)

Thromboxane A_2 (TXA_2), an eicosanoid, is another secondary mediator, which is synthesized by platelets upon their activation. Exposure to platelet agonists (collagen, ADP or thrombin) results in calcium and Protein kinase C (PKC) mediated activation of phospholipase A2 (PLA2) (Börsch-Haubold et al., 1995). Activated form of PLA2 converts membrane phospholipids to arachidonic acid, which is then metabolised by the enzymes COX-1 and thromboxane synthase to generate TXA_2 (Santilli et al., 2011; Paul et al., 1999). Being lipid soluble, it diffuses from platelets after its synthesis.

There are two TXA_2 receptors present on platelet membrane namely, TPα and TPβ, with TPα being the more active member. Both the receptors are coupled to $G\alpha_q$ and $G\alpha_{12/13}$, which functions in a manner as described previously for thrombin and ADP receptors leading to platelet shape change, degranulation and upregulation of αIIbβ3 (Offermanns, 2006; Djellas et al., 1999; Huang et al., 2004) (Figure 1.4). TP receptor-deficient mice were found to have prolonged bleeding time and lack of responsiveness to U46619 (a TXA_2 mimetic peptide). Collagen stimulated platelet aggregation was also delayed indicating the importance of TP receptors in platelet activation stimulated by a range of agonists (Thomas et al., 1998).

Aspirin inhibits platelet function by irreversibly blocking the affinity of COX enzyme towards its substrate arachidonic acid which inhibits the production of TXA_2. Aspirin has been regarded as the "gold standard" antiplatelet agent for prevention of arterial thrombosis (Awtry and Loscalzo, 2000; Schror, 1997).

1.5.2.4. Adenosine triphosphate (ATP)

Platelet dense granules also secrete ATP upon activation, which acts as an agonist for platelet P2X1 receptors. These receptors are essentially non-selective cation channels responsible for the ATP-induced movement of calcium (Mahaut-Smith et al., 2004). These receptors are prone to desensitisation during the process of platelet-suspension preparation for studying platelet functions (Rolf et al., 2001). Treatment of platelets with selective P2X1 agonist, α,β-methylene-ATP results in a rapid influx of calcium ions coupled with platelet shape change (Rolf et al., 2001). These receptors cannot stimulate aggregation independently, however, they have been shown to regulate platelet aggregation stimulated by collagen or U46619 through reversible ERK2 phosphorylation mediated via a Ca^{+2} and PKC-dependent pathway. The P2X1-PKC-ERK2 pathway promote dense granule release, and thus facilitate platelet aggregation on mild stimulation with collagen. (Oury et al., 2001; Erhardt et al., 2003; Toth-Zsamboki et al., 2003). In addition to this, the P2X1-stimulated role of ATP-gated calcium influx in early collagen instigated calcium signalling has also been demonstrated (Fung et al., 2005).

Studies conducted in $P2X1^{-/-}$ mice reported reduced collagen-induced platelet aggregation and secretion *in vitro*. Thrombus formation on the collagen-coated surface was also inhibited. Similar results were observed *in vivo*, where thrombus formation in small arteries characterised by high shear was attenuated, as was thromboembolism, while bleeding time was mildly prolonged (Hechler et al., 2003).

1.5.3. Calcium-mediated signalling

Elevation in cytosolic calcium levels is a common feature that is shared by activation pathways downstream of all platelet agonists (collagen, thrombin, ADP or TXA2) functioning through different receptors (Varga-Szabo et al., 2009). Calcium signalling plays an essential role in platelet activation process and controls cytoskeletal rearrangement, degranulation and increase in the affinity of integrin αIIbβ3 (Harper and Sage, 2017; Rink and Sage, 1990). In a resting state, platelets maintain a cytosolic calcium concentration of around 50-100 nM, which during activation phase can reach in the micromolar range (Harper and Sage, 2017). The rise in cytosolic calcium concentration is governed by two sources: (i) Agonist-induced release of calcium from the DTS, which is stimulated by IP3 binding to IP3 receptors (Ca^{+2} permeable ion channels) on the DTS. (ii) The influx of extracellular calcium across the plasma membrane through calcium channels (CRAC, Orai1, etc.) (Bergmeier and Stefanini, 2009).

Depletion of calcium from DTS facilitates a rapid influx of calcium across the plasma membrane via stromal interaction molecule 1 (STIM1) and Orai1; a phenomenon referred to as store-operated calcium entry (SOCE). STIM1 is a calcium sensor located on the membrane of DTS in a bound state with calcium through its EF-hand domain (Grosse et al., 2007). IP3 operated release of calcium from DTS results in a dissociation of calcium-EF domain complex. This directs the translocation of STIM1 to the plasma membrane where it interacts with Orai1, allowing the entry of calcium via this channel (Zhang et al., 2005; Lang et al., 2013). Furthermore, synthesis of DAG by thrombin stimulation activates transient receptor potential channel 6 (TRPC6), which is a non-selective cation channel and

promotes entry of calcium inside platelets in a SOCE independent manner (Hassock et al., 2002; Authi, 2007). Apart from these, purinergic P2X1 receptors are activated by ATP binding, which then participates in calcium influx by acting as non-selective cation channels (Oury et al., 2001).

1.5.4. Inside-out signalling

Integrin αIIbβ3 is the most abundant receptor expressed on platelet membrane with approximately 40,000–80,000 copies per platelet. Besides this, an additional pool of these receptors migrates from α-granules to surface upon platelet activation, which increases surface expression by approximately 25-50% (Quinn et al., 2003; Kauskot and Hoylaerts, 2012). Transformation of integrin αIIbβ3 from a low affinity to high-affinity state and its subsequent binding to fibrinogen is the common outcome arbitrated by all the platelet activation pathways (Plow et al., 2000). The underlying signalling that controls this event is referred to as the inside-out signalling. Defects or deficiency in expression of αIIbβ3 leads to Glanzmann thrombasthenia syndrome, characterised by significantly prolonged bleeding time (Nurden, 2006).

Inside-out signalling requires binding of cytosolic proteins, talin and kindlins, to the cytoplasmic domains of αIIbβ3, which disrupts the stable bond (salt-bridge) between αIIb and β3 subunits, leading to the activation of the receptor (Moser et al., 2009). These events begin with platelet agonist-induced rise in cytosolic calcium concentration and production of DAG. Both of which binds to Ca^{2+} and DAG-regulated guanine nucleotide exchange factor-I (CalDAG-GEFI),

which converts GDP-bound inactive form of Rap1 to its GTP-bound active form (Stefanini et al., 2009; Joo, 2012; Crittenden et al., 2004). This complex further interacts with Rap1-GTP–interacting adaptor molecule (RIAM), that recruits talin and facilitates its binding with integrin β3 cytoplasmic tail, resulting in affinity upregulation of αIIbβ3 (Li et al., 2010; Han et al., 2006; Banno and Ginsberg, 2008). However, recently it was proposed that the role of RIAM is dispensable for integrin αIIbβ3 activation in mouse platelets and there exists an alternative mechanism for the recruitment of talin-1 leading to integrin activation (Stritt et al., 2015).

The importance of talin in integrin αIIbβ3 was established by analysing platelets from conditional talin-1-deficient mice. These platelets did not induce αIIbβ3 activation in response to both GPVI and GPCR platelet agonists. Moreover, they did not adhere to any of the αIIbβ3 ligands and failed to aggregate (Petrich et al., 2007a; Nieswandt et al., 2007; Petrich et al., 2007b). In a similar manner, binding of kindlin-3 to integrin β3 cytoplasmic tail at a region distinct from that of talin is also crucial for integrin αIIbβ3 activation, which was confirmed using platelets devoid of kindlin-3 (Moser et al., 2008; Nieswandt et al., 2009).

An alternative pathway that operates independently of CalDAG-GEFI and involves PKC induced activation of Rap1 and αIIbβ3 has also been proposed (Cifuni et al., 2008).

1.5.5. Outside-in signalling

Binding of fibrinogen to activated integrin αIIbβ3 is not solely responsible for platelet aggregation and thrombus formation. It also results in αIIbβ3 clustering leading to the stimulation of intracellular signalling events known as 'outside-in' signalling. These signals are important for the stabilisation of aggregates and subsequent thrombus formation by facilitating platelet shape change, spreading and clot retraction (Li et al., 2010; Plow and Ma, 2007).

Binding of ligand to integrin αIIbβ3 induces receptor oligomerisation which initiates outside-in signalling through the coupling of Gα13 with the cytoplasmic β3 domain (Ma et al., 2007b). This activates SFKs, which can generate outside-in signals through 2 mechanisms (Gong et al., 2010; Li et al., 2010). Firstly, SFKs phosphorylate Y747 and Y759 motifs in the cytoplasmic domain of β3. Phosphorylation of Y747 suppresses talin binding to cytoplasmic domain, whereas, phosphorylation of Y759 defends β3 from calpain cleavage. This initiates platelet spreading (Anthis et al., 2009; Xi et al., 2006). SFKs also phosphorylate and activate focal adhesion kinases (FAK) and FAK-binding proteins (Harburger and Calderwood, 2009). Secondly, c-Src binds to a specific site on the β3 domain and phosphorylates p190 Rho GTPase-activating protein. This phosphorylated Rho GTPase inactivates RhoA. This is again important for platelet spreading. After the development of a stable thrombus, calpain cleaves the previously formed c-Src-β3 complex and thus relieves RhoA from its inhibitory influence. This activation of RhoA favours clot retraction (Arthur et al., 2000; Flevaris et al., 2007). Binding of fibrinogen to integrin αIIbβ3 also involves SFKs dependent activation of Syk, which then interacts with β3 cytoplasmic subunit. This leads to

the assembly of a signalosome made up of LAT, Slp-76, Btk and Vav, which activates PLCγ2 and favours platelet activation, in a manner which is similar to GPVI mediated signalling (Boylan et al., 2008; Abtahian et al., 2006; Woodside et al., 2001).

1.6. Negative regulation of platelet activation

Platelets play a crucial role in ensuring a normal haemostatic response. However, impairment in this process can cause inappropriate activation of platelets that can lead to thrombosis. Physiologically, there is a balance, which is maintained by ensuring a quiescent profile of platelets (in the absence of injury) with the aid from inhibitory agents such as prostacyclin (PGI_2) and nitric oxide (NO) that are released from the endothelial lining of the blood vessels (Mitchell et al., 2008). Both these molecules can generate robust inhibitory responses and also act to restrain excessive activation of platelets during an injury (Raslan and Naseem, 2014). Apart from these, several other inhibitory receptors have been identified in platelets such as platelet endothelial cell adhesion molecule-1 (PECAM-1), carcinoembryonic antigen cell adhesion molecule (CEACAM 1 and CEACAM 2), junctional adhesion molecule-A (JAM-A), G6b-B and nuclear receptors, which would now be discussed (Li et al., 2017).

1.6.1. Prostacyclin (PGI_2)

Prostacyclin is an eicosanoid, which is produced in endothelial cells through the metabolism of arachidonic acid and then released into the circulation. PGI_2 binds to IP receptors on platelets which are coupled to $G\alpha_s$ (Woulfe, 2005; Smolenski, 2012). This interaction stimulates adenylyl cyclase (AC) to generate cAMP by catalysing ATP. Phosphodiesterases (PDEs) enzymes limits the levels of cAMP by facilitating its hydrolysis to 5'AMP and thus regulate cyclic nucleotide signalling in platelets (Gresele et al., 2011). cAMP can subsequently activate protein kinase A (PKA), which has numerous targets. Most importantly, it

suppresses the IP3 receptors that are responsible for the release of calcium from the DTS (Quinton and Dean, 1992; Yan et al., 2009). It also inhibits platelet adhesion and spreading by phosphorylating GPIbβ subunit of GPIb-IX-V receptor and Gα13 (Bodnar et al., 2002; Manganello et al., 2003; Bye et al., 2016). PKA can inhibit the activation of Rap1b (important for integrin αIIbβ3 activation) by controlling its phosphorylation directly or in a CalDAG-GEFI-dependent manner (Subramanian et al., 2013; Altschuler and Lapetina, 1993) (Figure 1.4). Furthermore, vasodilator-stimulated phosphoprotein (VASP), a substrate for PKA is involved in platelet cytoskeleton remodelling through actin polymerisation. Phosphorylation of VASP by cAMP/PKA reduces its affinity for actin, which down-regulates platelet shape change (Harbeck et al., 2000; Jensen et al., 2004).

1.6.2. Nitric oxide (NO)

Nitric Oxide is synthesised from an amino acid, L-arginine, through the action of the enzyme endothelial nitric oxide synthase (eNOS) and released into the circulation (Tousoulis et al., 2012). NO, unlike other regulators lacks a well-defined platelet receptor. Instead, being a gaseous molecule, it rapidly diffuses across the plasma membrane and binds to its target, soluble guanylyl cyclase (sGC). This results in the formation of cGMP from GTP, which subsequently activate protein kinase G (PKG) (Du, 2007; Low and Bruckdorfer, 2004; Siess, 2004).

cGMP activity can negatively regulate the activity of phosphodiesterase-3, which is responsible for degradation of cAMP (Gkaliagkousi et al., 2007). It can also downregulate the activity of PI3K, which can influence αIIbβ3 upregulation

(Pigazzi et al., 1999). In a manner, which is similar to PKA, PKG has the ability to influence platelet activation by controlling IP3 receptor-mediated release of calcium from intracellular stores (Schlossmann et al., 2000) (Figure 1.4). PKG also phosphorylates the TxA_2 receptor, thereby inhibiting its function (Wang et al., 1998). VASP phosphorylation is also influenced by cGMP/PKG activity (Low and Bruckdorfer, 2004).

In general, inhibitory effects of cyclic nucleotide signalling are not restricted to any specific platelet activation pathway. They have the ability to down-regulate activation induced by all the platelet agonists functioning via GPCRs, GPVI, CLEC-2 or αIIbβ3.

1.6.3. Platelet endothelial cell adhesion molecule (PECAM-1)

PECAM-1 is a transmembrane glycoprotein and a member of Immunoglobulin (Ig) gene superfamily. PECAM-1 is estimated of having a copy number of 5000-8000 on the cell surface, which increases upon platelet activation following its release from α-granules (Jones et al., 2012). Apart from platelets, expression of PECAM-1 is seen in other haematopoietic cells (monocytes, neutrophils, T lymphocyte subsets) and endothelial cells (Woodfin et al., 2007). The endogenous ligand for PECAM-1 is PECAM-1 itself, which arises via homophilic interactions on adjacent cells, while, heterophilic interactions with non-PECAM-1 ligands (such as heparin-dependent proteoglycans and the integrin αvβ3) have been identified as well (Jackson, 2003). The principal structure of PECAM-1 includes two immunoreceptor tyrosine inhibitory-based motifs (ITIMs) in the cytoplasmic domain (L/I/V/S-x-Y-x-x-L/V). Activation of PECAM-1 induces

SFKs mediated phosphorylation of the 2 ITIM tyrosine residues, which recruits and induces binding of protein tyrosine phosphatases SHP1/SHP2 and SHIP1/SHIP2 that negatively regulate platelet activation mediated by collagen (Hua et al., 1998; Cicmil et al., 2002; Bruhns et al., 2000). Binding of these substrates leads to inactivation of tyrosine kinases such as Syk, LAT and PLCγ2. Inhibition in the activity of downstream components such as PI3K and Akt has also been reported, which further inhibits collagen or CRP-XL mediated platelet activation (Moraes et al., 2010a; Jones et al., 2001; Cicmil et al., 2002). In addition to inhibition of GPVI signalling, PECAM-1 activation has also been associated with the negative regulation of ADP and thrombin-stimulated effects (Jones et al., 2009). Falati *et al.* (2006) demonstrated that thrombi formed in PECAM-1$^{-/-}$ mice were larger and formed more rapidly than in control mice. They concluded that PECAM-1 exhibit an inhibitory role on circulating platelets in normal mice.

1.6.4. Carcinoembryonic antigen-related cell adhesion molecule (CEACAM)

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) is an ITIM bearing receptor expressed in platelets. CEACAM-1 can negatively regulate platelet-collagen interactions, thrombus growth *in vitro* and *in vivo*, and susceptibility to pulmonary thromboembolism (Wong et al., 2009). CEACAM-1 deficient mice were reported to exhibit prolonged bleeding time; moderate integrin αIIbβ3 mediated functional defects with reduced spreading on fibrinogen and fibrin clot retraction (Yip et al., 2016). The presence of CEACAM-2 in platelets has also been reported. Platelet from CEACAM-2$^{-/-}$ mice exhibited increased aggregation upon stimulation by CRP-XL, collagen or rhodocytin. Thrombi formed

in CEACAM-2 deficient mice were larger and more stable than wild-type controls. CEACAMs can therefore be regarded as negative regulators of GPVI and CLEC-2 induced platelet activation pathways (Alshahrani et al., 2014).

1.6.5. G6b-B

G6b-B is an isoform of G6B, which is another ITIM containing receptor expressed in platelets. The endogenous ligand of G6B is yet to be identified, although this receptor exists in a phosphorylated state in resting platelets, which undergoes an increase in tyrosine phosphorylation upon stimulation by collagen or CRP-XL (Senis et al., 2007). In a manner, which is similar to PECAM-1 and CEACAM, the tyrosine residues in ITIM motif of G6B binds to SHP1/SHP2 tyrosine phosphatases (de Vet et al., 2001; Mori et al., 2008). Newland *et al.* (2007) proposed that cross-linking of G6B with polyclonal antisera significantly inhibited platelet aggregation by agonists such as ADP and CRP-XL. It was later identified that G6b-B could interact with key signalling molecules involved in GPVI pathway including Csk, Src, Fyn, Syk, PLCγ2 and PI3K and thus inhibit platelet activation (Coxon et al., 2012). G6B-b deficient mice were macro-thrombocytopenic (low platelet counts with enlarged size) in nature and exhibited a bleeding diathesis, which suggested the crucial role of G6B-b in regulating megakaryocyte function and platelet production (Mazharian et al., 2012).

1.7. Nuclear receptors

Nuclear receptors (NRs) represent a large superfamily of intracellular transcription factors present in the cytoplasm or nucleus of eukaryotic cells. The human genome includes 48 such NR family members that include receptors for steroids, thyroid hormone and vitamins (Maglich et al., 2001). They regulate and control vital eukaryotic biological processes (Table 1) such as cell proliferation, differentiation, metabolism and homeostasis (Bain et al., 2007; Kiss et al., 2013). Any deviation from their normal function can lead to pathological manifestations such as cancer, diabetes, arthritis, obesity etc. (Khan and Lingrel, 2010).

Almost all the NRs share common structural features. They are long chain polypeptides, comprising three major domains: (i) variable N-terminal domain with a transcriptional activation function (AF-1), which interact with cofactors. (ii) Highly conserved DNA-binding domain (DBD) that directs the receptor to a highly specific DNA sequence called hormone response element (HRE) and enables its specific binding to the target gene. (iii) Well-conserved C-terminal ligand binding domain (LBD), which provides a binding site for the ligand and also mediates receptor dimerisation. The LBD also comprises of activation function-2 (AF-2) that regulate the ability of this site to activate transcription (Figure 1.5) (Huang et al., 2010; Jin and Li, 2010).

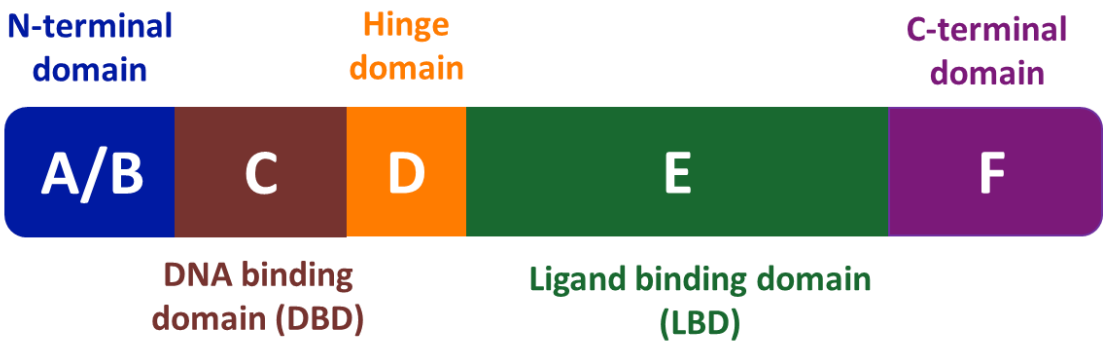


Figure 1.5: Structure of the nuclear receptors. Generally, all the NRs share common structural features that include up to six domains (A-F): the A/B domain represents the N-terminal domain and is regarded as the transcriptional activation domain. Its sequence and length vary between different NRs. The C-domain is a DNA binding domain (DBD), which binds to specific sequences of DNA called hormone response elements. The D-domain serves as a hinge region and links the DBD to the E-domain, which is the ligand binding domain (LBD). This site with a moderately conserved sequence and structure provide a binding site to ligands and also contribute to interactions between NRs that form heterodimers. The F domain is another highly variable region in NRs

NRs function by modulating transcription rate and subsequent gene expression (Aranda and Pascual, 2001; Ribeiro et al., 1995). Based on their mechanism of action, NRs are broadly categorized into two classes: Type I and Type II. Type I receptors are also referred to as steroid receptors and include the estrogen receptor, androgen receptor, progesterone receptor and glucocorticoid receptor. They are localised inside the cytosol in a bound state with the heat shock proteins (HSPs), generally HSP90. Upon ligand binding, the receptors dissociate from the HSPs, form a homodimer and enter the nucleus. Once inside, these homodimers bind to the DNA at the hormone response element (Leo and Chen, 2000; Leo et al., 2000). This is followed by recruitment of transcriptional co-activators such as the p160 family (Xu and Li, 2003), which can further promote or repress transcription (Figure 1.6).

Type II receptors are non-steroid binding NRs consisting of the retinoid X receptor (RXR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor (PPAR). They are situated inside the nucleus in a bound state with several co-repressor proteins and generally heterodimerise with the RXR. Binding of the ligand causes displacement of the co-repressor protein and employment of co-activators, which further activate or repress transcription (Figure 1.7) (Aranda and Pascual, 2001; Bain et al., 2007; Eckey et al., 2003; Ribeiro et al., 1995). An additional class of NRs exists, which is referred to as the orphan receptors. It includes receptors for which endogenous ligand have yet not been identified. (Shi, 2007).

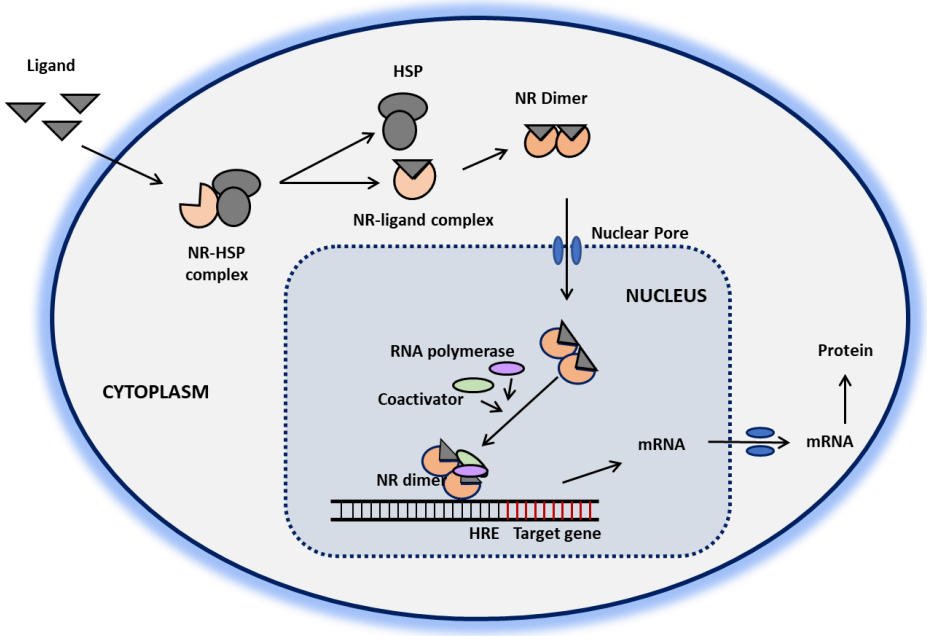


Fig 1.6: General mechanism of action for Type I nuclear receptors in nucleated cells. Type I NRs are localised inside the cytosol in a bound state with the heat shock proteins (generally HSP90). Upon activation by ligand, they dissociate from HSP, homodimerise and enter the nucleus. Once inside, these homodimers bind to specific DNA sequences (hormone response elements). This is followed by recruitment of transcriptional co-activators, which can further, promote or repress transcription in a ligand-dependent manner and thus alter gene expression.

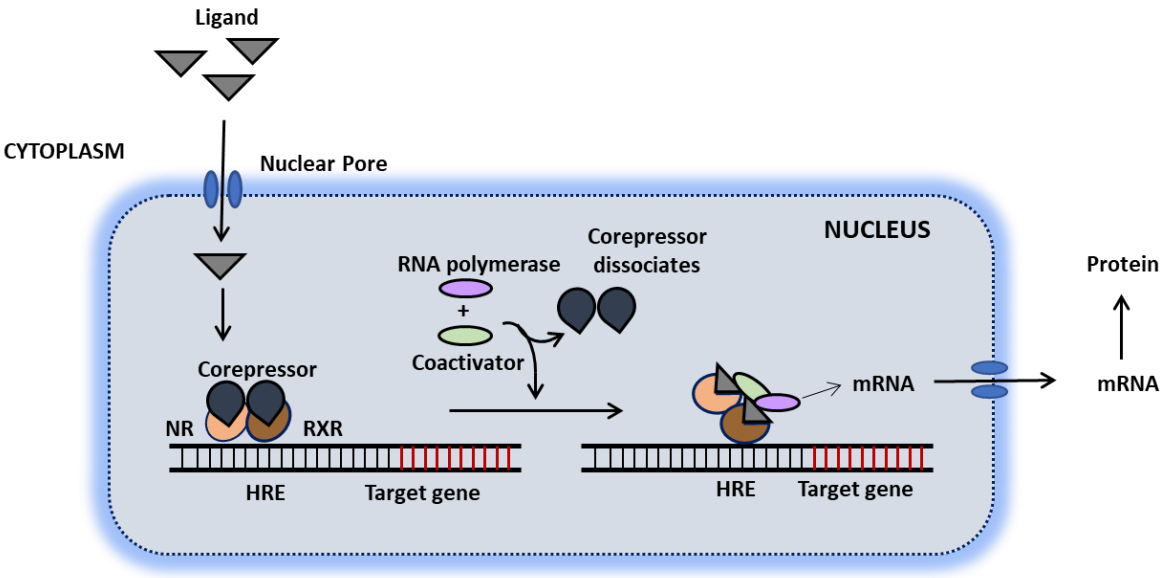


Fig 1.7: General mechanism of action for Type II nuclear receptors in nucleated cells. Type II NRs are situated inside the nucleus in a bound state with several co-repressor proteins. They generally heterodimerise with the RXR. Binding of the ligand causes displacement of the co-repressor protein and employment of co-activators, which further activate or repress transcription and thereby influence the gene expression.

1.7.1. Ligands of nuclear receptors

Ligands of NRs are lipophilic in nature, which enables them to diffuse through the plasma membrane with ease and bind to their respective receptors inside the cytosol or nucleus (Table 1). These molecules, generally are derivatives of retinoids, fatty acids, cholesterol, lipophilic hormones and vitamins, as well as antibiotics, xenobiotics and synthetic drugs (Sladek, 2011). Almost all the NRs with identified endogenous ligands are important targets for the development of drugs for the treatment of a large pool of diseases including cancer, diabetes and atherosclerosis (Burris et al., 2013).

1.7.2. Non-genomic actions of nuclear receptors

While the role of NRs towards the regulation of transcription and control of gene expression (genomic) is well known, non-genomic actions of NRs that function independently of transcriptional regulation have been uncovered recently. Unlike the genomic regulation, which can occur in a timeframe of few minutes to hours, non-genomic events occur in the time scale that ranges from seconds to a few minutes, which is considered too rapid to be attributed to the biosynthesis of mRNA or protein and is often unaffected by inhibitors of transcription or translation.

Commonly reported non-genomic actions of NRs include activation of ion channels, adenylyl cyclase, kinases, phosphatases, production of secondary messengers and rise in intracellular calcium concentrations (Nadal et al., 2001; Losel and Wehling, 2003; Losel et al., 2003; Hammes and Levin, 2007). Due to the

underlying features that differentiate the genomic and non-genomic functions, non-genomic effects are more commonly observed in cell types that lack a functional nucleus such as erythrocytes and platelets (Losel and Wehling, 2003).

Table 1.1. Nuclear receptors and their biological functions (Flora et al., 2018)

Nuclear receptor	Ligands	Biological function
GR	**Natural:** Glucocorticoid **Synthetic:** RU38486, A348441	Lipolysis, glucose metabolism
ER	**Natural:** Estrogen, including estrone (E1), estradiol (E2) and estriol (E3)	Development of the female reproductive system and secondary sexual characteristics
AR	**Natural:** Dihydrotestosterone, testosterone **Synthetic:** Mibolerone	Development of the male reproductive system and secondary sexual characteristics
LXR	**Natural:** Oxysterols, Cholesterol **Synthetic:** T0901317, GW3965	Lipid and carbohydrate metabolism
FXR	**Natural:** Bile acids **Synthetic:** GW4064, Farnesol, CDCA	Bile acid homeostasis
PPARα	**Natural:** Polyunsaturated Fatty Acids **Synthetic:** Fibrates (Gemfibrozil, fenofibrate, clofibrate)	Fatty acid oxidation and lipid metabolism
PPARβ	**Natural:** Unsaturated/saturated fatty acids, eicosanoids, prostacyclin **Synthetic:** GW501516	Cholesterol metabolism
PPARγ	**Natural:** 15-Deoxy-12,14 Prostaglandin J2 (15d-PGJ2) **Synthetic:** Thiazolidinedione (Ciglitazone, Pioglitazone, Rosiglitazone)	Lipid and glucose metabolism
RAR	**Natural:** all-trans retinoic acid	Cell growth, differentiation and organogenesis
RXR	**Natural:** 9-cis-retinoic acid, docosahexaenoic acid **Synthetic:** Methoprene acid, Rexinoids (LG100268)	Cellular proliferation and differentiation, glucose, fatty acid and cholesterol metabolism
VDR	**Natural:** Calcitriol **Synthetic:** Maxacalcitol, Calcipotriol	Calcium homeostasis, cell proliferation and differentiation

1.7.3. Nuclear receptors are acute regulators of platelet function

Platelets have been reported to express both the classes of NRs, Type I or the steroid hormone receptors and Type II or the non-steroid receptors. This includes the androgen receptor (Khetawat et al., 2000; Campelo et al., 2012), estrogen receptor (Khetawat et al., 2000; Akarasereenont et al., 2006; Bar et al., 1993; Valera et al., 2012) glucocorticoid receptor (Moraes et al., 2005; Liverani et al., 2012), farnesoid X receptor (Moraes et al., 2016) , liver X receptor (Spyridon et al., 2011), peroxisome proliferator-activated receptor (PPARs) (Akbiyik et al., 2004; Ali et al., 2009a; Ali et al., 2006; Ali et al., 2009b; Du et al., 2014; Li et al., 2005; Moraes et al., 2010b; Unsworth et al., 2017b), retinoic acid receptor (Rondina et al., 2016), retinoid X receptor (Moraes et al., 2007; Unsworth et al., 2017c) and vitamin D receptor (Silvagno et al., 2010; Cumhur Cure et al., 2014). Both natural and synthetic ligands of these NRs possess the ability to modulate platelet function through different mechanisms.

1.7.3.1. Type I nuclear receptors

Mechanisms that govern the activities of type I NRs (GR, ER and AR) in platelets are not entirely clear. This might be attributed to the fact that plasma levels of steroid hormones targeting these NRs are under constant fluctuation (especially in females and under certain pathological conditions) (Frye and Rhodes, 2008; Richard et al., 2014; Güncü et al., 2005). Consequently, it is difficult to study the functions of type I NRs in platelets as frequent variations in steroid hormone concentrations may lead to inaccurate assessments in acute vs chronic study and might explain the existence of conflicting data.

1.7.3.1.1. Glucocorticoid receptor

Glucocorticoid receptors (GR) are well-known regulators of inflammation and glucose homeostasis and can be activated by glucocorticoid and anti-inflammatory hormones (Bledsoe et al., 2002). Treatment of platelets with prednisolone (synthetic glucocorticoid derived from cortisol) has been reported to attenuate the level of ADP or U46619-mediated aggregation and thromboxane B_2 (TxB_2) production, which was reversed following treatment with a GR antagonist mifepristone (Moraes et al., 2005; Liverani et al., 2012). This inhibition was found to be independent of the activity of cyclic nucleotides - cAMP or cGMP, key mediators of inhibitory platelet signalling (Liverani et al., 2012). Both adhesion and thrombus formation on collagen *in vitro* was found to be inhibited following prednisolone treatment, which might likely be due to reduced ADP or TxA_2 stimulated-platelet aggregation (Liverani et al., 2012). Prednisolone has also been shown to regulate platelet-monocyte interactions following stimulation by ADP, which is attributed to attenuation of platelet activity and not to inhibition of monocytes (Liverani et al., 2012). However, it is interesting to note that alternative GR ligands - dexamethasone, fludrocortisone and triamcinolone were not able to elicit anti-platelet effects under the experimental conditions used in these studies (Liverani et al., 2012; Moraes et al., 2005). This difference in activation is thought to be due to the formation of a heterodimeric complex between GR and the mineralocorticoid receptor (MR) that is susceptible to differential activation by specific receptor ligands. The mechanism that leads to diminished secondary mediator signalling by the GR ligand prednisolone requires further investigation, although, there is evidence to suggest that this might be

mediated via the down-regulation of signalling proximal to the P2Y12 receptor (Liverani et al., 2012).

1.7.3.1.2. Estrogen receptor

Estradiol-17β (E2) and estrogen receptors (ERs) play vital roles in regulating reproductive and sexual development and are also known to have an influence on the cardiovascular health (Murphy, 2011). Human megakaryocytes and platelets have been shown to express ERβ but not ERα (Khetawat et al., 2000). The effects of several forms of estrogen, including estrone (E1), estradiol (E2) and estriol (E3) on platelet function have been evaluated and the results are conflicting. While, acute treatment of platelets *ex vivo* with either E1 or E3 was found to increase adrenaline or ADP-mediated aggregation (Akarasereenont et al., 2006), chronic treatment with estrogen (in patients undergoing estrogen replacement therapy) was found to be associated with a significant reduction in adrenaline-induced platelet aggregation and ATP release, in comparison to the control groups (Bar et al., 1993). Supporting this, chronic treatment with high levels of estradiol in mice exhibited substantial reduction in platelet activation both *ex vivo* and *in vivo*, with both, an increase in bleeding time and resistance to thromboembolism being observed (Valera et al., 2012). However, it is important to note these effects on platelet reactivity are also due to modulation of the expression of platelet proteins (such as β1 tubulin) during haematopoiesis that then alters platelet production and activation (Valera et al., 2012), rather than a direct consequence of non-genomic effects on platelet function.

1.7.3.1.3. Androgen receptor

The expression of androgen receptor (AR) has been reported in both megakaryocytes and platelets but its role in the regulation of platelet function is not well understood (Khetawat et al., 2000). Testosterone and dihydrotestosterone are the physiological activators of AR. A few studies have identified augmented platelet aggregation response in males compared to female rats due to higher levels of androgenic steroids (Johnson et al., 1975), since platelet aggregation was found to be inhibited following castration in male rats and these effects were noted to be reversed following treatment with testosterone (Johnson et al., 1977). In another study, acute treatment of rat or human PRP with testosterone was noted to exhibit heightened platelet aggregation upon stimulation with ADP, adrenaline, collagen, arachidonic acid or calcium ionophore, indicating its rapid non-genomic responses (Pilo et al., 1981). Additionally, two independent studies also confirmed that testosterone causes a significant increase in TXA_2 receptor density on the platelet surface, thereby, indirectly increasing platelet responsiveness (Ajayi et al., 1995; Matsuda et al., 1994). In contrast to these studies, treatment with testosterone has also been associated with inhibition of platelet aggregation. However, these effects were reported to be attributable to the effects of endothelium-derived NO synthesis and therefore they might not represent a direct effect of testosterone on the platelet androgen receptor and platelet activity (Campelo et al., 2012).

1.7.3.2. Type II nuclear receptors

1.7.3.2.1. Liver X Receptor

Liver X receptors (LXR) play a fundamental role in the regulation of fatty acid, cholesterol and glucose homoeostasis. Its endogenous ligands include oxysterols such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, while GW3965 and T0901317 are widely used synthetic ligands (Gabbi et al., 2014; Wójcicka et al., 2015). Ligands for LXR have been proposed to demonstrate anti-inflammatory and atheroprotective properties.

The presence of LXRβ has been reported in platelets (Spyridon et al., 2011) and their treatment with synthetic ligand GW3965 was associated with reduced platelet aggregation, calcium mobilisation, secretion and integrin activation following stimulation by collagen, CRP-XL or thrombin. Furthermore, GW3965-treated mice were also found to form smaller, less stable thrombi following laser injury of the cremaster muscle arterioles. Exposure to GW3965 caused a negative regulation of GPVI-mediated signalling, which was identified to be an outcome of a direct interaction of LXR with Syk and PLCγ2. In support of this, another study reported the ability of endogenous LXR ligand 22(R)-OH-cholesterol (but not its stereoisomer 22(S)-OH-cholesterol) to inhibit collagen-induced platelet aggregation and shape change (Schaffer et al., 2013). LXR has also been reported to form a heterodimer with RXR in platelets (Unsworth et al., 2017c).

During thrombus formation, two distinct populations of platelets appear, co-aggregated platelets, which support thrombus growth, and loosely attached

pro-coagulant platelets that expose phosphatidylserine and support coagulation. The procoagulant state is a feature that represents platelet hyper-reactivity and is usually associated with pathological conditions such as hyperlipidemia, obesity and high cholesterol. Treatment of platelets with LXR ligands, GW3965 and T0901317 and natural ligands, 27-OH-cholesterol and 24-(S)-hydroxyl-cholesterol have also been shown to cause platelet inhibition to several agonists through the conversion of platelets to procoagulant coated platelets state (Unsworth et al., 2017d). LXR ligand stimulated coated-platelets expose phosphatidylserine at platelet surface and also retain high levels of fibrinogen (which is converted to fibrin) and other alpha granule components at the platelet membrane. Conversion to the state of coated-platelet is believed to support coagulation but it makes platelets less responsive to platelet agonists, through the closure of integrin αIIbβ3. Similar observations were made in platelets treated with LXR ligands. The mechanism underlying this effect of GW3965 seems to be an outcome of reduced intracellular calcium signalling, depolarisation of the mitochondrial membrane potential independently of cyclophilin D and via generation of reactive oxygen species (ROS) (Unsworth et al., 2017d). Therefore, platelet dysfunction observed in patients with high cholesterol, hyperlipidaemia, metabolic syndrome and obesity might be due to altered LXR signalling in platelets.

1.7.3.2.2. Farnesoid X Receptor

Farnesoid X receptor (FXR) functions physiologically to regulate bile acid and cholesterol homoeostasis. Its expression has been reported in both human and mouse platelets and their treatment with synthetic FXR ligand

GW4064 resulted in a decrease in sample turbidity (Moraes et al., 2016; Unsworth et al., 2017d). This was later associated with platelet swelling and conversion of platelets to a procoagulant coated-platelets state (Unsworth et al., 2017d). In a manner similar to LXR, treatment with FXR ligands results in the formation of coated platelets, prior to platelet agonist stimulation. This is characterised by the exposure of phosphatidylserine, retention of fibrinogen, fibrin and alpha granule proteins on the platelet surface, cyclophilin D dependent depolarisation of the mitochondrial membrane, deregulation of calcium signalling, generation of ROS and closure of integrins at the platelet surface (Unsworth et al., 2017d). The closure of platelet integrins is believed to underlie the observed reduction in platelet aggregation to platelet agonists. In a mouse model of thrombosis, the initial kinetics of thrombus formation were elevated in GW4064 treated mice, but thrombus stability was considerably reduced in comparison to control mice (Moraes et al., 2016). The inhibition of platelet activity by FXR ligands were found to an outcome of increased intracellular levels of cGMP, leading to a down-regulation of platelet signalling. Furthermore, treatment of FXR-deficient mice with FXR ligands did not alter platelet activation, which confirms their selective non-genomic action being arbitrated via FXR (Moraes et al., 2016).

1.7.3.2.3. Peroxisome proliferator-activated receptors

Three isoforms of peroxisome proliferator-activated receptors (PPARs) exist, PPARα, PPARβ and PPARγ, and are recognised for their role in cell development, differentiation, cholesterol and fatty acid metabolism, and glucose

homoeostasis. All the three isoforms of PPARs, upon binding to their ligands can exhibit inhibition of platelet activation in a non-genomic manner.

PPARα

PPARα ligands such as fenofibrate or statins (simvastatin) have been reported to attenuate ADP-stimulated platelet activation by inducing a rise in intracellular levels of cAMP via a PPARα-dependent mechanism. In alignment with this, the inhibition was found to be reversed following treatment with PPARα antagonist GW6471 (Ali et al., 2009a). The role of PPARα in regulating platelet activation was further supported using mice deficient in PPARα, which upon treatment with fenofibrate did not alter platelet activation and bleeding time. The inhibition of platelet activity following exposure to fenofibrate was associated with an upregulation of cAMP levels via inhibition of PKCα, a key mediator of platelet signalling, through interaction between PPARα and PKCα. This interaction was believed to prevent binding of PKCα with its substrates and thus causing an inhibition of platelet functions (Ali et al., 2009a). These findings identify PPARα as a key mediator of statin and fenofibrate-mediated anti-platelet activity.

PPARβ/δ

Synthetic ligands of PPARβ/δ such as GW0742 and L-165041 have been observed to attenuate platelet aggregation and calcium mobilisation in response to stimulation by a range of platelet agonists (Ali et al., 2006). It is known that PPARβ/δ can also be activated by the prostaglandin PGI_2. Consequently the possibility of PGI_2 eliciting some of its inhibitory effects on platelets through

PPARβ/δ (in addition to the prostaglandin IP receptor) cannot be ruled out and requires further investigation (Ali et al., 2006). In a manner similar to PPARα, incubation of platelets with PPARβ/δ ligands causes an elevation in intracellular cAMP levels and potential interaction of the receptor with PKCα has been identified as a plausible mechanism by which PPARβ/δ regulates platelet reactivity (Ali et al., 2009b). PPARβ/δ ligands have been identified to reduce the development of atherosclerosis and prevent plaque formation (Lee et al., 2003). The activity of platelets to promote the initiation and progression of atherosclerosis is well known. Therefore, these suggested antiplatelet effects of PPARβ/δ ligands may partly explain anti-atherosclerotic properties of its ligands.

PPARγ

Of all the isoforms of PPARs reported in platelets, PPARγ is the most extensively explored. This is primarily because of its direct connection with several cardiovascular diseases including diabetes mellitus, atherosclerosis and thrombosis (Beckman et al., 2002; Chinetti et al., 2000; Moraes et al., 2006). PPARγ is an important therapeutic target and its synthetic ligands, the thiazolidinediones (pioglitazone, rosiglitazone, lobeglitazone etc) are recognised for their cardioprotective properties and widely used for the treatment of type-2 diabetes (Chandra et al., 2017; Yue, 2003).

The antiplatelet activity of PPARγ ligands may provide a mechanistic basis that in part underlies these observations. For instance, rosiglitazone in a clinical study (conducted on patients suffering from coronary heart disease) was reported to exhibit long-term anti-platelet effects through the inhibition of P-selectin

exposure and granule secretion (Sidhu et al., 2004). The endogenous (15d-PGJ_2) and synthetic (rosiglitazone and ciglitazone) ligands of PPARγ have been shown to down-regulate platelet function *ex vivo* upon stimulation by several agonists that target GPCR (thrombin and ADP) (Akbiyik et al., 2004), GPVI (collagen and CRP-XL) (Moraes et al., 2010b) and integrin αIIbβ3 receptors (Unsworth et al., 2017b). Treatment with 15d-PGJ_2 or rosiglitazone reduced granule secretion and TxB_2 synthesis in response to thrombin or ADP (Akbiyik et al., 2004). These ligands were also associated with inhibition of collagen/CRP-XL-induced platelet aggregation, granule secretion and mobilisation of intracellular calcium. Exposure to PPARγ ligands also caused a reduction in early GPVI signalling events such as decreased phosphorylation of Syk and LAT (Moraes et al., 2010b). Furthermore, a direct interaction of PPARγ with Syk and LAT was identified upon stimulation with collagen in the absence PPARγ ligands. The interaction was disrupted upon treatment with PPARγ ligands (Moraes et al., 2010b). These ligands also regulate integrin αIIbβ3 outside-in signalling through the upregulation of PKA activity. Incubation of platelets with PPARγ ligand inhibit β3 phosphorylation and several other downstream signalling molecules of the integrin αIIbβ3 signalling pathway including Syk, $PLC\gamma_2$, PKC, FAK and PI3K (Unsworth et al., 2017b). PPARγ ligands, therefore, possess the ability to modulate platelet activation through the regulation of several different mechanisms. In another study, synthetic PPARγ ligand, pioglitazone was also found to delay intra-arterial thrombus formation in rats (Li et al., 2005).

1.7.3.2.4. Retinoic Acid Receptor

Three isoforms of retinoic acid receptors (RARs) exist; RARα, RARβ and RARγ that play a principal role in several biological processes, including development, reproduction, immunity, organogenesis and homoeostasis (Duong and Rochette-Egly, 2011). While RARβ/γ display a tissue-specific distribution, the expression of RARα is ubiquitous and its presence in platelets and megakaryocytes has also been reported (Dolle, 2009). Endogenously, RARs are activated by retinoids (metabolites of vitamin A), while few synthetic ligands also exist (Duong and Rochette-Egly, 2011).

RARα has been identified to directly interact with actin-related protein-2/3 complex (Arp2/3) subunit 5 (Arp2/3s5) in platelets, which is required for the regulation of platelet cytoskeletal processes. Treatment of platelets with the endogenous RARα ligand, all-trans retinoic acid (atRA), disrupts the RARα-Arp2/3 interactions resulting in an inhibition of cytoskeletal rearrangements and platelet spreading (Rondina et al., 2016). Recently, RARα was observed to regulate protein synthesis (including microtubule-associated protein-1 light chain 3 beta 2) in human platelets through its binding to a subset of mRNAs and thereby blocking translation (Schwertz et al., 2017). It was also identified that prolonged treatment of platelets with RARα ligand (for several hours) significantly altered the levels of protein synthesis compared to controls (Schwertz et al., 2017).

1.7.3.2.5. Vitamin D receptor

The vitamin D receptor (VDR) is another ligand-activated transcription factor that mediates the actions of vitamin D and its metabolites. VDR is also

known to form a heterodimer with the RXR and regulate calcium homoeostasis, cell growth and differentiation, detoxification of xenobiotics, and modulation of adaptive and innate immunity (Kato, 2000). Although anticoagulant effects of vitamin D have been reported and VDR signalling has been characterised in monocytes and vascular cells, the role of the VDR in platelet function remains unknown. Both human platelets and megakaryocytes have been found to express the VDR. Biochemical fractionation studies along with immuno-electron microscopy analysis identified VDR to be localised in the soluble and mitochondrial compartment of human platelets and mature megakaryocytes (in addition to its normal localisation in the nucleus) (Silvagno et al., 2010). Although little is known about the role for vitamin D and the VDR in platelet function, a patient study identified a strong association between low vitamin D plasma levels and a high mean platelet volume (MPV), a marker of platelet hyperactivity (Cumhur Cure et al., 2014).

1.7.3.2.6. Retinoid X Receptor

The retinoid X receptor (RXR) due to their ability to form heterodimers with almost a quarter of the known human NRs (PPAR's, LXR, FXR, PXR etc.) is considered as one of the most important receptors in the NR superfamily (Evans and Mangelsdorf, 2014). The presence of RXR homodimers has also been reported (Sato et al., 2010). The fundamental role of RXR is to regulate several vital biological processes such as cell proliferation, differentiation, apoptosis, haematopoiesis, metabolism (glucose, fatty acid and cholesterol) and pattern formation during embryogenesis (Ahuja et al., 2003). Moraes et al. (2007)

reported the expression of RXRα and RXRβ (but not RXRγ) in human platelets and megakaryocytes. Treatment of platelets with the endogenous ligand of RXR, 9-*cis*-retinoic acid or the synthetic ligand, methoprene acid, was found to inhibit platelet functions stimulated by ADP, U46619 (Moraes et al., 2007) or thrombin (Unsworth et al., 2017c) that initiate signalling via Gq coupled GPCRs. Down-regulation of GPCR mediated platelet activation by RXR has been associated with its direct interaction with Gαq in a ligand-dependent manner that inhibits Gq induced Rac activation and intracellular Ca^{2+} mobilisation (Moraes et al., 2007).

1.7.3.2.7. Pregnane X receptor

The Pregnane X receptor is a member of NR superfamily, which is predominantly expressed in liver and intestines. It is well characterised for its role as a sensor of xenobiotic and toxic endogenous compounds. Upon encountering such compounds, PXR up-regulates the expression of proteins such as cytochrome P450 3A (CYP3A) that are involved in metabolism, detoxification and subsequent elimination of these compounds from the body (Iyer et al., 2006; Ma et al., 2008). PXR is also involved in regulation of steroid hormone and bile salt metabolism (Krasowski et al., 2005).

Despite being a member of NR superfamily, several features of PXR have been identified that are different from other NRs. Firstly, in contrast to other NRs, PXR display the broadest range of specificity towards ligands owing to its large and flexible ligand-binding pocket. PXR ligands are therefore structurally diverse and encompass prescription drugs (such as rifampicin), herbal medicines (such as hyperforin or St. John's wart), dietary supplements, environmental pollutants, and

endobiotics (bile acids) (Honkakoski et al., 2003; Ma et al., 2008) (Fig 2). Secondly, unlike classic steroid hormone receptors, high-affinity (sub-nanomolar) ligands for PXR have not been discovered. The lowest EC_{50} values of steroids that activate PXR are low-micromolar, generally two to three orders of magnitude higher than concentrations found circulating in plasma (Iyer et al., 2006; Moore et al., 2002; Staudinger et al., 2011). Thirdly, there exists a high level of sequence and functional divergence of PXR among different species. The ligand binding domain of human and mouse PXR share a sequence similarity of only 77%, while, 76% exist between human and rat PXR (Carnahan and Redinbo, 2005; Jones et al., 2000). This represents the lowest sequence similarity by any NR in the superfamily, as other NRs have comparable identities between species. This variation in LBD sequences between species has resulted in cross-species differences in the ligands that activate PXR. For example, human PXR can be activated by ligands such as rifampicin, SR12813 and hyperforin, while, they have little or no effect on mouse PXR. In a similar manner, PXR ligand pregnenolone 16α-carbonitrile (PCN) is highly specific to rodents only (Iyer et al., 2006; Krasowski et al., 2005; Timsit and Negishi, 2007). The species-specific activation of PXR by its ligands pose a major challenge to conduct studies for the development and evaluation of candidate drugs targeting human PXR. This has also led to the development and characterisation of the humanised PXR mice in which the mouse PXR was genetically replaced by its human counterpart (Scheer et al., 2008; Scheer et al., 2010; Ma et al., 2007a; Xie et al., 2000). As can be anticipated, humanised PXR mice were responsive to human-specific PXR ligand rifampicin, while lacked a response to mouse-specific inducer PCN.

Conflicting results exist with respect to the protective role of PXR towards cardiovascular diseases, especially, atherosclerosis. It is well known that the low-density lipoprotein cholesterol (LDL) and very low-density lipoprotein cholesterol (VLDL) promote the progression of atherosclerosis, whilst, high-density lipoprotein cholesterol (HDL) attenuates it (Natarajan et al., 2010; Barter, 2005). Numerous studies have suggested the ability of PXR to evoke synthesis of HDL-cholesterol. Bachmann *et al.* (2004), reported PXR ligand-mediated increase in plasma HDL-cholesterol in wild-type mice, but not in PXR-knockout mice (Bachmann et al., 2004). PXR could also induce HDL production and its major constituent apolipoprotein (Apo)A1 in C57BL/6 and ApoE*3-Leiden (human lipoprotein metabolism model) mice (de Haan et al., 2009). PXR expression in mice was also found to antagonize the cholic acid-mediated downregulation of plasma HDL-cholesterol (Li et al., 2007). Additionally, PXR has also been shown to promote cholesterol efflux and HDL synthesis by up-regulating the level of cytochrome CYP27A1 (Li et al., 2007). Cholic acid-mediated down-regulation of plasma HDL cholesterol and ApoA-1 levels were reported to be abolished in humanised PXR transgenic mice (Masson et al., 2005). In contrast to these findings, Sui et al. (2011) reported that the deficiency of PXR attenuates atherosclerosis development by reducing lipid uptake in macrophages. Activation of PXR was associated with a high increase in atherosclerotic lesions in $ApoE^{-/-}$ mice. Although in the same study, $PXR^{-/-}$ mice displayed higher VLDL levels in comparison with wild-type indicating the potential involvement of PXR in maintaining low levels of VLDL endogenously (Zhou et al., 2009a).. Anti-diabetic properties of PXR have also been observed in fasting mice where its activation has been shown to decrease serum glucose levels. It also suppresses the expression of

genes involved in gluconeogenesis, by interacting with glucagon responsive transcription factors or by competing for their coactivators (Gao and Xie, 2010).

Recently, the presence of PXR was reported in the vasculature (human, rat and mouse blood vessels, and human and rat aortic endothelial and smooth muscle cells), where it regulated the expression of drug metabolizing enzymes CYP 3A, 2B and 2C (Swales et al., 2012). This suggests the ability of vasculature to detoxify circulating toxins and avert vascular damage. PXR was also observed to strengthen anti-oxidative defence by inducing glutathione-s-transferase and glutathione peroxidase enzymes, thereby, providing a protective role against oxidative stress that might develop in the vascular system (Swales et al., 2012). Thus, based on these findings, PXR might have the ability to exhibit cardioprotective effects.

Table 1.2. A summary of nuclear receptors identified in platelets and their molecular mechanism of action (Flora *et al*., 2018)

Nuclear Receptor	Ligands	Effect on Platelet Function	Mechanisms of Action
GR (Moraes et al., 2005; Liverani et al., 2012)	• Prednisolone	Negative regulation of platelet secondary mediator stimulated effects (ADP and TXA_2) (*in vitro*)	Mechanism unknown
ER (Bar et al., 1993; Valera et al., 2012)	• Estrogen - estrone (E1), estradiol (E2) and estriol (E3)	Reduction in platelet responsiveness, however, conflicting results exist (*in vitro*, *ex vivo* and *in vivo*)	Mechanism is unknown
AR (Johnson et al., 1975; Johnson et al., 1977; Pilo et al., 1981)	• Testosterone • Dihydrotestosterone	Potentiation of platelet aggregation (*in vitro* and *ex vivo*)	Mechanism is unknown
LXR (Spyridon et al., 2011)	• GW3965 • T0901317 • 24(S)-OH-cholesterol • 27-OH-cholesterol	Inhibition of platelet function and thrombosis (*in vitro* and *in vivo*) Conversion of platelets to the procoagulant state (*in vitro*)	• Reduced phosphorylation of early GPVI signalling components – Syk, LAT and PLCγ2 Increase LXR-Syk and LXR-PLCγ2 interaction • Formation of coated platelets, including PS exposure, mitochondrial membrane depolarisation
FXR (Moraes et al., 2016)	• GW4064 • Chenodeoxycholic acid • 6α-ethyl-chenodeoxycholic acid	Inhibition of platelet function, thrombosis and haemostasis (*in vitro* and *in vivo*) Conversion of platelets to the procoagulant state (*in vitro*)	• Cyclophilin D dependent formation of coated platelets and closure of surface integrins. • Associated with PS exposure and mitochondrial membrane depolarisation • Augmented cGMP levels which promote PKG activity and phosphorylation of VASP S239
PPARα (Ali et al., 2009a)	• Fenofibrate • Statins	Inhibition of platelet function (*in vitro*)	• Increase in cAMP levels • PPARα-PKCα interaction and attenuation of PKCα
PPARβ/δ (Ali et al., 2009b)	• GW0742 • L-165041	Inhibition of platelet function (*in vitro*)	• Increase in cAMP levels • PPARα-PKCα interaction and attenuation of PKCα

Table 1.2 ***(Continued)***

Nuclear Receptor	Ligands	Effect on Platelet Function	Mechanisms of Action
PPARγ (Moraes et al., 2010b; Unsworth et al., 2017b)	• 15d-PGJ2 • Thiazolidinediones (Rosiglitazone, Ciglitazone, Pioglitazone)	Inhibition of platelet function, thrombosis and haemostasis (*in vitro* and *in vivo*)	• Inhibition in phosphorylation of Syk and LAT to reduce GPVI signalling • Reduced PPARγ-Syk and PPARγ-LAT interaction upon PPARγ ligand treatment • Negative regulation of integrin αIIbβ3 outside-in via upregulation of PKA activity and inhibition β3 phosphorylation
RAR (Rondina et al., 2016)	• all-trans retinoic acid	Inhibition of cytoskeletal rearrangements and platelet spreading (*in vitro*)	Disruption of RARα-Arp2/3 interactions.
RXR (Moraes et al., 2007; Unsworth et al., 2017c)	• 9-*cis*-retinoic acid • Methoprene acid • Docosahexaenoic acid	Inhibition of platelet function, thrombosis and haemostasis (*in vitro* an *in vivo*)	• RXR-Gq interaction and negative regulation of Rac activation to inhibit GPCR mediated platelet activation • Upregulation of PKA activity and phosphorylation of VASP S157 in cAMP and NFκβ dependent manner
VDR (Cumhur Cure et al., 2014)	• Vitamin D and its metabolites	Low vitamin D plasma levels cause high mean platelet volume, a marker of platelet hyperactivity (*in vivo*)	Mechanism is unknown

1.8. Hypothesis

The nuclear receptors, PXR and RXR exhibit non-genomic effects to regulate platelet functions and signal transduction stimulated by a range of agonists and thus modulate thrombus formation.

1.9. Aims of the study

The past few decades have seen a steady rise in the number of patients suffering from cardiovascular diseases (CVDs), making CVDs a major health risk globally (Grundy et al., 2004; Luepker, 2011). Platelets are regarded as important targets for the treatment of CVDs. Therefore, some of the current treatment strategies include use of drugs/therapies that inhibit platelet functions by targeting different platelet activation mechanisms (Capodanno et al., 2013; Metharom et al., 2015; Thachil, 2016). These treatment regimens have been successful in reducing the overall mortality or morbidity, but still exhibit numerous side effects such as bleeding and drug resistance that limits their use (Nathan et al., 2017). Therefore, there is a need to devise newer strategies that are safe yet potent in treating or preventing CVDs with minimal side effects.

As discussed earlier, the presence of several kinds of NRs have been reported in platelets with their ligands being able to down-regulate platelet functions. The pregnane X receptor has been recently discovered in the cardiovascular system (blood vessels, aortic endothelial and smooth muscle cells) (Swales et al., 2012). It has also been reported to display anti-atherosclerotic potential in murine models of atherosclerosis (Li et al., 2007; Bachmann et al.,

2004; de Haan et al., 2009). Anti-atherosclerotic properties are common features shared by most of the NRs identified in platelets such as RXR, LXR, FXR and PPARs (Lalloyer et al., 2006; Duval et al., 2002; Hageman et al., 2010; Calkin and Tontonoz, 2010). Moreover, in nucleated cells, PXR acts as an active dimer partner of RXR, whose expression in platelets has already been reported (Moraes et al., 2007). Based on these considerations, the presence of PXR in platelets and implications of its ligands in regulating platelet functions and signalling were explored.

In addition to this, RXR is another NR identified in platelets and its ligands have been found to exhibit anti-thrombotic effects. The inhibitory effects of RXR ligands on platelets were reported to be due to the down-regulation of platelet activation stimulated by secondary mediators, ADP and TXA_2 (Moraes et al., 2007). However, their role on collagen and thrombin-mediated platelet activation were unclear. This is important because collagen is the principal agonist responsible for initiating platelet activation upon vascular injury. Whereas, thrombin is a highly potent platelet agonist, generated via the coagulation cascade and is also secreted from platelets. Therefore, further research is required to better understand the effects of RXR ligands on collagen and thrombin-stimulated platelet activation.

Key objectives

1. To explore the presence of PXR and localisation of PXR and RXR in human and mouse platelets.
2. To examine if RXR forms a heterodimer with other NRs (PPARs, LXR, PXR etc.).
3. To evaluate the ability of PXR and RXR ligands to regulate platelet function.

4. To explore the effects of RXR and PXR ligands on thrombosis and haemostasis.
5. To study the effects of PXR and RXR ligands on platelet signalling to better understand underlying mechanisms of action.

Chapter-2

Materials and Methods

2.1. Materials

2.1.1. Platelet agonists

Horm-Chemie collagen (collagen fibres from equine tendons) was from Nycomed (Munich, Germany). Thrombin from bovine plasma was from Sigma (Poole, UK) and cross-linked collagen-related peptide (CRP-XL) was provided by Professor Richard Farndale (University of Cambridge, UK). U46619, a thromboxane A2 analog, was from Tocris Biosciences (Bristol, UK). Adenosine diphosphate (ADP) was from Sigma (Poole, UK).

2.1.2. Nuclear receptor ligands

PXR ligands rifampicin and 5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN) were purchased from Sigma Aldrich (Poole, UK), while, SR12813 was from Abcam (Cambridge, UK). RXR ligands 9-*cis*-retinoic acid and methoprene acid were purchased from Sigma Aldrich (Poole, UK) respectively.

2.1.3. Antibodies

Information regarding the primary and secondary antibodies used for this study is listed in Table 2.1 and Table 2.2, respectively, along with their applications and concentrations used.

2.1.4. Animals

C57BL/6 mice were from Envigo (Huntingdon, UK) and humanised PXR mice were purchased from Taconic Biosciences (Lille Skensved, Denmark).

Table 2.1. List of primary antibodies used for this study

Antibody	Host	Application	Dilution	Source and catalog no.
Polyclonal anti-PXR	Rabbit	Western blotting	1:1000	SantaCruz (Calne, UK) **sc-25381**
Polyclonal anti-PXR	Rabbit	Immunofluorescence	1:100	Abcam (Cambridge, UK) **ab85451**
Monoclonal anti-PXR	Mouse	Immunoprecipitation	1: 200	Abcam **ab41930**
Monoclonal anti-RXR α/β/γ	Mouse	Western blotting Immunoprecipitation Immunofluorescence	1:1000 1:200 1:100	SantaCruz **sc-46659**
Polyclonal anti-LXR	Rabbit	Western blotting	1:1000	Abcam **ab28479**
Polyclonal anti-PPARα	Rabbit	Western blotting	1:1000	SantaCruz **sc-9000**
Polyclonal anti-PPARγ	Goat	Western blotting	1:1000	SantaCruz **sc-1984**
Monoclonal anti-GPIb	Mouse	Immunofluorescence	1:100	ThermoFisher (Loughborough; UK) **PM6/248**
Polyclonal anti-GPIb	Goat	Immunofluorescence	1:100	SantaCruz **sc-6602**
Monoclonal anti-Integrin alpha2 chain (GPIa)	Rat	Flow cytometry	1:50	EMFRET Analytics (Würzburg, Germany) **M070-1**
Monoclonal anti-GPVI	Rat	Flow cytometry	1:50	Emfret Analytics **M011-1**
Monoclonal anti-Integrin αIIbβ3	Rat	Flow cytometry	1:50	Emfret Analytics **M025-2**
Monoclonal anti-GPIba	Rat	Flow cytometry	1:50	Emfret Analytics **M040-2**

Table 2.1 primary antibodies (continued)

Antibody	Host	Application	Dilution	Source and catalogue no.
FITC conjugated polyclonal anti-human fibrinogen	Rabbit	Flow cytometry	1:50	Dako (Glostrup, Denmark) **F0111**
PE/Cy5 anti-human CD62P monoclonal antibody	Mouse	Flow cytometry	1:50	BD Biosciences (New Jersey, USA) **#551142**
Phospho-VASP (Ser157)	Rabbit	Western blotting	1:1000	Cell Signalling Technology (Hitchin, UK) **#3111**
Phospho-VASP (Ser239)	Rabbit	Western blotting	1:1000	Cell Signalling Technology **#3114**
Anti-phospho-tyrosine 4G10	Mouse	Western blotting	1:1000	Merck Millipore (Watford, UK) **#05-321**
Anti-phospho-tyrosine PKC	Rabbit	Western blotting	1:1000	Cell Signalling Technology **#2261**
Phospho-Src (Y418)	Rabbit	Western blotting	1:1000	ThermoFisher **#44-660G**
Phospho-Lyn (Y396)	Rabbit	Western blotting	1:1000	Abcam **ab226778**
Phospho-Syk (Y525/526)	Rabbit	Western blotting	1:1000	Abcam **ab58575**
Phospho-LAT (Y200)	Rabbit	Western blotting	1:1000	Abcam **ab68139**
Phospho PLCγ2 (Y1217)	Rabbit	Western blotting	1:1000	Cell Signalling Technology **#3871**

Table 2.1 primary antibodies (continued)

Antibody	Host	Application	Dilution	Source and catalogue no.
Phospho-MLC (Ser19)	Rabbit	Western blotting	1:1000	Cell Signalling Technology **#3671**
Monoclonal anti-14-3-3 ζ	Mouse	Western blotting	1:1000	SantaCruz **sc-293415**
Polyclonal anti-actin	Goat	Western blotting	1:1000	SantaCruz **sc-1615**
Monoclonal anti-GAPDH	Mouse	Western blotting	1:1000	Abcam **ab8245**
DyLight 649 anti-GPIbα	Rat	*in vivo* thrombosis assay	0.2 µg/gm weight of mice	EMFRET Analytics **M040-3**

Table 2.2. List of secondary antibodies used for this study

Antibody	Host	Application	Dilution	Source and catalogue no.
AlexaFluor 488 anti-mouse IgG	Donkey	Immunofluorescence Western blotting	1:500 1:1000	Life Technologies (Paisley, UK) **A-21202**
AlexaFluor 488 anti-goat IgG	Donkey	Immunofluorescence Western blotting	1:500 1:1000	Life Technologies **A-11055**
AlexaFluor 568 anti-rabbit IgG	Donkey	Immunofluorescence	1:500	Life Technologies **A10042**
AlexaFluor 647 anti-mouse IgG	Donkey	Immunofluorescence Western blotting	1:500 1:1000	Life Technologies **A-31571**
AlexaFluor 647 anti-rabbit IgG	Donkey	Immunofluorescence Western blotting	1:500 1:1000	Life Technologies **A-31573**
Cy5 anti-rabbit IgG	Goat	Western blotting	1:1000	Life Technologies **A-10523**
VeriBlot for IP Detection	Proprietary (Abcam)	Western blotting	1:1000	Abcam **ab131366**

2.1.5. Other Reagents

Chronolume kit was from Chronolog (PA, USA). Clear and black 96-well flat bottom plates were from Greiner Bio-One (Frickenhausen, Germany). The poly-L-lysine coated-12mm coverslips were obtained from VWR and ProLong Gold Antifade Mountant was from Life Tech (Carlsbad, CA, USA). Glass microscope slides were from Thermo-Fisher Scientific (Loughborough; UK). Fibrinogen from human plasma was from Sigma (Poole, UK). Paraformaldehyde (16%, methanol-free) was from Agar Scientific (Essex, UK). Alexa Fluor 488 phalloidin was from Thermo-Fisher Scientific (Loughborough; UK). GPRP (Gly-Pro-Arg-Pro) was from Sigma (Poole, UK). Protease-free bovine serum albumin (BSA) was from First Link (Wolverhampton, UK). Phosphate-Buffered Saline (PBS) tablets were from Sigma (Poole, UK). Thromboxane B_2 competitive ELISA Kit was purchased from Cayman chemical (Cambridge, U.K.). Protein A/G Magnetic Beads for immunoprecipitation assays was from Thermo-Fisher Scientific (Loughborough; UK). Protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, sodium orthovanadate and pepstatin-A) were from Sigma (Poole, UK). Cangrelor and indomethacin were from Sigma (Poole, UK). MRS2179 was purchased from Abcam (Cambridge, UK). 10% and 4-20% gradient Mini-PROTEAN® TGX™ precast polyacrylamide gels [10 well (50 µl) and 15 wells (15 µl)], polyvinylidene difluoride (PVDF) membranes and dual-stained molecular weight markers were obtained from Bio-rad (Hemel Hempstead, UK). Whatman 3MM chromatography paper was from Thermo Fisher Scientific (Waltham, MA, USA). Dimethylsulphoxide (DMSO) was from Sigma (Poole, UK). Fura-2-AM and Tris were from Thermo-Fisher Scientific (Waltham, MA, USA). Digitonin and EGTA

were from Sigma (Poole, UK). 98% DioC6(3) iodide (3,3'Dihexyloxacarbocyanine iodide) was from Sigma (Poole, UK). Vena8 Fluoro+ biochips were from Cellix Ltd microfluidic solutions (Dublin, Ireland).

2.2. Methods

2.2.1. Human platelet preparation

Human blood was taken from consenting, drug-free volunteers on the day of the experiment according to the methodology approved by the University of Reading Research Ethics Committee. Blood was taken using 3.8% (w/v) sodium citrate and Acid Citrate Dextrose (ACD; 110 mmol/L glucose, 80 mmol/L citric acid, 120 mmol/L sodium citrate) as an anticoagulant. Whole blood was centrifuged at 102*g* for 20 minutes at 20°C to yield platelet-rich plasma (PRP). Where washed platelets were required, they were isolated from the PRP by further centrifugation at 1413*g* for 10 minutes at 20°C in the presence of 0.1 μg/ml prostacyclin to prevent activation. The supernatant was discarded in Klorsept disinfectant (Medentech, Wexford, Ireland) and the platelet pellet was resuspended in 25ml of modified Tyrodes-HEPES buffer (134 mmol/L NaCl, 0.34 mmol/L Na_2HPO_4, 2.9 mmol/L KCl, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, 5 mmol/L glucose, 1 mmol/L $MgCl_2$, pH 7.3) and 3 ml of ACD in the presence of 0.1 μg/ml prostacyclin. Platelets were centrifuged at 1413*g* for 10 minutes at 20°C and resuspended to a density of $4x10^8$ cells/ml in modified Tyrodes-HEPES buffer using a platelet count obtained with a Z Series Coulter Counter (Beckman Coulter, CA, USA). Washed platelets were rested for at least 30 minutes at 30°C prior to the experiment to allow responses to recover.

ADP-sensitive washed platelets were prepared by collecting blood into 3.8% (w/v) sodium citrate and centrifugation at 102*g* for 20 minutes at 20°C to yield PRP (without the addition of ACD). Platelets were isolated from the PRP by further centrifugation at 350*g* for 20 minutes. The slower speed of centrifugation minimises the chance of ADP release from platelets during preparation that can lead to receptor desensitisation, caused by higher centrifugation speeds. The supernatant was discarded, and the platelet pellet was re-suspended to a density of $4x10^8$ cells/ml in modified Tyrodes-HEPES buffer.

2.2.2. Mouse platelet preparation

The PRP was obtained by centrifuging blood (supplemented with 1ml HEPES-buffered Tyrode's solution) at 203g for 8 minutes. Where washed platelets were required, they were isolated from the PRP by further centrifugation at 1028g for 6 minutes in the presence of PGI_2 (12.5ng/ml). The resulting platelet pellet was re-suspended in modified HEPES-buffered Tyrode's solution at a concentration of $4x10^8$ cells/ml and was left to rest at 30°C for 30 minutes.

2.2.3. Immunofluorescence microscopy

Human blood was collected in vacutainers containing sodium citrate and mouse blood was collected in 3.8% (w/v) sodium citrate (1:10) as described previously. Phosphate buffer saline (containing, 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) was prepared by dissolving one PBS tablet in 200 mL of deionized water. Mouse blood was diluted

again 1:1 with PBS:citrate to prevent the formation of clots. Both human and mouse blood was centrifuged at 100g for 20 minutes to collect PRP. Resting or activated platelets (stimulated with 5 μM U46619; in the presence of 4 μM integrillin) in PRP were fixed with an equal volume of 8% paraformaldehyde-PBS (PFA-PBS) to make a final concentration of 4% (v/v) and incubated for 15 min. Thereafter, platelets were centrifuged at 950g for 10 minutes. The supernatant was removed, and platelet pellet was resuspended in 2 ml of PBS-ACD (pH 6.1) for washing. Platelets were centrifuged for 10 minutes at 950g and resuspended in 1 ml of PBS-ACD to concentrate platelets. Platelets were centrifuged again at the same speed for 10 minutes and then resuspended in 500μl of 1% (w/v) BSA-PBS, to concentrate platelets even more. Poly-L-lysine coated-12mm coverslips (VWR micro cover glass No.1.5) were put in 6x6 culture plate and 90μl of platelets were added on each coverslip. Culture plates were placed at 37^{o}C for 90 minutes. After 2-3 washes with PBS, samples were blocked with 0.2% (v/v) Triton-X-100, 2% (v/v) serum from same species as secondary antibody and 1% (w/v) protease-free BSA for 1h. Thereafter, primary antibodies diluted (1:100) in 0.2% (v/v) Triton-X-100, 2% (v/v) serum from the same species as secondary antibody and 1% (w/v) protease-free BSA were added and left overnight. The following day, samples were washed with PBS (2-3 times) and secondary antibodies (1:200) were added for 1 hour at room temperature. The unbound antibodies were washed off with PBS (2-3 times) and samples were fixed using 4% (v/v) PFA-PBS for 5 minutes. The coverslips were washed again with PBS (2-3 times). Coverslips were placed on glass slides after adding ProLong Gold Antifade mounting media (Life technologies). The slides were kept at room temperature until mounting

media dried and then kept in the fridge until they were imaged using a Nikon A1-R confocal microscope (100x oil immersion).

2.2.4. Platelet aggregometry

Light transmission aggregometry (LTA) was performed in an optical platelet aggregometer (Chrono-Log, PA, USA, and Helena Biosciences Europe, Gateshead, UK), as originally described by Born (1962). 222.5 µl of washed platelets ($4x10^8$ cells/ml) were stimulated in the presence of 25 µl agonist (collagen, CRP-XL, thrombin, U46619 or ADP) with continuous stirring (1200 rpm at 37^oC) for 5 minutes and aggregation was measured as an increase in light transmittance. The effects of NR ligands on platelet aggregation were measured by incubating washed platelets with 2.5 µl of NR ligand dissolved in DMSO (final DMSO concentration in sample of 0.1% v/v) or vehicle control (containing, DMSO 0.1% v/v) for 10 or 20 minutes prior to the addition of agonist. The aggregation was recorded for 5 minutes.

2.2.5. Flow cytometry

Fibrinogen binding and P-Selectin exposure were measured using FITC-conjugated polyclonal rabbit anti-human fibrinogen antibody and PE/Cy5 mouse anti-human CD62P antibody, respectively, in a 96-well flat bottom plate. PRP was treated with NR ligands or vehicle control for 10 minutes (containing, DMSO 0.1% v/v). 1 µl each of anti-fibrinogen and anti-CD62P antibody was added per 50 µl sample prior to stimulation with agonists (CRP-XL or thrombin) for 20 minutes with occasional gentle mixing. GPRP (25 µg/ml) was added in samples stimulated

with thrombin to prevent fibrin polymerization. Reactions were stopped by adding 0.2% (w/v) formyl saline.

The levels of integrin α2β1, αIIbβ3, GPVI and GPIb were evaluated using flow cytometry in resting and activated (CRP-XL stimulated) mouse platelets (in PRP) in a similar manner by incubating platelets with monoclonal anti-mouse integrin α2β1 (FITC-conjugated), integrin αIIbβ3 (FITC-conjugated), GPVI (Cy5-conjugated) and GPIb (Cy5-conjugated) antibodies respectively.

To measure PXR within platelets, resting and activated (with 1 μg/ml CRP-XL in the presence of integrilin) human washed platelets (200 μl) at $4x10^8$ cells/mL were fixed by adding an equal volume 2% (w/v) formyl saline and permeabilised using 400 μl of BD Phosflow Perm Buffer III (BD Bioscience, Oxford, UK) for 1 h in ice. Platelets were then incubated with rabbit anti-PXR primary antibody (SantaCruz; sc-25381) for an hour. Following washing at 550g for 20 min, platelets were resuspended in HEPES buffer saline. Thereafter, platelets were incubated with an appropriate secondary Cy5-conjugated antibody (Invitrogen, Paisley UK) for an hour. Negative controls were set using an appropriate isotype control.

Analyses were performed by flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK), and data were collected from 10,000 events [gated on platelets using FSC (forward scatter, limited between 1520-16000000) and SSC (side scatter, limited between 152-1600000)] and analysed using inbuilt BD Accuri C6 plus software, version 1.0.264.21.

2.2.6. Dense granule secretion

Secretion of ATP from dense Granule upon agonist stimulation was measured in washed platelets ($4x10^8$ cells/ml) using a Lumi-aggregometer (model 700, Chronolo-Log, PA, USA) (Feinman et al., 1977). 225 µl of washed platelets were added to a glass cuvette and incubated with the Chronolume reagent for 2 minutes, while stirring using the aggregometer. 2 nM ATP was added to this stirred suspension of platelets to set the ATP response baseline. The luminescence increase was observed using the AggroLink 8 software (Chrono-Log, PA, USA), with the luminescent gain adjusted until the ATP response was within the manufacturer-instructed range of 20-60%. These settings were saved and used for the rest of the experiment. Thereafter, 197.5 µl of washed platelets were incubated with 2.5 µl of NR ligands or vehicle control (containing, DMSO 0.1% v/v) at 37°C for 20 minutes under non-stirring conditions. 2 minutes prior the end of incubation period, 25 µl of Chronolume reagent was added and stirred using the aggregometer. Washed platelets were then stimulated by the addition of 25 µl of agonist (collagen or thrombin) and the baseline was set. ATP release from dense granule was recorded for 5 minutes following the addition of agonist using the AggroLink 8 software, which calculates ATP secretion levels from the 2nM ATP standard.

2.2.7. Thromboxane B_2 ELISA assay

TxB_2 assays were performed using a TXB_2 immunoassay kit obtained from Cayman Chemical (Cambridge, UK) and following the manufacturer's protocols. Briefly, 222.5 µl of human washed platelets ($4x10^8$ cell/ml) were

added to a glass cuvette and incubated with 2.5 µl of NR ligand or vehicle control (containing, DMSO 0.1% v/v) for 20 minutes before their activation with 25 µl of agonist (CRP-XL or thrombin) for 5 minutes. The reaction was discontinued by the addition of STOP solution [containing, EGTA (1 mM) and indomethacin 20 µM)] and immediately centrifuged (12,000 rpm, 2 min, RT) to isolate washed platelet supernatants, which were frozen immediately at -80°C. Samples were later thawed and diluted 1:40 in ELISA buffer [containing, phosphate (100 mM), BSA (0.1% w/v), NaCl (400 mM), EDTA (1 mM), sodium azide (0.01% w/v)] and 50 µl added to wells of a polyclonal goat anti-mouse IgG-coated plate. 50 µl of TxB_2 standards were aliquoted to determine the relationship between absorbance and TxB_2 concentration. 50 µl of TxB_2-acetylcholinesterase tracer and 50 µl of anti-TxB_2 mouse monoclonal antibody were added to each well and incubated for 2 hours at room temperature. Wells were washed four times with wash buffer and incubated with 200 µl of Ellman's Reagent under dark conditions. Absorbance at 405 nm was measured periodically using a Novostar plate reader. Absorbance values for TxB_2 standards were used to make a standard curve and test sample values were converted to TxB_2 concentrations using the inverse function.

2.2.8. Measurement of intracellular calcium mobilisation

The mobilisation of intracellular calcium from intracellular stores was evaluated using a dual excitation fluorescent dye Fura-2 AM, which binds free intracellular calcium. PRP was incubated with Fura-2AM (2 µM) for 1 hour at 30^{o}C and was followed by centrifugation at 350g for 20 minutes. The platelet pellet was resuspended in modified Tyrodes-HEPES buffer ($4x10^8$ cells/ml). Thereafter,

Fura-2 AM loaded washed platelets were incubated with NR ligands or vehicle control (containing, DMSO 0.1% v/v) for 10 minutes at 37^{o}C prior to addition of platelet agonists (CRP-XL or thrombin). Fluorescence measurements (excitation 340 and 380 nm, emission 510 nm) were recorded for 5 minutes (1 measurement every 1.5s) using a NOVOstar plate reader. Dual excitation (at 340 and 380 nm) allows quantification of $[Ca^{2+}]_i$; peak excitation of unbound Fura-2AM occurs at approximately 380 nm, whereas calcium-bound Fura-2 peak excitation is at ~340 nm (Bootman et al., 2013). $[Ca^{2+}]_i$ was estimated by using the ratio of the 340 nm and 380 nm excited signals. Calibration was performed by treating an untreated sample with digitonin (50 μM) to lyse the platelets, which releases the Fura-2AM into the Tyrodes buffer, containing $CaCl_2$ (2 mM), allowing measurement of the maximum fluorescence ratio. To calculate the minimum fluorescence ratio, Ca^{2+} ions were chelated by addition of 10 mM ethylene glycol-bis(β-aminoethyl ester)-N,N,N',N'-tetraacetic acid (EGTA) and 10 mM TRIS base (added to ensure an alkaline pH for optimal Ca^{2+} buffering by EGTA). Auto-fluorescence was measured using unloaded platelets. Using these calibration values (maximum, minimum and autofluorescence), experimental $[Ca^{2+}]_i$ concentrations were calculated using the following equation:

$$[Ca^{2+}]_i = K_d \times \frac{S_f}{S_b} \times \frac{R - R_{min}}{R_{max} - R}$$

Where K_d is the dissociation constant of Fura-2AM (~224 nM). S_f and S_b are the values of the fluorescence at 380nm excitation (corrected to background auto-fluorescence), with zero or saturating $[Ca]^{2+}$ respectively. R is the 340/380nm fluorescence ratio, corrected for background fluorescence. R_{min} and R_{max} are the

ratio limits at zero or saturating $[Ca]^{2+}$, respectively, adjusted using a viscosity constant of 0.85. This corrects for the effects of the cellular environment on the fluorescence of Fura-2.

2.2.9. Platelet adhesion and spreading

Glass coverslips were placed in 6 well plates and coated with collagen or fibrinogen (100 μg/ml each) (in modified PBS) for 1 hour. 1% (w/v) BSA was then added onto coverslips and incubated for 1 hour to prevent platelets binding to the glass. The coverslips were washed 3 times with PBS. Washed platelets at a density of $2x10^7$ cells/ml were treated with NR ligand or vehicle control (containing, DMSO 0.1% v/v) for 20 minutes, and then added onto coverslips and incubated for 45 minutes at 37°C. The supernatant was then removed from the coverslips, which were again washed 3 times with PBS. Platelets were then fixed with 0.2% (w/v) PFA for 10 minutes, the supernatant removed, and coverslips washed 3 times with PBS. Platelets were then permeabilised with 0.2% (v/v) Triton-X-100 for 5 minutes, and then the supernatant was removed and coverslips washed 3 times again with PBS. Alexa-Fluor 488 phalloidin was then added onto the coverslips for 1 hour, incubated in the dark, to label platelet F (filamentous) actin. The supernatant was removed, and coverslips washed 3 times with PBS. Coverslips were then mounted onto slides with the addition of Prolong Gold Antifade mounting media to preserve fluorescence. Samples were imaged, using a 100X oil immersion lens on a Nikon A1-R confocal microscope (Nikon, Tokyo, Japan). Fluorescence was excited at 488 nm with an argon laser and emitted at 500-520 nm, with images captured in one focal plane. Platelet adhesion data were obtained

by counting the number of platelets on 5 images of each coverslip that were captured randomly. Platelets were scored as adhered (not spread), spreading (defined as extending filopodia) or spread fully (lamellipodia formed), and the relative frequency of each population was determined using ImageJ software.

2.2.10. Clot retraction

To measure thrombin-stimulated fibrin clot retraction, PRP was obtained as described earlier. 198 µl of PRP was incubated with 2 µl of NR ligands or vehicle control (containing, DMSO 0.1% v/v) for 20 minutes. 785 µl of modified Tyrodes-HEPES buffer was added to test tubes, along with 5 µl of red blood cells, to allow visualization of the clot. This was followed by the addition of 200 µl PRP treated with NR ligand or vehicle control. Clot formation was initiated by adding 10 µl of thrombin (final concentration 1 U/ml) to the test tubes. A glass pipette was added to the centre of each test tube, around which the clot would form, and samples were placed in an incubator chamber at 37^{o}C. Photographs were taken every 10 minutes and the assay was terminated after 60 minutes at which time the clot in the vehicle-treated samples were seen to have retracted completely. Clot weight was measured as a marker for clot retraction. Clots were removed from the glass pipettes and transferred into the pre-weighed microfuge tubes. Clot mass was determined by subtracting the weight of pre-weighed microfuge tubes from the weight of microfuge tubes containing clot.

2.2.11. SDS-PAGE and western blotting

Human or mouse washed platelets were prepared at a density of $8x10^8$ cells/ml as described earlier and lysed by adding 6X Laemmli sample reducing buffer [4% (w/v) SDS, 20% (v/v) glycerol, 0.5M Tris, 0.001% (w/v) Brilliant Blue R and 10% (v/v) 2-mercaptoethanol]. Samples were heated to 95^{o}C for 5 minutes before storing at -20^{o}C until use.

To study cell signalling, human washed platelets were prepared at a density of $4x10^8$ cells/ml under non-aggregation conditions [indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). These platelets were treated with NR ligands or vehicle control (containing DMSO, 0.1% v/v) for 20 minutes and then stimulated with agonists (CRP-XL, thrombin or U46619) in the aggregometer. Unstimulated or stimulated samples were lysed with 6X Laemmli sample reducing buffer and heated to 95^{o}C for 5 minutes before storing at -20^{o}C until use.

Proteins were separated by SDS-PAGE as described previously by Laemmli (1970), using 10% or 4-20% Mini-PROTEAN TGX precast protein gels. Samples were heated to 95^{o}C for 5 minutes again prior to loading into gels, which were submerged in 1X Tris/Glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) within a Mini-PROTEAN tetra vertical electrophoresis cell (Bio-Rad, CA, USA). Electrophoresis was run for 45 minutes or 1 hour at a constant voltage of 150V.

The separated proteins on gels were transferred to a polyvinylidene difluoride (PVDF) membrane using semi-dry western blotting (Trans-Blot SD

Semi-Dry Transfer Cell; BioRad, CA, USA). A single piece of PVDF membrane soaked in methanol was placed below the resolving gel in the transfer cell. This arrangement of gel and PVDF membrane was sandwiched between 4 sheets of 3MM filter paper soaked in cathode buffer (25 mM Tris-base, 40 mM 6-amino-N-hexanoic acid; pH 9.4) placed at the top and 4 sheets of 3MM filter paper soaked in anode buffer (300 mM Tris-base, 20% (v/v) methanol; pH 10.4) placed at the bottom. A constant voltage of 15V was applied to this setup for 2 hours to facilitate efficient transfer of proteins from gel to membrane.

PVDF membranes were then transferred into a 5% (w/v) solution of bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween 20 (TBS-T) (20 mM Tris, 140 mM NaCl, 0.1% Tween, pH 7.6) to block the membrane for 1 hour at room temperature. Primary antibodies (concentrations for antibodies used are described in Table 2.1) were added into a 2% (w/v) solution of BSA (dissolved in TBS-T) and membranes were incubated with these solutions overnight at $4^{o}C$ on a rotator. Primary antibody solutions were removed from the PVDF membranes the next day and membranes were washed three times for 10 minutes each with TBS-T. Secondary antibodies (concentrations for antibodies used are described in Table 2.2) were added to a 2% (w/v) BSA (dissolved in TBS-T) solution, which was then added to PVDF membranes and incubated in the dark at room temperature for 1 hour. PVDF membranes were washed three times again for 5 minutes each with TBS-T. PVDF membranes were scanned using a Typhoon FLA 9500 (Amersham Biosciences, Buckinghamshire, UK), and quantification of the fluorescence intensity of individual bands was determined using Image Quant software version 8.1 (GE healthcare).

2.2.12. Immunoprecipitation

Immunoprecipitation was used to isolate proteins of interest from platelet lysates. Washed human platelets were prepared ($8x10^8$ cells/ml) as described previously. Cells were lysed on ice using an equal volume of 2X NP40 buffer (300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2% v/v NP40; pH=7.3) containing protease inhibitors [Leupeptin (10 µg/ml), aprotinin (10 µg/ml), phenylmethylsulphonyl fluoride (1 mM) sodium orthovanadate (1mM) and pepstatin-A (25 µg/ml)]. The lysed platelets in NP40 buffer (1X) were incubated with an appropriate primary antibody (concentrations for antibodies used are described in Table 2.1) and Protein A/G magnetic beads (20 µl per 500 µl of lysate) at 4°C overnight. The following day, the beads were collected in Eppendorf tube using a magnetic stand and washed twice with NP40 buffer (1X) containing protease inhibitors and once with TBST. Thereafter, 100 µl of 2X Laemmli sample reducing buffer was added to the beads. The samples were then heated to 95°C for 5 minutes and kept at -20°C for use in Western blotting

2.2.13. *In vitro* thrombus formation under flow

Human or mouse whole blood was incubated at 30°C with 5 µM of the lipophilic dye DiOC6 for 1 hour. Vena8 BioChip microfluidic channels were coated with type I collagen (100 µg/ml) for one hour and excess collagen was washed with modified Tyrodes-HEPES buffer. Whole blood was incubated with NR ligands or vehicle control (containing, DMSO 0.1% v/v DMSO) for 10 minutes (RXR ligands) or 20 minutes (PXR ligands) prior to perfusion through the collagen-coated microfluidic channels at an arteriolar shear stress of 20 Dyne/cm^2 (shear

rate: 500 s^{-1}). Fluorescence was excited at 488 nm with an argon laser and emission detected at 500-520 nm. The thrombus formation on the microfluidic chip was observed using a Nikon A1-R confocal microscope with a 20X objective and images (focused on a single section) were captured every 1 second for 600 seconds. Mean thrombus fluorescence intensity was calculated using NIS Elements software (Nikon, Tokyo, Japan).

2.2.14. Genotyping of the hPXR mice

To evaluate the presence of the hPXR gene, genotyping of each mouse was performed as per the manufacturer's protocol (Taconic Biosciences) prior to their use in experiments. Ear-clip tissue samples from humanised PXR mice were collected and used for DNA extraction for genotyping. 300 µl of the extraction mixture containing 1 mM EDTA (protects DNA from intracellular DNAase) and 1 mM NaOH (breaks open the cell membrane) was added to tissue samples and heated to 95^{o}C for 10 minutes. For Polymerase Chain Reaction (PCR), the mastermix (Table 2.3) containing appropriate primers (Table 2.4) was added to 5 µl of DNA sample irrespective of the concentration of the genomic DNA extracted from the samples. The DNA in the master mix was amplified using optimised conditions for PCR (Table 2.5). The amplified DNA was visualised (using Sybr safe DNA gel stain) following separation on 1.5% (w/v) agarose gel in TAE (Tris-base 96.8 g, acetic acid 22.84 mL, EDTA 40 mL (stock 0.5 M) in 1L) for 30 minutes at 150V. The gels were visualised using Typhoon FLA 9500 and analysed using the Image Quant software.

Table 2.3. Contents of the Mastermix

Reaction Mix		Volume (each reaction)
2X Reddy Mix contains:		12.5 µl
0.625 units	ThermoPrime *Taq* DNA Polymerase	
75 mM	Tris-HCl (pH 8.8 at 25°C)	
20 mM	$(NH_4)_2SO_4$	
1.5 mM	$MgCl_2$	
0.01% (v/v)	Tween 20	
0.2 mM	each of dATP, dCTP, dGTP and dTTP	
10 µM Primer (forward/reverse)		0.5 µl
DNA samples		5 µl
UltraPure DEPC treated H_2O		7 µl
TOTAL		**25 µl**

Table 2.4. Primers used for genotyping

Mouse type	Primers used	PCR product size (bp)
hPXR	Forward primer 5'-GGA CTT GCC CAT CGA GGA C- 3' Reverse Primer 5- ACA GGA TGG AGG GGC AGC- 3'	364
C57/BL6	Forward primer 5'-GCT TCT CAT TTC TCC CTC CTG-3' Reverse Primer 5'-TGA TCC TTT CCT GGG CAG C- 3'	733

Table 2.5. Specification of PCR thermal cycling

Step	Temp (°C)	Time (min)	No. of cycles
Hot Start	**95**	**15:00**	**1**
Denature	**94**	**0:45**	**35**
Anneal	**60**	**1:00**	
Extension	**72**	**1:00**	
Final Extension	**72**	**5:00**	**1**

2.2.15. *In vivo* thrombus formation

Thrombus formation *in vivo* was studied as described by Falati *et al.* (2002). On the day of the experiment, mice were anaesthetised by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg) and atropine (0.25 mg/kg). Anaesthesia was maintained with 5 mg/kg pentobarbital as and when required. The cremaster muscle was exteriorized and the connective tissue removed, after which an incision was made, allowing the cremaster muscle to be affixed over a glass slide as a single sheet; the muscle preparation was hydrated throughout with buffer (135mM NaCl, 4.7mM KCl, 2.7mM $CaCl_2$, 18mM NaHCO3, pH 7.4). NR ligands, vehicle control (containing, DMSO 0.1% v/v) and DyLight 649 anti-GPIbα antibody (0.2 μg/g mouse weight; for platelet labelling) was infused into the mouse circulation through carotid artery cannula prior to the injury (performed using a Micropoint Ablation Laser Unit; Andor Technology PLC, Belfast, Northern Ireland). Thrombus formation was visualised after 10 minutes (RXR ligands) or 20 minutes (PXR ligands) of the infusion of NR ligands or vehicle control using an Olympus BX61W1 microscope (Olympus Corporation, Tokyo, Japan). The images were captured both prior to and after the injury, using a

Hamamatsu digital camera C9300 (Hamamatsu Photonics UK Ltd, Hertfordshire UK) charge-coupled device (CCD) camera in 640 x 480 format. Images were analysed using Slidebook 6 software (Intelligent Imaging Innovations, CO, USA). Following the procedure, mice were sacrificed in accordance with Home office licences and approval from the University of Reading local ethics review panel and Animal welfare and Ethics Research Board. Procedures on these mice require micro-surgery expertise and were therefore performed in collaboration with Dr P. Sasikumar.

2.2.16. Tail bleeding assay

On the day of the experiment, C57/BL6 or hPXR mice were anesthetised by intraperitoneal injection of ketamine (125 mg/kg) and xylazine (12.5 mg/kg) and NR ligands or vehicle control (containing, DMSO 0.1% v/v) was injected via the femoral vein. 10 minutes (RXR ligands) or 20 minutes (PXR ligands) later, 0.5 mm of the tail tip was removed with a scalpel and the tail was immediately placed into tubes containing saline, in a manner that prevented the cut end of the tail from contacting the side of the tube. The time of bleeding was recorded until the blood flow had ceased. Following the procedure, or after 20 minutes, mice were sacrificed in accordance with Home office licences and approval from the University of Reading local ethics review panel and Animal welfare and Ethics Research Board. This assay was performed in collaboration with Ms T. Sage or Dr L. Holbrook.

2.2.17. Statistical Analyses

Statistical significance was assessed using one-way ANOVA (with post-hoc Dunnett's multiple comparison test) where more than two groups were studied. For 2-grouped comparisons, student *t*-test was performed. Two-way ANOVA (with Sidak's multiple comparisons test) was used to analyse *in vitro* thrombus formation assay. The nonparametric Mann-Whitney U test was used to analyse non-normally distributed data (tail bleeding and *in vivo* thrombosis assay). All the data are presented as mean ± SEM and $P \leq 0.05$ were considered to be statistically significant. Statistical analysis was performed using Prism software (GraphPad, San Diego, CA, version 7.00).

Chapter-3

The pregnane X receptor exhibits non-genomic effects to regulate platelet functions

3.1. Introduction

The pregnane X receptor (PXR; NR1I2) is a member of nuclear receptor (NR) superfamily, predominantly expressed in liver and intestines, primary organs responsible for metabolism and elimination of xenobiotics (pharmaceuticals, environmental, dietary, and occupational chemicals) and toxic endogenous compounds (such as bilirubin and bile salts) in mammalian species (Ma et al., 2008). Upon encountering such compounds, PXR becomes activated and regulates the activity of phase I and phase II drug/xenobiotic metabolising enzymes and transporters involved in detoxification process (Kliewer, 2003).

NRs, in general, are known to be highly selective to their ligand. PXR, however, is an exception to this rule and displays a high level of promiscuity with respect to the choice of ligand. This is because of the presence of a large and flexible ligand-binding pocket (size >1300 $Å^3$), which can accommodate a diverse spectrum of lipophilic substances that includes prescription drugs, dietary supplements, environmental pollutants, endogenous hormones, and bile acids (Timsit and Negishi, 2007). The level of promiscuity is reflected in the receptor affinity. Consequently, the concentration of ligands needed to activate PXR is generally two or three orders of magnitude higher than concentrations found circulating in plasma (Iyer et al., 2006). The requirement of higher concentrations is an adaptive response, for instance, lithocholic acid is a hepatotoxic secondary bile acid, which at lower concentrations does not activate PXR and helps to solubilise fats for absorption (Ajouz et al., 2014). However, at higher concentrations of around 100 μM, PXR activation by lithocholic acid upregulates

CYP3A enzymes that stimulate its detoxification and elimination, and hence prevent its hepatotoxic effects (Iyer et al., 2006; Staudinger et al., 2001).

A critical step that regulates the activity of PXR in nucleated cells is its interaction with the retinoid X receptor (RXR) to form a heterodimer, the absence of which affects its binding to DNA, which ultimately modulates the rate of transcription of target genes (Ihunnah et al., 2011). PXR shares structural features that are typical to all NRs, consisting of an N-terminal domain containing a highly conserved DNA binding domain, a hinge domain followed with a C-terminal ligand binding domain (LBD) (Orans et al., 2005).

Increasing evidence suggests that PXR, beyond drug metabolism, can also regulate several physiological (glucose, lipid and bile acid metabolism) and pathophysiological processes such as metabolic disorders (type 2 diabetes and obesity) and cardiovascular diseases (atherosclerosis) (di Masi et al., 2009; Gao and Xie, 2010; Wallace and Redinbo, 2013). PXR ligands have been proposed to promote cholesterol efflux and HDL production, both of which are credited with anti-atherosclerotic effects (de Haan et al., 2009; Li et al., 2007; Zhou et al., 2009a; Masson et al., 2005). Moreover, recently, the presence of PXR was reported in the human vasculature (Swales et al., 2012). Given the central role of platelets towards the initiation of cardiovascular disorders such as atherosclerosis and presence of several NRs in platelets (and its binding partner RXR) (Moraes et al., 2007), we explored whether PXR is present in human platelets and evaluated the potential role of its ligands in regulating platelet function.

3.2. PXR is expressed in human and mouse platelets

In humans, PXR is encoded by the NR1I2 (Nuclear receptor subfamily 1, group I, member 2) gene. PXR exist in two spliced isoforms – PXR.1 and PXR.2. Of these, PXR.1 comprises 434 amino acid residues and is widely distributed and characterised for its functions. The PXR.2 isoform has 37 fewer amino acid residues in its ligand-binding domain (LBD), which reduces its ligand-binding ability. The physiological role of PXR.2 is not yet fully understood (Lamba et al., 2004; Lin et al., 2009). Similarly, two isoforms of PXR have been identified in mouse. PXR.1 is composed of 431 amino acid residues and the truncated PXR.2 LBD (in this case, 41 amino acid residues shorter) makes it less responsive towards its ligands (Kliewer et al., 1998; Laudet and Gronemeyer, 2002).

To study the functions of PXR ligands in platelets, we firstly investigated the expression of PXR in both human and mouse platelets. An immunoblot analysis using a rabbit polyclonal antibody (SantaCruz; sc-25381) raised against PXR amino acids 101-260 of human origin confirmed the presence of PXR in human and mouse platelets. HEK-293 cells transfected with human PXR (PXR-293) (SantaCruz; sc-158906) were used as a positive control. As shown in figure 3.1, a protein band of approximately 45 kDa was observed in PXR-293 cell lysates. Bands of similar size (next to the positive control) detected in human (figure 3.1a) and mouse platelet lysates (figure 3.1b), suggested the expression of PXR in human and mouse platelets. However, the signal of PXR observed was quite weak, therefore, the presence of PXR in human platelets was further validated by immunoprecipitation (IP) assay, where PXR was immunoprecipitated using a mouse monoclonal anti-PXR antibody (Abcam; ab41930), targeting amino acids 1-

40 and blotted with a rabbit polyclonal antibody (SantaCruz; sc-25381) targeting amino acids 101-260 of PXR. The antibody used for IP was used as a negative control (Figure 3.1c). In the IP sample of human platelets, a band of approximately 70 kDa was obtained as against 45 kDa seen in the immunoblot analysis. This may perhaps be due to the possibility of PXR existing in a bound form with other proteins, which upon treatment with a mild NP40 buffer used during the IP assay cause PXR to be pulled out in a complex form. No band was observed in the negative control, which excludes IgG contamination.

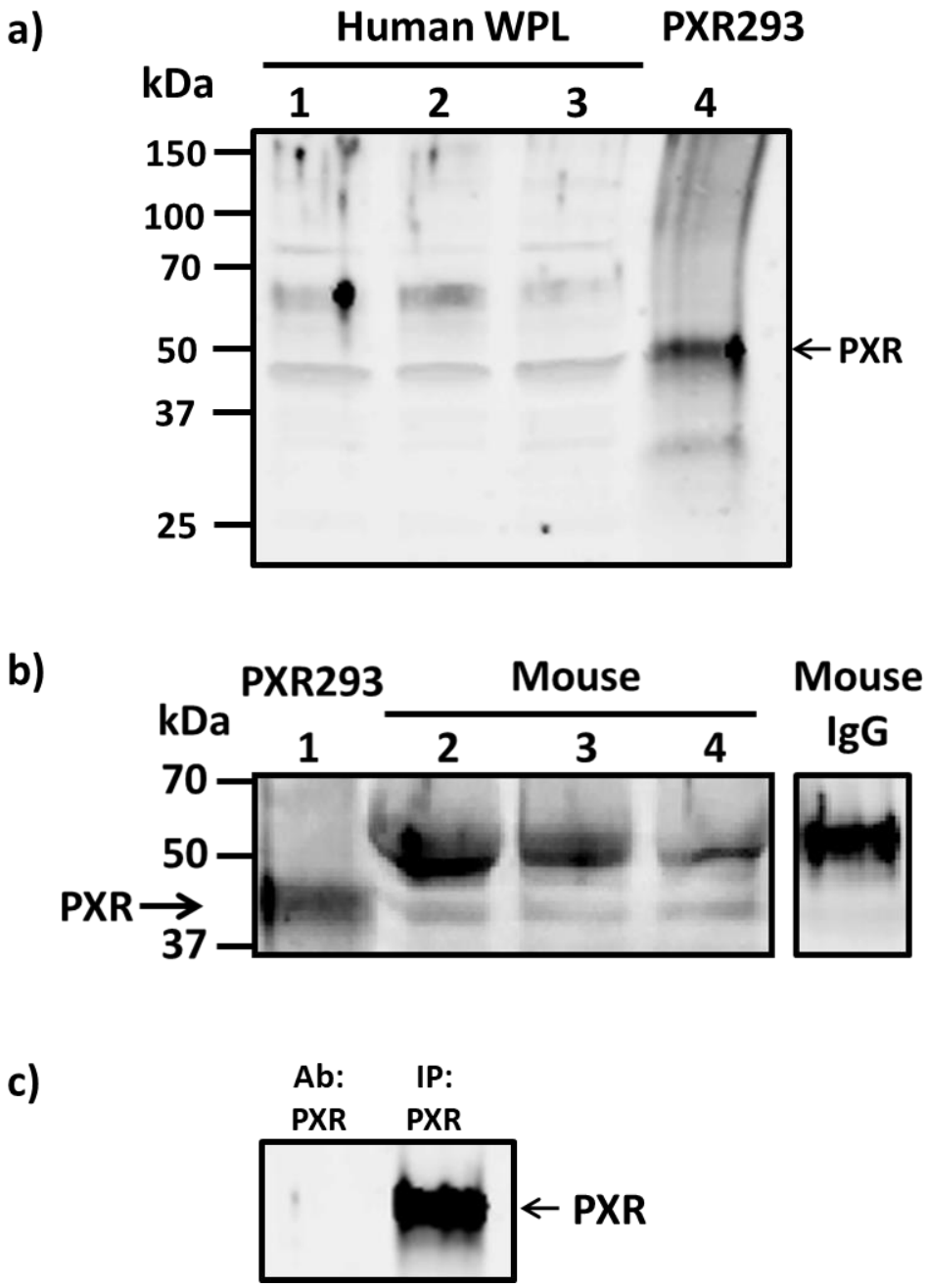


Figure 3.1: PXR is present in human and mouse platelets. (a) The presence of PXR was examined by immunoblot analysis of human whole platelet lysates (WPL) using a rabbit polyclonal anti-PXR antibody (targeting amino acids 101-260). Human PXR transfected 293 lysate was used as a positive control. **(b)** The presence of PXR was also explored in mouse platelet lysates using the same antibody and human PXR transfected 293 lysate was used a positive control. **(c)** Additionally, PXR was immunoprecipitated (IP) from human platelets (IP: PXR) using a mouse monoclonal antibody (targeting amino acids 1-40) and blotted with a rabbit polyclonal antibody (targeting amino acids 101-260). The antibody used for IP was used as a negative control (Ab: PXR). Data are representative of 3 separate experiments using different donors/mice.

3.3. The localisation of PXR in human and mouse platelets

Following the discovery of the presence of PXR in human and mouse platelets, its sub-cellular localisation was investigated in resting and activated human platelets using immunofluorescence microscopy. There is a substantial difference in the morphology and activity of resting and activated platelets, which may influence the distribution of PXR. Therefore, studying the localisation of PXR under both these conditions would be important in understanding its cellular functions.

Resting and activated (with 5 μM U46619 in the presence of integrilin) platelets (in PRP) were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton X-100. U46619 was used as an agonist because it stimulates gentle activation of platelets with minimal shape change, which is helpful in studying the distribution of NRs. Samples were then incubated with a rabbit polyclonal anti-PXR antibody (Abcam, ab85451) to identify the distribution of PXR, while; platelets were stained using a mouse monoclonal anti-GPIb antibody (ThermoFisher; PM6/248), which marks the surface of platelets. The secondary antibodies conjugated with Alexa Fluor 647 and Alexa Fluor 488 were used for visualisation of PXR and GPIb respectively. Human platelets without any primary antibody treatment were used as a negative control. The samples were visualised using a Nikon A1-R confocal microscope (100X oil immersion lens).

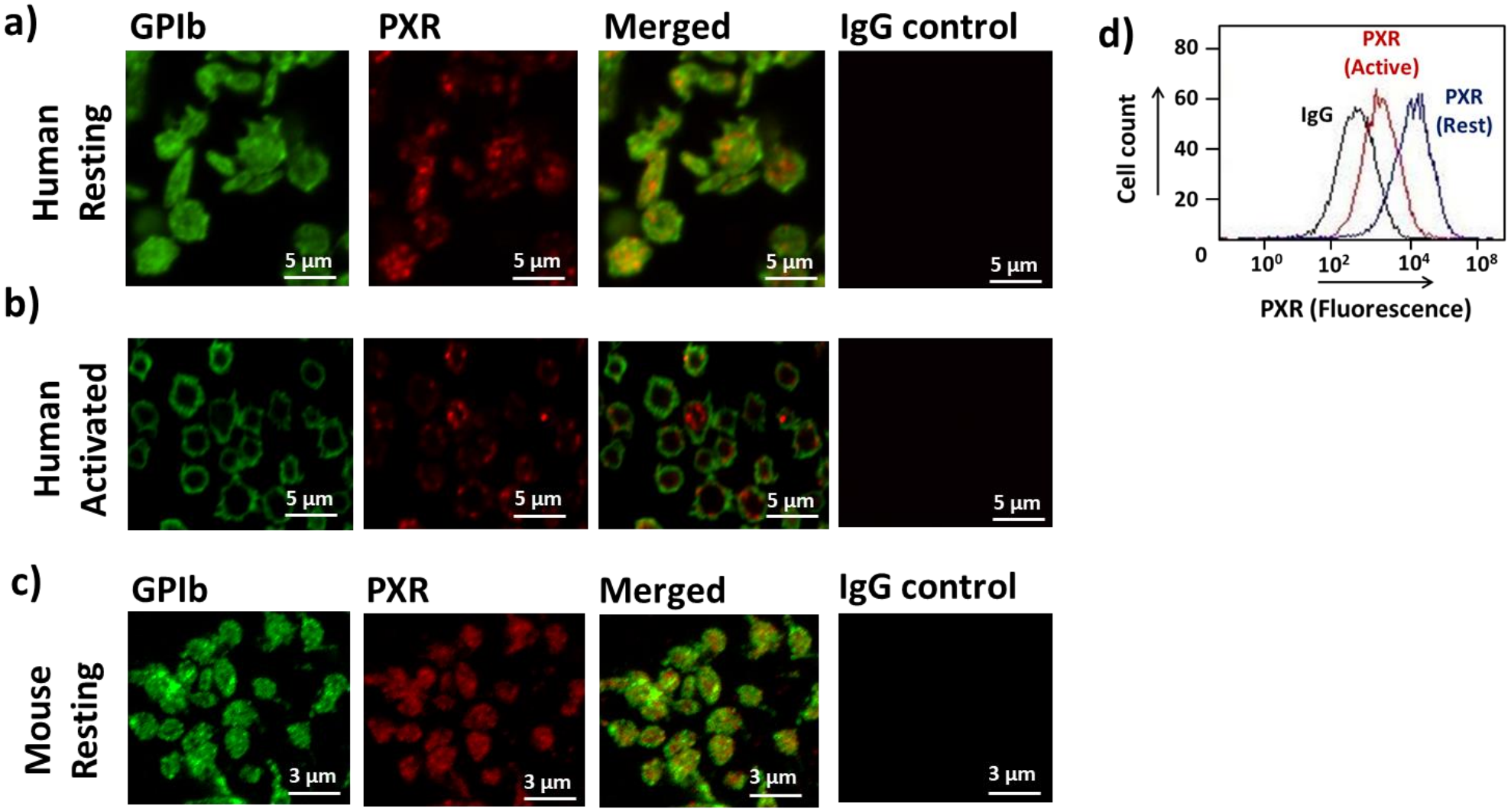
Under resting conditions, PXR (red) was found to be uniformly distributed inside the cytosol of platelets (green colour marks the surface of platelets) in a punctate arrangement (Figure 3.2a). Upon activation of platelets, PXR appeared to

relocate towards the plasma membrane along with a reduction in staining (Figure 3.2b). These observations are in alignment with recent findings on FXR, which displayed a punctate arrangement inside the resting platelet cytosol, while a translocation towards the plasma membrane was observed with reduced staining in activated platelets (Moraes et al., 2016).

The apparent reduction in staining was attributed to the possibility that PXR is released from platelets upon their activation. Indeed, the release of NRs such as RXR and PPARγ in the form of microparticles from activated platelets (stimulated by thrombin, collagen or ADP) has been reported (Ray et al., 2008). These findings were further validated by flow cytometry analysis. Resting and activated permeabilised platelets were incubated with a PXR antibody (Abcam; ab85451) or the equivalent rabbit IgG control and the median fluorescence was examined. It was observed that the level of fluorescence associated with PXR in activated platelets (0.1 U/ml thrombin) was lower (a shift in fluorescence profile towards left) when compared with resting platelets (Figure 3.2d). This suggests a reduction in the number of PXR molecules present inside the platelets post activation by thrombin, consistent with its release.

Immunofluorescence studies performed on resting and permeabilised mouse platelets incubated with rabbit polyclonal anti-PXR (SantaCruz; sc-25381) and goat polyclonal anti-GPIb (Santa Cruz; sc-6602) antibodies displayed a similar kind of punctate arrangement of PXR inside the platelet cytosol (Figure 3.2c).

Figure 3.2: Trafficking of PXR in human platelets. The localisation of PXR in human resting, activated (with 5 μM U46619 in the presence of integrilin) and resting mouse platelets (resting) was investigated using immunofluorescence microscopy. Platelets were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton-X-100. PXR (in red) and membrane GPIb receptors (in green) were stained using anti-PXR and anti-GPIb antibodies. Secondary antibodies conjugated to Alexa-647 and Alexa-488 were used to visualize PXR and GPIb, respectively. Platelets without primary antibody treatment were used as negative controls. The samples were visualised using a Nikon A1-R confocal microscope (100X oil immersion lens). Figures represent the distribution of PXR in **(a)** resting and **(b)** activated human platelets. **(c)** The localisation of PXR in resting mouse platelets. **(d)** The fluorescence level of PXR was measured in permeabilised resting and activated (with 0.1 U/ml thrombin) human platelets using flow cytometry. Data are representative of >3 separate experiments.


a)
GPIb
PXR
Merged
IgG control
Human Resting
5 µm
b)
Human Activated
c)
Mouse Resting
3 µm
d)
PXR (Active)
PXR (Rest)
IgG
Cell count
PXR (Fluorescence)

3.4. PXR and RXR form a heterodimer in platelets

The formation of heterodimers between the RXR and numerous other non-steroid NRs (such as PPARs, FXR, PXR, LXR etc.) has been shown to occur in a range of cell types (Evans and Mangelsdorf, 2014). Binding of the NRs with RXR, forming a heterodimeric complex is pivotal for the attachment of NRs to their respective specialised sites on DNA, dedicated towards the initiation of transcription and genomic regulation (Rastinejad et al., 2013). While the presence of such heterodimers in nucleated cells is well studied for their functions, little is known about their existence in the anucleated cell such as platelets. One previous study by Ray *et al.* (2008) reported the existence of RXR-PPARγ complex in resting and activated human platelets. Based on this, we investigated, whether interactions exist between RXR and PXR in human platelets.

Coimmunoprecipitation (Co-IP) studies were performed, where an anti-RXR mouse monoclonal antibody (SantaCruz; sc46659) was used to isolate RXR from resting and activated human platelets. This was followed by a western blot analysis using an anti-PXR rabbit polyclonal antibody (SantaCruz; sc-25381) to determine whether PXR and RXR interact with each other. An equivalent amount of anti-RXR antibody was used a negative control. A secondary antibody (Abcam; ab131366) that does not identify denatured IgG was used for targeting the primary antibodies to avoid the detection of any IgGs that may have been present in the samples. The PXR was found to coimmunoprecipitate with RXR from both resting and activated platelets (Figure 3.3). No significant difference in the level of PXR associated with RXR in resting and activated platelets was observed.

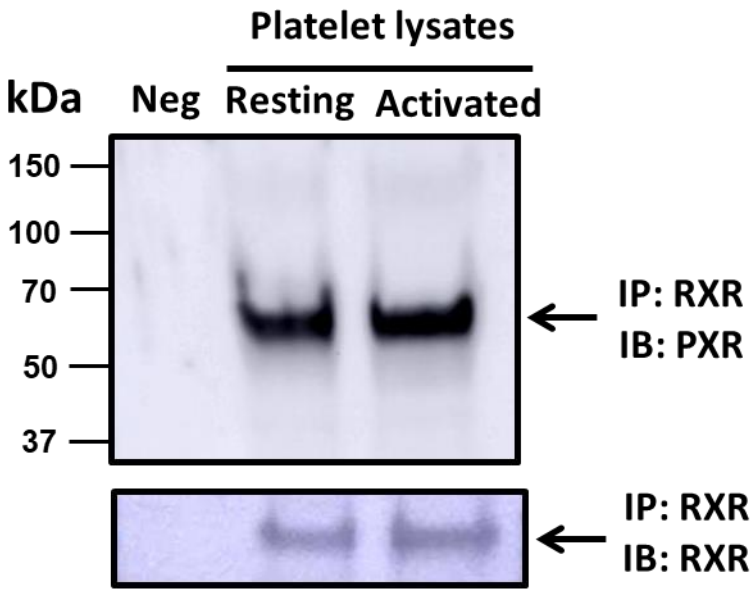


Figure 3.3: PXR is associated with RXR in resting and activated human platelets. Presence of RXR-PXR heterodimers was investigated in human platelets using a Co-IP assay. Human washed platelets ($8x10^8$ cells/ml) were lysed in NP40 buffer before immunoprecipitation of RXR using a mouse monoclonal anti-RXR antibody overnight at 4°C in the presence of protein A/G magnetic beads. Isolated proteins were subjected to SDS–PAGE and then western blotted onto a PVDF membrane. Immunoblot analysis was followed with the addition of a rabbit polyclonal anti-PXR antibody and its detection using a secondary antibody that does not recognize denatured IgG. Presence of RXR was also confirmed in the same samples. An equivalent amount of anti-RXR antibody was used as a negative control to exclude IgG contamination (Neg). Data are representatives of 3 separate experiments using platelets from different donors.

To further verify these findings, immunofluorescence microscopy was performed on resting and activated permeabilised human platelets to determine whether both proteins co-localise in platelets. The resting and activated (with 5 μM U46619 in the presence of integrilin) platelets (in PRP) were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton X-100. Samples were then incubated with a mouse monoclonal anti-RXR antibody (Santa Cruz; sc46659) and rabbit polyclonal anti-PXR antibody (Abcam; ab85451) to identify the distribution of RXR and PXR respectively, while, platelets were stained using a goat polyclonal anti-GPIb antibody (Santa Cruz; sc-6602), which marks the surface of platelets. The secondary antibodies conjugated with Alexa Fluor 647, Alexa Fluor 568 and Alexa Fluor 488 were used for visualisation of RXR, PXR and GPIb respectively. The samples were visualised using a Nikon A1-R confocal microscope (100X oil immersion lens).

Consistent with our previous findings, in resting platelets, both RXR and PXR were observed to be distributed uniformly in the cytosol in a characteristic punctate arrangement (Figure 3.4a), whereas, they appeared to migrate towards the plasma membrane upon activation with a reduction in staining (Figure 3.4b). A high degree of colocalisation between RXR (stained in red) and PXR (stained in blue) was seen in both resting and activated platelets (stained green for GPIb), which is indicated by pink colour in the merged image.

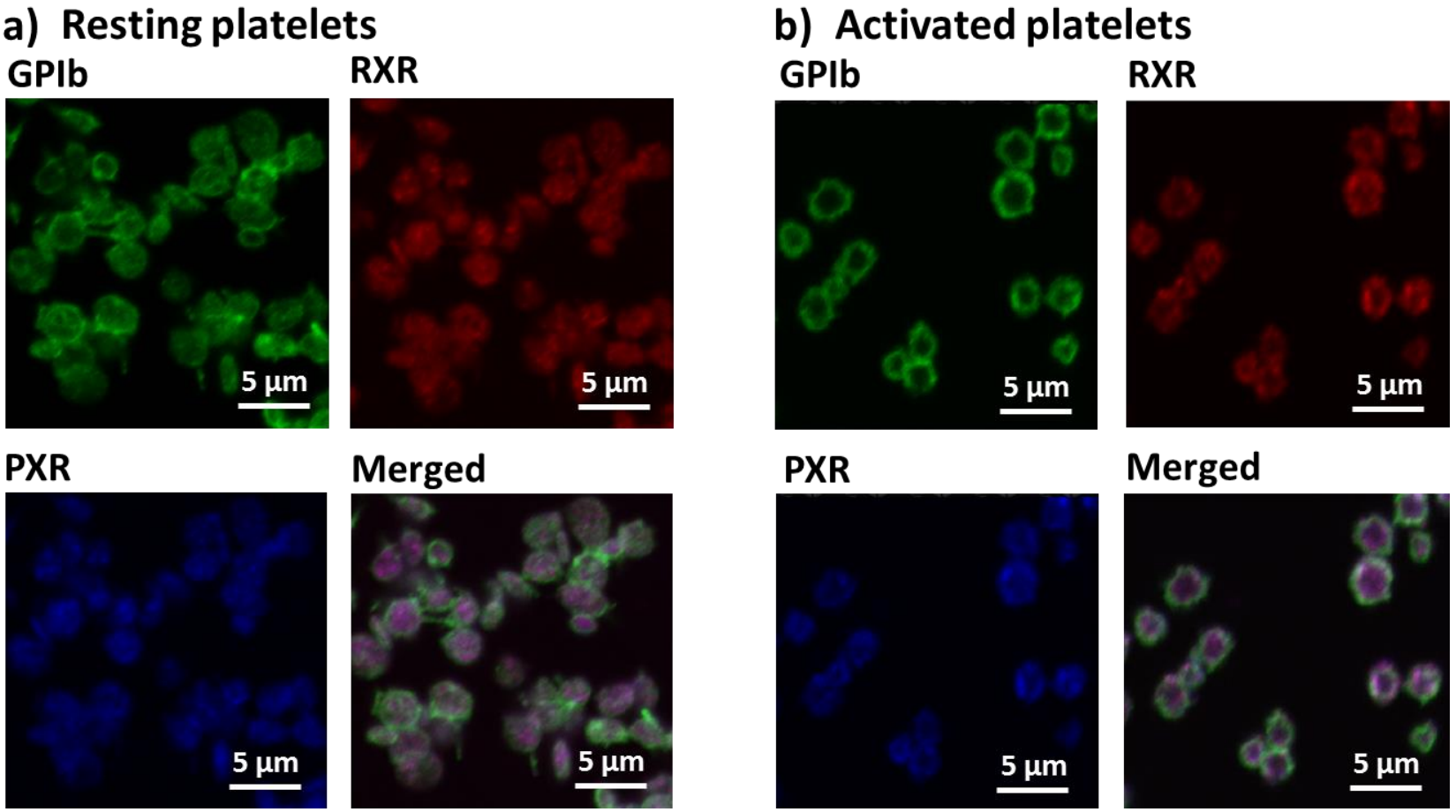


Figure 3.4: PXR and RXR are colocalised within human platelets. The potential colocalisation of PXR and RXR in resting and activated (with 5 µM U46619 in the presence of integrilin) human platelets was investigated using immunofluorescence microscopy. Platelets were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton-X-100. RXR (in red), PXR (in blue) and membrane GPIb receptors (in green) were stained using anti-RXR, anti-PXR and anti-GPIb antibodies respectively. Secondary antibodies conjugated to Alexa-647, Alexa-568 and Alexa-488 were used to visualize RXR, PXR and GPIb, respectively. The samples were visualised using a Nikon A1-R confocal microscope (100X oil immersion lens). Figures represent the distribution of RXR and PXR in **(a)** resting and **(b)** activated platelets. Data are representative of >3 separate experiments.

The results of colocalisation were analysed using scatter plots, which graphically represent the fluorescence intensity of one colour plotted against the fluorescence intensity of the second colour associated with each pixel. In case of a proportional colocalisation, the points of the scatterplot cluster around a straight line (45 degrees to either axis) and the slope represent the ratio of two fluorescence intensities. Whereas, lack of colocalisation results in a distribution of points into two separate, unrelated groups on either side of the straight line (Dunn et al., 2011). A high level of colocalisation was observed between RXR and PXR in both resting and activated platelets, with a distribution of fluorescence intensity points clustering proportionally around a straight line as represented in the scatter plot (prepared using NIS element software, Nikon) (Figure 3.5a).

The Pearson correlation coefficient (PCC) is another parameter, which is used to quantify the degree of colocalisation between different fluorophores. The PCC ranges between -1 to +1. A value of 0 represents a lack association between the two fluorescence signals, a value greater than 0 indicates a positive association (proportional increase between the two fluorescence intensities), and a value less than 0 indicates a negative association (an increase in the value of one variable is followed by a decrease in the value of other) (Adler and Parmryd, 2010). Average values of the PCC were found to be 0.94 and 0.92 between RXR and PXR in resting and activated platelets respectively, representing a high degree of colocalisation (Figure 3.5b). Moreover, there appeared to be a significant difference in the PCC values corresponding to RXR-GPIb and PXR-GPIb colocalisation in resting and activated platelets. Activated platelets displayed a higher value of PCC (0.76±0.02 for RXR-GPIb and 0.71±0.02 for PXR-GPIb) in contrast to resting (0.57±0.03 for

RXR-GPIb and 0.56±0.03 for PXR-GPIb), consistent with the migration of NRs towards the plasma membrane upon platelet activation.

These findings reinforce the conclusion that PXR becomes translocated in activated platelets and moves from a uniform punctate distribution in the cytosol, towards the plasma membrane.

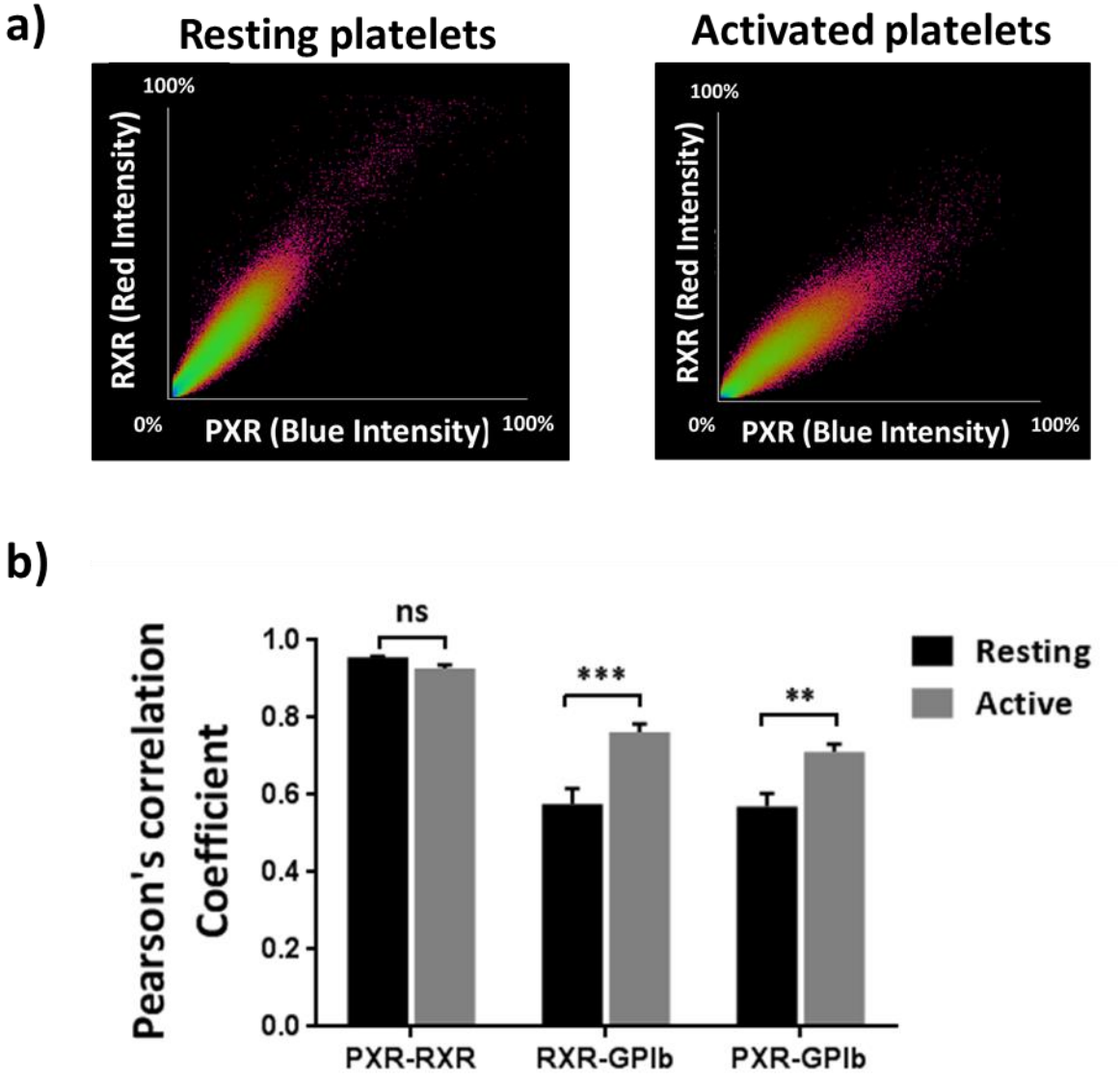


Figure 3.5: PXR and RXR are colocalised within human platelets. (a) The extent of colocalisation represented using scatter plots between the fluorescence intensity points of RXR and PXR in resting and activated platelets. **(b)** The Pearson correlation coefficient (PCC) representing the degree of colocalisation between RXR-PXR, RXR-GPIb and PXR-GPIb in resting and activated (5 μM U46619) platelets. PCC was quantified for >10 platelets using different fields. Data represent mean ± SEM, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by Student T-test.

3.5. PXR ligands inhibit platelet aggregation

Having established the presence and location of PXR in platelets, we sought to determine whether this receptor is fundamental in the cells and thereby modulate their function. Upon vascular injury, collagen in the subendothelial matrix gets exposed and initiates platelet aggregation process. The potential effects of PXR ligands, rifampicin and SR12813 on platelet aggregation in response to collagen were therefore evaluated.

Both the PXR ligands used, rifampicin and SR12813 are structurally distinct with rifampicin having a larger structure and high molecular weight (822.94 g/mol) in comparison to SR12813 (504.53 g/mol). Rifampicin is regarded as a classical activator of PXR, whereas, SR12813 is a bisphosphonate ester used to experimentally lower serum cholesterol levels (Goodwin et al., 2002). Both the ligands are strong activators of PXR and share comparable levels of potency with SR12813 being slightly more potent than rifampicin (EC_{50} of rifampicin - 0.7 μM and SR12813 - 0.2 μM) determined through cell-based reporter assays (Jones et al., 2000; Moore and Kliewer, 2000). It is important to note here that these EC_{50} values were determined based on the genomic regulation of PXR by these ligands. Figure 3.6 shows the structure of commonly used PXR ligands.

To maintain the consistency of results amongst donors, the concentration of collagen used to stimulate platelets was optimized for each donor to attain 50% of the maximum level of aggregation (EC_{50}) in 5 minutes (actual concentrations ranged between 0.5-0.8 μg/ml). Human washed platelets ($4x10^8$ cells/ml) were incubated with a range of concentrations of SR12813 (10, 20, 50 and 100 μM) or

vehicle (containing DMSO, 0.1% v/v) for 10 minutes prior to stimulation with collagen.

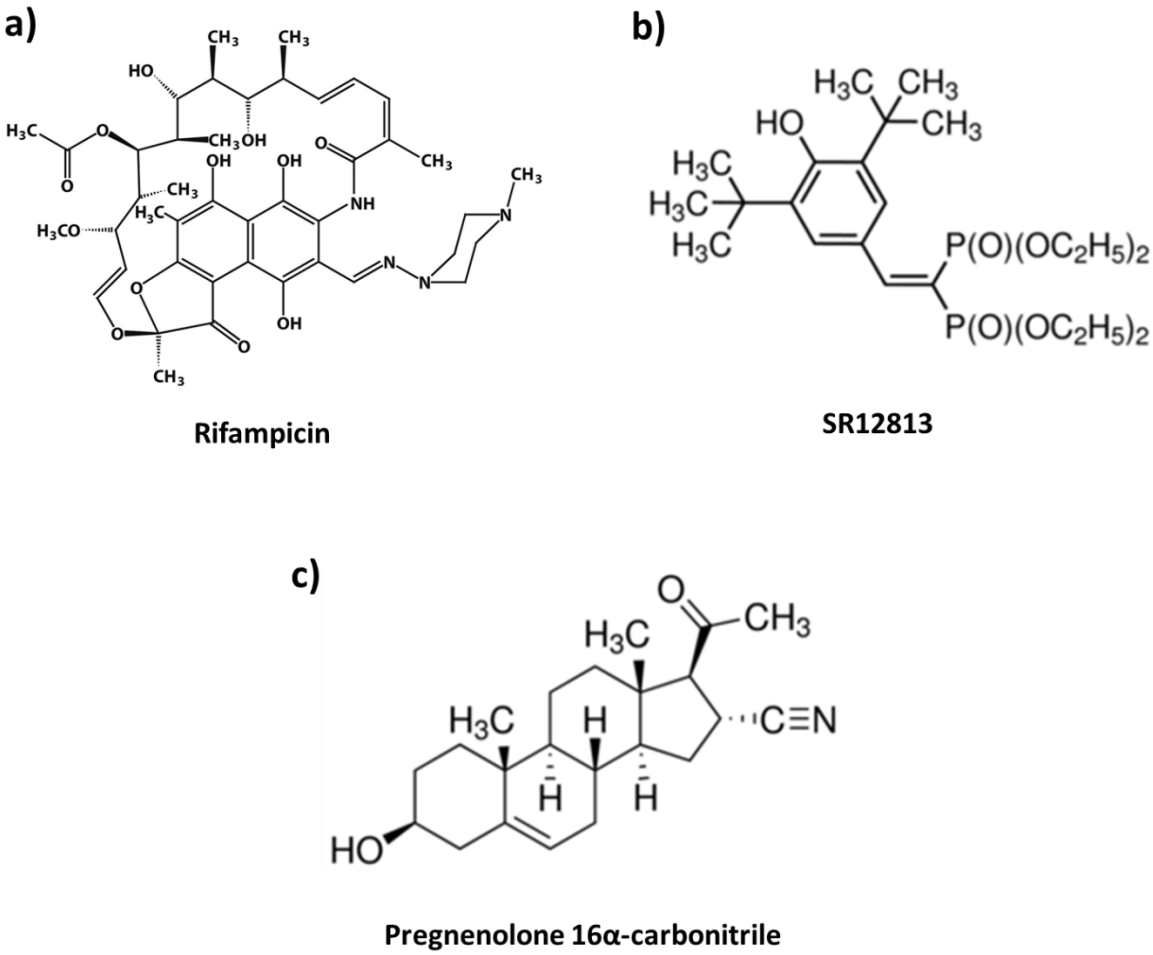


Figure 3.6: Structure of PXR ligands. (a) Rifampicin is an antibiotic, which is used for the treatment of tuberculosis and is regarded as the classical activator of human PXR. **(b)** SR12813 is an experimentally used cholesterol-lowering drug and a potent activator of human PXR. **(c)** Pregnenolone 16α-carbonitrile is a strong activator of mouse PXR.

Aggregation responses were recorded using an optical aggregometer with constant stirring (1200 rpm) for 5 minutes at 37^{o}C. Approximately, 27% and 39% reduction in aggregation was observed with 50 and 100 μM of SR12813 respectively in comparison to vehicle-control (containing DMSO, 0.1% v/v) (Figure 3.7 ai, aii). An increase in the incubation period of SR12813 from 10 to 20 minutes enhanced the degree of inhibition to 41% and 62% with 50 and 100 μM respectively (Figure 3.7 bi, bii). Aggregation responses recorded for 5 minutes demonstrated this effect to represent inhibition rather than a delay in aggregation. Furthermore, aggregation responses in the presence of 50 and 100 μM SR12813 (20 mins incubation) were found to be reversible, probably due to a lack of secretion caused by these ligands.

To ascertain that the effects displayed by SR12813 are mediated through PXR, the second and structurally unrelated ligand, rifampicin was tested. Human platelets incubated for 10 minutes with rifampicin (10, 20, 50 and 100 μM) or vehicle (containing, DMSO, 0.1% v/v) inhibited collagen-mediated platelet aggregation by 20% and 25% at 50 μM and 100 μM, respectively (Figure 3.7 ci, cii). In support of previous observations, a higher incubation period of 20 minutes with rifampicin resulted in a stronger inhibition of 43% and 67% at 50 μM and 100 μM respectively (Figure 3.7 di, dii). Therefore, the extent of inhibition by both PXR ligands was extremely similar with each ligand exhibiting reversible aggregation. It can be speculated that such robust effects of PXR ligands noticed with prolonged incubation periods reflect their rate of transit across the plasma membrane.

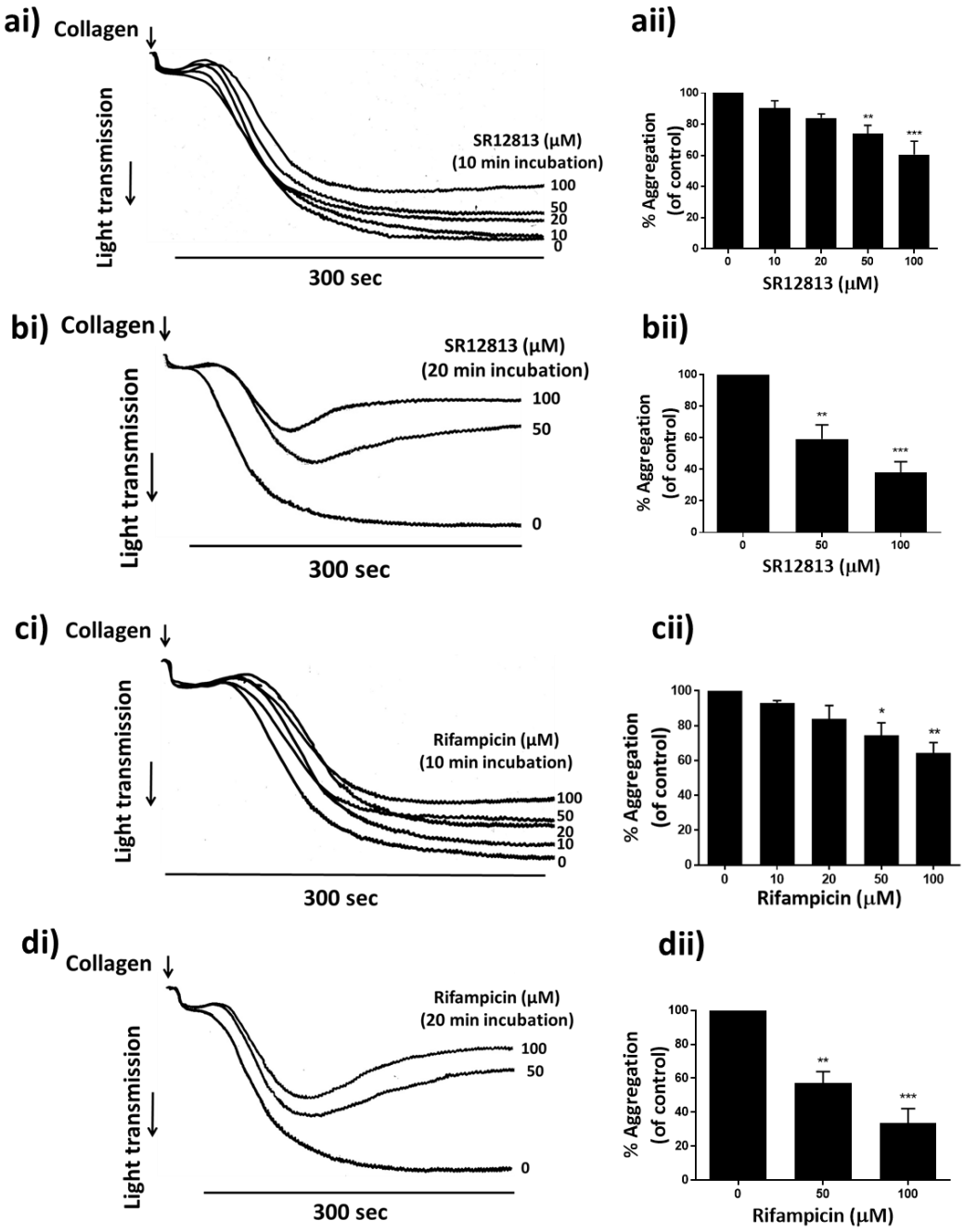


Figure 3.7: PXR ligands inhibit collagen-mediated platelet aggregation. Washed human platelets (4×10^8 cells/mL) were incubated with SR12813, rifampicin or vehicle (containing DMSO, 0.1% v/v) prior to their stimulation with collagen (EC_{50} ranged between 0.5 - 0.8 μg/ml). Aggregation was measured as a change in light transmission and monitored for 300 seconds at 37°C under constant stirring (1200 rpm). Representative aggregation traces of platelets treated with **(ai, bi)** SR12813 or **(ci, di)** rifampicin for 10 and 20 minutes and stimulated with collagen are shown. Quantified data displays the percentage of aggregation for **(aii, bii)** SR12813 or **(cii, dii)** rifampicin treated samples (vehicle-treated samples represent 100% aggregation) at the end of 5 minutes. Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

Platelets release numerous pro-thrombotic substances upon activation by collagen *in vivo* (such as thrombin, ADP and TxA_2). Apart from their release from activated platelets, thrombin is also generated locally at the site of injury via the coagulation cascades. All these agonists potentiate the aggregation response and platelet plug formation by activating more platelets circulating in the vicinity. These agonists signal via GPCRs present on the platelet surface. Therefore, we investigated the effects of PXR ligands on these GPCR agonists in order to determine whether the actions of PXR are restricted to GPVI receptor-stimulated signalling or represent a general mechanism of platelet inhibition.

The effects of PXR ligands on aggregation stimulated by thrombin were evaluated. Similar to collagen, the concentration of thrombin was optimized for each donor to produce 50% aggregation (EC_{50}) as maximum aggregation response in 5 minutes (actual concentration ranged between 0.03 – 0.04 U/ml). 10 minutes of treatment with 50 and 100 μM of SR12813 inhibited thrombin-evoked platelet aggregation by 17% and 26% respectively in 5 minutes, in comparison to vehicle-control (containing DMSO, 0.1% v/v) (Figure 3.8 ai, aii). Whereas, a reduction of 27% and 38% was achieved by 50 and 100 μM of rifampicin respectively in 5 minutes (Figure 3.8 bi, bii). Increase in incubation period had no further effect on the degree of platelet aggregation. Also, a slight increase in the thrombin concentration above the EC_{50} (for instance, 0.05 U/ml), almost completely overcame the inhibitory effects of PXR ligands. So, the inhibitory effects of PXR ligands were visible only at lower concentrations of thrombin.

Also, both SR12813 and rifampicin exhibited a stronger inhibition for up to 3 minutes from the beginning of aggregation, in contrast to 5 minutes. At 3-minutes post stimulation by thrombin, SR12813 was noted to diminish aggregation by 36% and 48% (Figure 3.8 aiii), while; rifampicin exhibited inhibition of 38% and 45% at 50 and 100 μM respectively, in comparison to vehicle-control (Figure 3.8 biii). This stronger inhibition exhibited by SR12813 and rifampicin for up to 3 minutes of thrombin stimulation might be due to the effects of PXR ligands in reducing the release of pro-thrombotic secondary mediators (such as ADP and TxA_2).

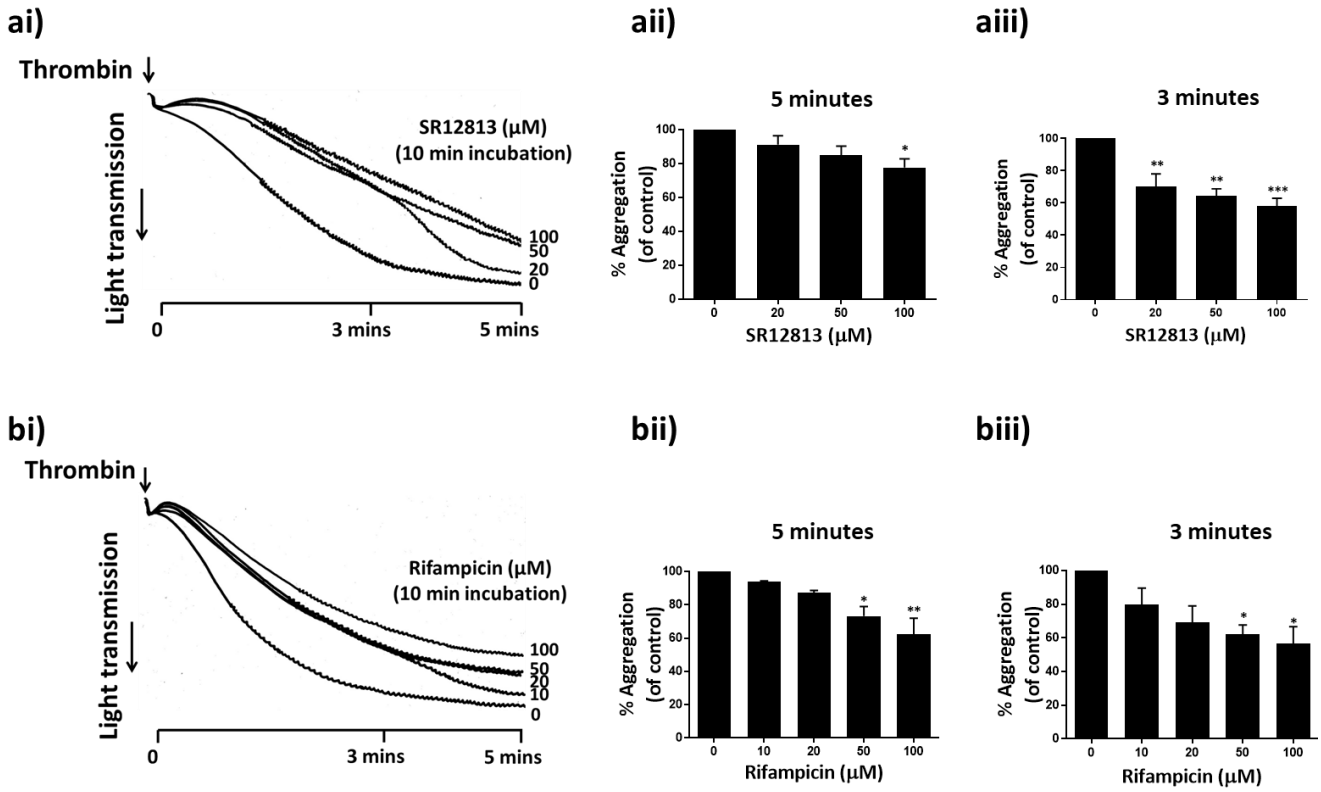


Figure 3.8: PXR ligands inhibit thrombin-mediated platelet aggregation. Washed human platelets (4×10^8 cells/mL) were incubated with SR12813, rifampicin or vehicle (containing, DMSO 0.1% v/v) prior to their stimulation with thrombin (EC_{50} ranged between 0.03–0.04 U/ml). Aggregation was measured as a change in light transmission and monitored for 300 seconds at 37°C under constant stirring (1200 rpm). Representative aggregation traces of platelets treated with **(ai)** SR12813 or **(bi)** rifampicin and stimulated with thrombin are shown. Quantified data displays the percentage of aggregation attained by **(aii, aiii)** SR12813 or **(bii, biii)** rifampicin treated samples in 5 mins and 3 mins (vehicle-treated samples represent 100% aggregation). Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

Besides thrombin, the effects of PXR ligands on aggregation instigated by other GPCR agonists, ADP and TxA_2 were also studied. Both ADP and TxA_2 are considered as weaker agonists in comparison to collagen and thrombin. However, they play a vital role in amplifying the aggregation responses. Defects in their release can significantly reduce the aggregation response of platelets *in vivo*. Since, TxA_2 has a short half-life, the TP receptor agonist, U46619 was used instead to evaluate the effects of PXR ligands on stimulation through this pathway. Both SR12813 (Figure 3.9 ai, aii) and rifampicin (Figure 3.9 bi, bii) were able to attenuate U46619- (0.2 μM, EC_{50} determined for each donor) mediated platelet aggregation by approximately 35% and 55% at 50 μM and 100 μM respectively, in comparison to vehicle-treated control (containing DMSO, 0.1% v/v). Additionally, treatment with SR12813 (Figure 3.9 ci, cii) or rifampicin (Figure 3.9 di, dii) negatively regulated platelet aggregation stimulated by ADP (5-10 μM, EC_{50} range determined for each donor) by approximately 50% and 65% at 50 and 100 μM respectively, in comparison to vehicle-control. An increase in the incubation period of PXR ligands (20 minutes) did not cause additional inhibition of ADP or U46619-mediated platelet aggregation. Similar to collagen and thrombin, the effects of PXR ligands were visible only at EC_{50} concentrations, while little or no effects were observed at concentrations higher than EC_{50}.

Based on these findings, we conclude that stimulation by PXR ligands negatively regulates platelet aggregation evoked by a range of agonists that function through different receptors. Since an increased incubation period (20 minutes) of PXR ligands resulted in additional inhibitory effects on collagen-stimulated platelet aggregation only, it might be plausible that their effects on

platelet activation are mediated more prominently downstream of the GPVI receptor.

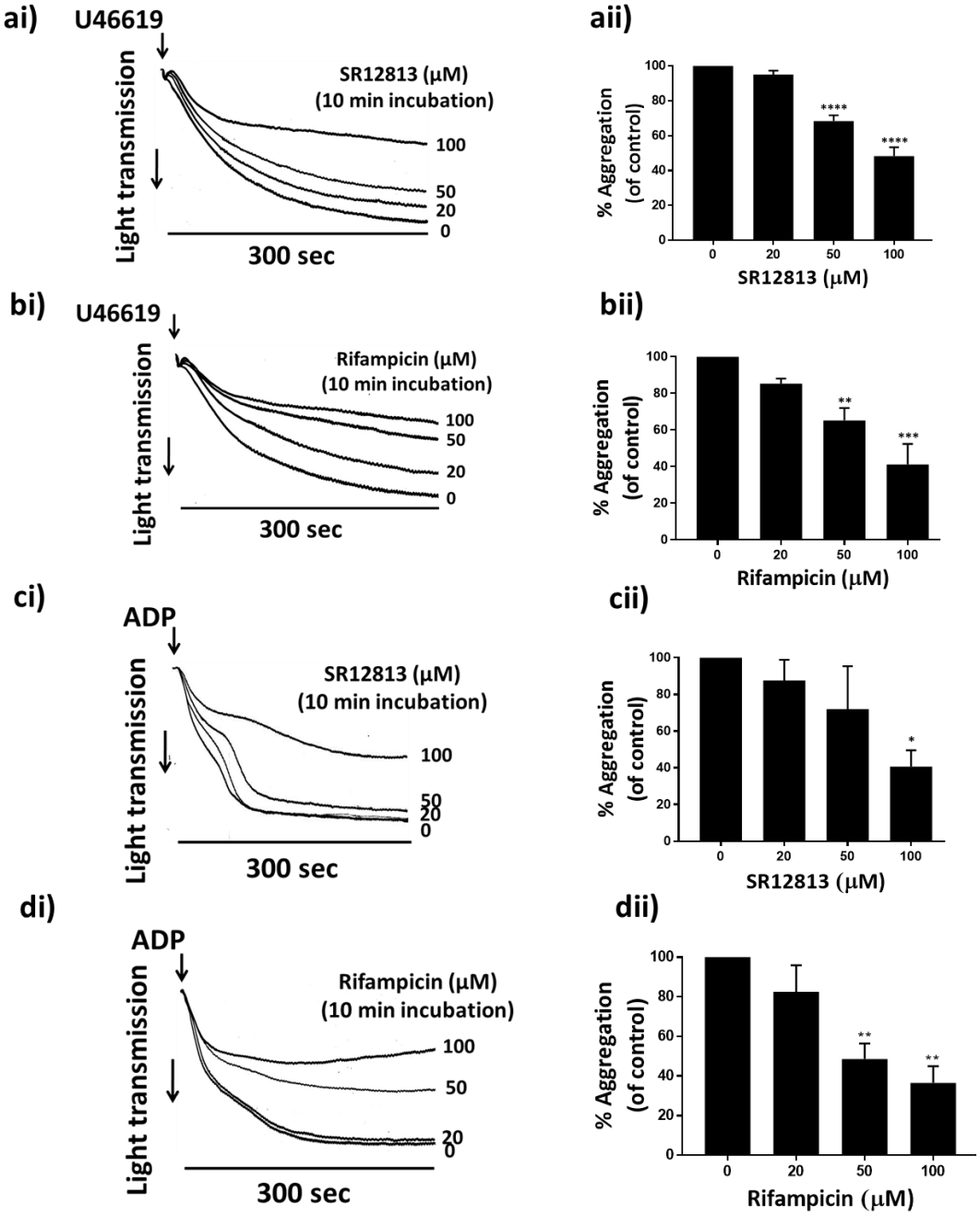


Figure 3.9: PXR ligands inhibit U46619 and ADP mediated platelet aggregation. Washed human platelets (4×10^8 cells/mL) were incubated with SR12813, rifampicin or vehicle (containing DMSO, 0.1% v/v) prior to their stimulation with U46619 (EC_{50} 0.2 μM) or ADP (EC_{50} ranged between 5-10 μM). Aggregation was measured as a change in light transmission and monitored for 300 seconds at 37°C under constant stirring (1200 rpm). Representative aggregation traces of platelets treated with SR12813 or rifampicin and stimulated with **(ai, bi)** U46619 or **(ci, di)** ADP are shown. Quantified data displays the percentage of aggregation attained by SR12813 or rifampicin treated samples upon stimulation with **(aii, bii)** U46619 or **(cii, dii)** ADP in 5 mins (vehicle-treated samples represent 100% aggregation). Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

3.6. PXR ligands negatively regulate integrin αIIbβ3 activation

Integrin αIIbβ3 is the primary receptor that allows platelets to aggregate to form a platelet plug. In resting platelets, integrin αIIbβ3 maintains a low-affinity state with a lower copy-number on the platelet surface. The transition of integrin αIIbβ3 from a low-affinity to a high-affinity state is the final outcome of platelet activation and a common feature, which is shared by all the platelet agonists to a greater or lesser degree, irrespective of their mechanism of action. This event, as described previously in chapter-1 (section 1.5.4) is mediated by inside-out signalling. Activated platelets display greater numbers of high-affinity integrin αIIbβ3 molecules on their surface, which bind to extracellular soluble ligands present in plasma, such as fibrinogen or von Willebrand factor. This facilitates platelet-platelet interactions through the formation of bridges between adjacent platelets, allowing them to aggregate together and form a platelet plug. Defects in the expression or activation of integrin αIIbβ3 have been characterised by significantly prolonged bleeding time, a clinical condition termed as Glanzmann thrombasthenia (Nurden, 2006). Over the past few years, several integrin αIIbβ3 antagonists such as abciximab, eptifibatide, and tirofiban have been developed and used clinically for the treatment and prevention of thrombosis (Bledzka et al., 2013).

Given the inhibitory effects of PXR ligands on platelet aggregation, their effects on primary events contributing to the formation of platelet aggregates, i.e. integrin αIIbβ3 activation were examined. The extent of fibrinogen binding to integrin αIIbβ3 on activated platelets was measured using flow cytometry, which

provides an indirect estimation of the extent of integrin αIIbβ3 molecules present in an activated state of the platelet surface.

To investigate the effects of PXR ligands on the extent of fibrinogen binding to integrin αIIbβ3, human PRP was incubated with PXR ligands (10, 20, 50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes prior to addition of FITC-conjugated anti-human fibrinogen antibody. PRP was stimulated with the GPVI-specific agonist cross-linked collagen-related peptide (CRP-XL; 0.25 μg/ml) for 20 minutes at room temperature, with occasional gentle mixing. CRP-XL is a GPVI selective agonist, made of a triple helical peptide containing ten GPO repeats, a prominent repeated sequence present in collagens (Smethurst et al., 2007). The GPO strands are crosslinked to provide stability to the structure. Collagen cannot be used for this assay because of its fibrillar structure that obstructs the flow of platelets through the cytometer. Samples were then fixed with 0.2% (v/v) formyl saline and fluorescence was measured using a flow cytometer for 10,000 events gated onto the platelet population. Similar to aggregation, the concentration of CRP-XL used to stimulate platelets was determined based on EC_{50} responses (50% of maximal fibrinogen binding). This would enable comparison between different assays (Figure 3.10 ai).

Treatment of PRP with CRP-XL (0.25 μg/ml) caused a dramatic rise in the level of fibrinogen binding to integrin αIIbβ3, which was attenuated by treatment with SR12813 or rifampicin. A significant reduction of 52% was achieved by 100 μM of SR12813 in comparison to vehicle-control (containing, DMSO 0.1% v/v) (Figure 3.10 aii). Similarly, rifampicin treatment was also associated with inhibition of 30% and 55% at 50 and 100 μM respectively (Figure 3.10 aiii).

The effects of PXR ligands on fibrinogen binding evoked by thrombin (0.05 U/ml) were also examined. The concentration of thrombin used was determined using EC_{50} response (Figure 3.10 bi). Fibrinogen binding to integrin αIIbβ3 was observed to be attenuated by 40% in samples treated with 100 μM of SR12813 in comparison to vehicle-treated control (Figure 3.10 bii). Rifampicin also down-regulated fibrinogen binding in a concentration-dependent manner with 25% and 50% reduction caused by 50 and 100 μM of rifampicin respectively (Figure 3.10 biii).

Taken together, these data indicate that PXR ligands prevent integrin αIIbβ3 from attaining a high-affinity state towards fibrinogen following CRP-XL or thrombin-mediated platelet stimulation. Similar to aggregation responses, mostly the higher concentrations of PXR ligands (50 and 100 μM) were found to be effective in reducing the level of fibrinogen binding. As explained earlier, the degree of platelet aggregation is dependent on the extent of fibrinogen binding to integrin αIIbβ3. Therefore, these data can explain the reductions observed with platelet aggregation after treatment with PXR ligands, which might be due to diminished fibrinogen binding to integrin αIIbβ3.

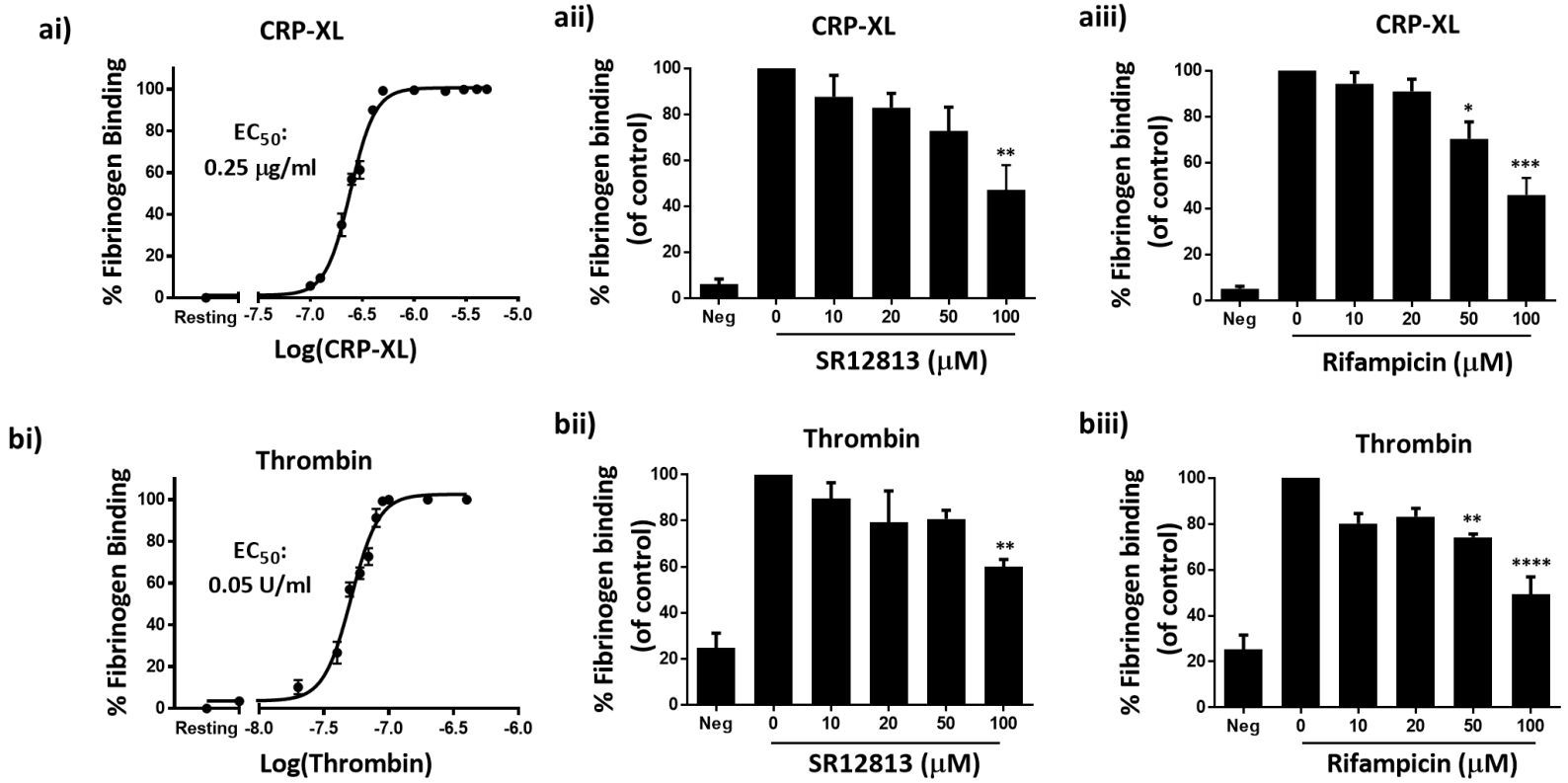


Figure 3.10: PXR ligands attenuate fibrinogen binding to integrin αIIbβ3. Human PRP was incubated with SR12813 or rifampicin (10, 20, 50 and 100 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 minutes. EC_{50} values for **(ai)** CRP-XL and **(bi)** thrombin-mediated fibrinogen binding were determined by treating PRP with a range of concentrations for 20 minutes in the presence of FITC-labelled rabbit anti-fibrinogen antibody. Post-stimulation, samples were fixed with 0.2% (v/v) formyl saline and analysed by flow cytometry. The effects of PXR ligands on samples stimulated with **(aii, aiii)** CRP-XL (0.25 μg/mL) or **(bii, biii)** thrombin (0.05 U/ml) are shown. Data represent percentage fibrinogen binding compared with vehicle-treated control, which is defined as 100% fibrinogen binding. The response of unstimulated samples treated with FITC-conjugated anti-human fibrinogen antibody is also shown (Neg). Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

3.7. PXR ligands attenuate α-granule secretion

α-granules contain a diverse range of proteins that are released once platelets become activated. Besides amplifying the activation process, the contents of the α-granules directly contribute towards platelet adhesion, aggregation and subsequent thrombus formation. The α-granule secretome includes membrane proteins (integrin αIIbβ3, PECAM-1 and GPVI), soluble proteins (fibrinogen, fibronectin and vWF), chemokines such as platelet factor 4 (PF4), coagulation factors and growth factors including platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β).

P-selectin (CD62P), which is present in α-granule membranes is a transmembrane protein that is translocated to the surface as secretion occurs. It facilitates platelet-leukocyte interactions, which contribute towards thrombus development and is also associated with inflammatory responses (Franks et al., 2010). Exposure of P-selectin on the platelet surface is regarded as a classical marker to study defects in α-granule secretion and it is also an indirect measure of platelet activation (Nagy et al., 2013). Given the down-regulation of platelet aggregation and fibrinogen binding, we evaluated whether PXR ligands also modulate earlier stages of platelet activation such as secretion from α-granules (Blair and Flaumenhaft, 2009; Whiteheart, 2011).

Human PRP was incubated with PXR ligands (10, 20, 50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes. Following this, the anti-CD62P Cy5/PE conjugated antibody was added and the samples were stimulated with CRP-XL (0.25 μg/mL) or thrombin (0.05 U/ml) for 20 minutes,

with occasional gentle mixing. To maintain consistency between results same concentrations (as used for the determination of fibrinogen binding) of CRP-XL and thrombin were used for stimulating platelets to measure P-selectin exposure. Reactions were terminated by fixing samples with 0.2% (v/v) formyl saline and fluorescence was recorded by a flow cytometer for 10,000 events gated onto the platelet population.

Stimulation with CRP-XL caused an extensive increase in P-selectin exposure on the platelet surface, which was reduced by 40% upon treatment with 100 μM of SR12813 in comparison to vehicle-treated control (containing, DMSO 0.1% v/v) (Figure 3.11 ai). Rifampicin at 100 μM, also caused a reduction by 27% in comparison to vehicle-control (Figure 3.11 aii). The inhibitions observed were slightly less pronounced in comparison with the reductions noted with CRP-XL mediated fibrinogen binding. Although both these parameters can regulate each other, they are two independent parameters of platelet activation. It is therefore possible that the effects of PXR ligands are dedicated towards a lesser extent in modulating α-granule secretion in comparison to fibrinogen binding to integrin αIIbβ3.

In addition to the inhibition of P-selectin exposure observed with CRP-XL stimulation, both PXR ligands down-regulated thrombin-mediated P-selectin exposure. Treatment of PRP with 100 μM of SR12813 attenuated thrombin-mediated P-selectin exposure by approximately 30% (Figure 3.11 bi). A similar inhibitory profile was replicated by rifampicin treatment with 100 μM of rifampicin reducing P-selectin exposure by approximately 50% in comparison to vehicle-control (Figure 3.11 bii).

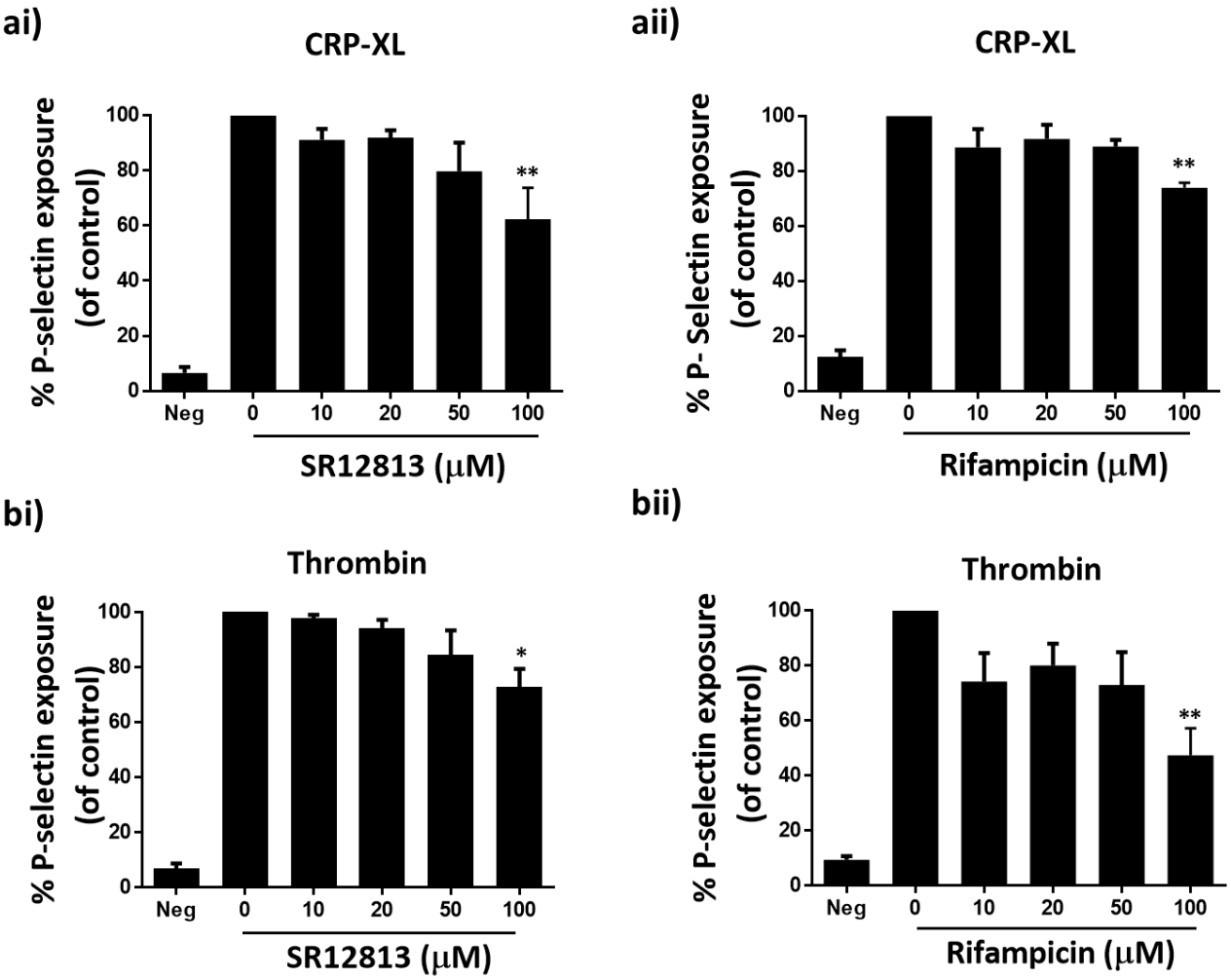


Figure 3.11: PXR ligands down-regulate P-selectin exposure. Human PRP was incubated with SR12813 or rifampicin (10, 20, 50 and 100 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 minutes. This was followed by the addition of anti-CD62 Cy5/PE conjugated antibody and stimulation of samples with **(ai, aii)** CRP-XL (0.25 μg/mL) or **(bi, bii)** thrombin (0.05 U/ml) for 20 minutes with occasional gentle mixing. Post-stimulation, samples were fixed with 0.2% (v/v) formyl saline and analysed by flow cytometry. Data represent percentage P-selectin exposure compared with vehicle-treated control, which is defined as 100% P-selectin exposure. The response of unstimulated samples treated with anti-CD62P Cy5/PE conjugated antibody is also shown (Neg). Data represent mean ± SEM (n≥3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by one-way ANOVA.

3.8. Secretion from dense-granules is inhibited by PXR ligands

Human platelets contain 3-8 dense granules per platelet, which are rich in pro-thrombotic and inflammatory components such as serotonin, histamine, polyphosphates, calcium, ADP and ATP. The release of these constituents from dense granules (primarily ADP) recruits more platelets to the site of injury and thus provides positive feedback by amplifying initial platelet activation signals. Hence, dense granule secretion plays a pivotal role in the growth of the thrombus (McNicol and Israels, 1999; Youssefian et al., 1997) and defects in their biogenesis or secretion have been associated with bleeding disorders (Ambrosio et al., 2012). Platelet aggregation, therefore, depends at least partly on the release of contents from dense granules. Given the negative regulation of platelet aggregation and α-granule secretion by PXR ligands, the effects of PXR ligands on dense granules were investigated by studying the release of ATP using lumi-aggregometry (Paniccia et al., 2015).

The bioluminescence assay used to monitor ATP release from dense granules utilise a reaction in which the enzyme luciferase converts luciferin into oxyluciferin in the presence of magnesium, using ATP as a source of energy (released from dense granules upon stimulation with an agonist). This interaction produces light, which is proportional to ATP concentration and is quantified using a lumiaggregometer (Paniccia et al., 2015; Feinman et al., 1977).

Washed human platelets ($4x10^8$ cells/ml) in the presence of chronolume reagent (containing firefly luciferase and D-luciferin) were incubated with PXR ligands SR12813 or rifampicin (20, 50 and 100 μM) or vehicle (containing, DMSO 0.1% v/v) for 20 minutes prior to stimulation with collagen (1 μg/ml). A higher

concentration of collagen was used to ensure maximum release of ATP from the dense granules. Consequently, the incubation time with PXR ligands was prolonged because of high concentration of the agonist used. ATP release from dense granules was monitored for 5 minutes. SR12813 inhibited collagen-stimulated ATP release from dense granules in a concentration-dependent manner with 48% reduction observed at 100 μM, in comparison to vehicle-control (Figure 3.12 ai, aii). A comparable level of inhibition was exhibited by platelets incubated with 50 μM and 100 μM of rifampicin, resulting in a 35% and 55% reduction respectively (Figure 3.12 bi, bii).

As described previously, thrombin-stimulated platelet aggregation in the presence of PXR ligands were inhibited to a stronger extent for up to 3 minutes from the initiation of aggregation. This was attributed to a reduction in secretion of pro-thrombotic secondary mediators (such as ADP and TxA_2) from platelets. To examine this, we looked at the effects of PXR ligands on thrombin-induced (0.05 U/ml) dense granule secretion over a period of 5 minutes. Similar to collagen-stimulation, a higher concentration of thrombin was used to ensure maximum release of ATP from the dense granules and therefore, the incubation time with PXR ligands was prolonged to 20 minutes. SR12813 caused a concentration-dependent inhibition of ATP release with a significant reduction of approximately 20% and 40% being achieved at 50 μM and 100 μM respectively, in comparison to vehicle-control (containing, DMSO, 0.1% v/v) (Figure 3.12 ci, cii). Rifampicin treatment also reduced ATP secretion by 50% at the highest concentration of 100 μM (Figure 3.12 di, dii). Therefore, the attenuation in the thrombin-stimulated dense granule secretion (rich in ADP) by PXR ligands may be one of the underlying

reasons that can explain stronger inhibition exhibited by PXR ligands during the initial stage (3 minutes) of thrombin-evoked platelet aggregation and reduced platelet activation overall.

The inhibitory effects of PXR ligands were also noted to be slightly more potent on ATP release in contrast to P-selectin exposure. This might be attributed to the fact that there are only 3-4 dense granules per platelet in comparison to 60-80 alpha granules, which might account for higher amounts of secretion detected from alpha granules. Additionally, secretion from α-granules has been suggested to be more sensitive to agonist stimulation than dense granules (Mirlashari et al., 1996).

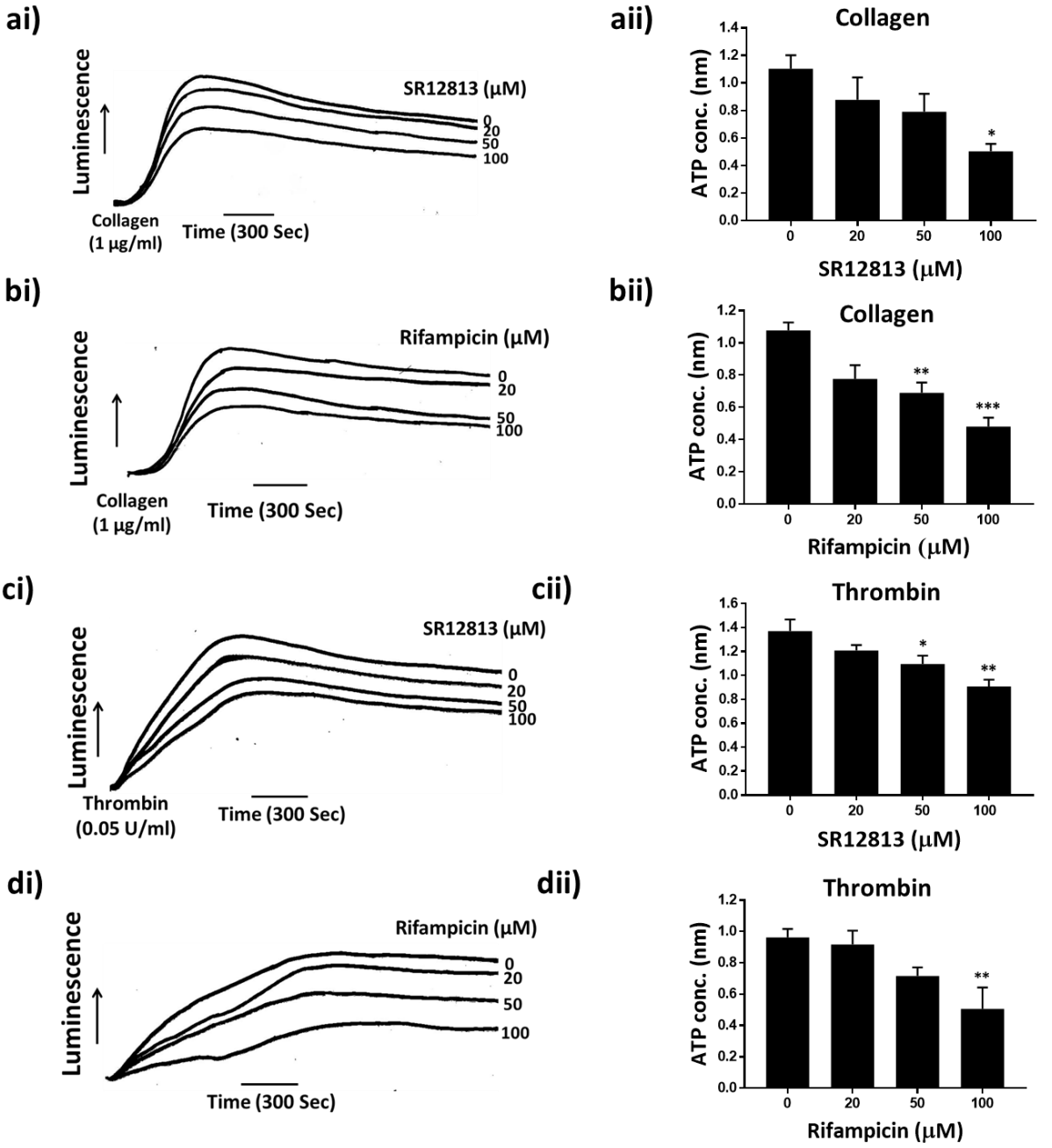


Figure 3.12: PXR ligands reduce ATP secretion from dense granules. Washed platelets ($4x10^8$ cells/mL) were incubated with SR12813 or rifampicin (20, 50 or 100μM) or vehicle (containing, DMSO 0.1% v/v) for 20 minutes prior to stimulation with collagen (1 μg/ml) or thrombin (0.05 U/ml). Luciferase reagent was added 2 minutes before the addition of agonist. Changes in ATP release were monitored using an optical lumi-aggregometer for 5 minutes. **(ai, bi)** Representative traces display a collagen-mediated increase in luminescence upon ATP secretion and its inhibition by SR12813 or rifampicin. **(aii, bii)** Quantified data of collagen-mediated ATP secretion. **(ci, di)** Representative traces of thrombin-mediated ATP release for samples incubated with SR12813 or rifampicin. **(cii, dii)** Quantified data of thrombin-evoked ATP secretion. Vehicle-treated samples represent 100% ATP secretion. Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

3.9. PXR ligands attenuate TxB_2 production

Thromboxane A_2 (TxA_2) is pro-thrombotic lipid mediator that is synthesised and released by activated platelets. As explained in chapter 1, TxA_2 is the primary product of COX-1-dependent metabolism of arachidonic acid and exerts its actions via G protein-coupled thromboxane prostanoid receptors (TP receptors). This provides positive feedback regulation by amplifying platelet aggregation through recruitment of more platelets at the site of injury. Its actions physiologically are therefore exerted mostly in an autocrine or paracrine fashion (Fontana et al., 2014). Consequently, the TxA_2 pathway is one of the major therapeutic targets for the treatment and prevention of thrombosis. Aspirin is an irreversible blocker of COX-1, which is one of the widely used antiplatelet drug (Warner et al., 2011).

Having established the role of PXR ligands in negative regulation of platelet aggregation and secretion from both alpha and dense granules, their effects on TxA_2 production were studied. TxA_2 is marked by a short half-life (approximately 30 secs) and undergoes non-enzymatic hydrolysis to its inactive form of TxB_2. Therefore, the levels of TxB_2 were examined, which gives an indirect estimation of the concentrations of TxA_2 (Seidel et al., 2011). Washed platelets treated with SR12813 or rifampicin (50 and 100 μM) or vehicle (containing DMSO, 0.1% v/v) for 20 minutes were stimulated with collagen (1 μg/ml) or thrombin (0.05 U/ml) in an aggregometer for 5 minutes. Higher concentration of platelet agonists was used to ensure maximum synthesis and release of TxB_2 from activated platelets. Consequently, incubation time with PXR ligands was prolonged because of high concentration of the agonists used. Samples without stimulation with agonists were used as a negative control. The samples (treated and untreated with PXR

ligands) after stimulation with collagen or thrombin in an aggregometer were centrifuged at 12,000 rpm for 2 min to collect the supernatant for analysis. The levels of TxB_2 in plasma samples were evaluated using competitive ELISA assay. A series of standards were run to enable a standard curve to be drawn based on which the concentration of samples were established.

No detectable levels of TxB_2 were observed in unstimulated samples. Upon stimulation by collagen (1 μg/ml), a sharp rise in TxB_2 levels was observed, which was reduced upon treatment with both SR12813 or rifampicin. Incubation with 100 μM of SR12813 (Figure 3.13 ai) or rifampicin (Figure 3.13 aii) displayed inhibition of 35% and 20% respectively in comparison to vehicle-control (containing DMSO, 0.1% v/v).

Thrombin-mediated increase in TxB_2 levels was also diminished by SR12813 (Figure 3.13 bi) or rifampicin (Figure 3.13 bii) treatment with both 50 and 100 μM representing approximately 55% reduction in contrast to vehicle-control. Interestingly, the concentration of 50 μM and 100 μM of both the PXR ligands were equally potent in reducing thrombin-mediated TxB_2 synthesis. This suggests the stronger efficacy of PXR ligands in mitigating TxB_2 synthesis stimulated by thrombin in comparison to collagen. Moreover, this further adds to the observation that the effects of PXR ligands towards thrombin-mediated platelet aggregation might be predominantly due to a reduction in the release of pro-thrombotic mediators.

Thus, it can be concluded that PXR ligands, in general, possess the ability to negatively regulate the release of autocrine and paracrine factors from platelets, contributing to reduced platelet aggregation and potentially thrombus formation.

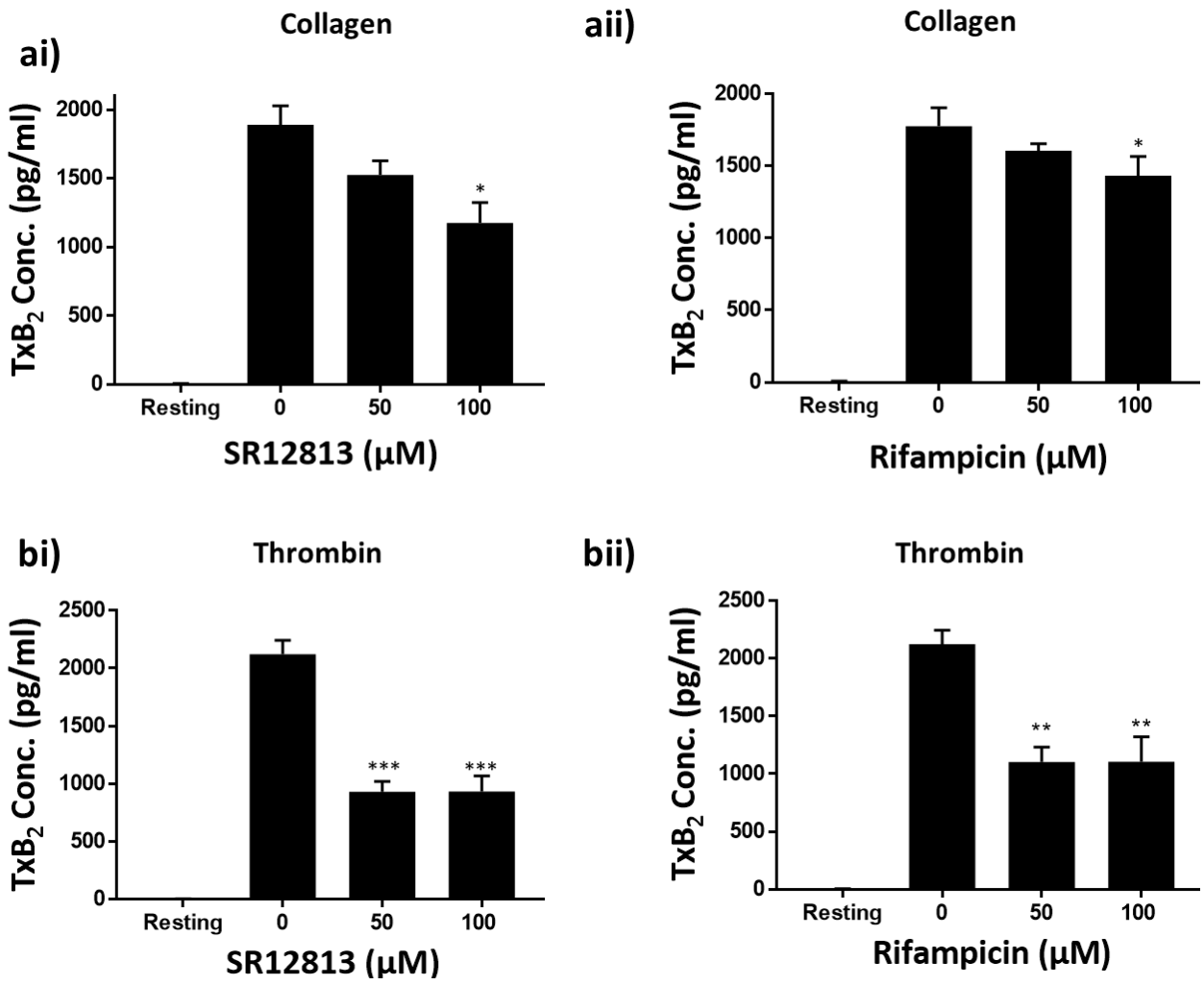


Figure 3.13: PXR ligands inhibit TxB_2 production. Washed human platelets ($4x10^8$ cells/ml) were pre-incubated with SR12813 or rifampicin or vehicle (containing, 0.1% v/v DMSO) for 20 min and stimulated by collagen (1 µg/ml) or thrombin (0.05 U/ml) in an aggregometer for 5 minutes at 37°C. The reactions were terminated by the addition of a STOP solution (containing, 1 mM EGTA and 20 µM indomethacin) and centrifuged (12,000 rpm, 2 min, RT) to isolate supernatants, which were immediately frozen at -80°C. The amount of TxB_2, a stable metabolite of TxA_2, was determined using a TxB_2 ELISA kit according to the manufacturer's protocol. The levels of TxB_2 obtained (pg/ml) after incubation of platelets with SR12813 or rifampicin and stimulation with **(ai, aii)** collagen or **(bi, bii)** thrombin are shown. Data represent mean ± SEM (n=4), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

3.10. Reduction in collagen-stimulated aggregation by PXR ligands is not solely dependent on attenuation of ADP and TxA_2 stimulated effects.

Sustained platelet activation downstream of collagen or thrombin is partially dependent on the release of pro-thrombotic secondary agonists such as ADP and TXA_2 (Nieswandt and Watson, 2003). Previously it was observed (section 3.5) that PXR ligands inhibit platelet aggregation stimulated by ADP and U46619 (TxA_2 mimetic peptide). Based on these observations, we examined whether the inhibitory effects of PXR ligands on collagen or thrombin-stimulated platelet aggregation are solely due to their ability to inhibit the actions of TxA_2 and ADP secreted after stimulation, or whether they act through inhibition of other aspects of platelet function.

To study this, collagen-mediated platelet aggregation assay was performed on washed platelets ($4x10^8$ cells/ml) treated with COX-1 inhibitor - indomethacin (I) and ADP receptor antagonists – cangrelor (C) and MRS2179 (M) for 5 minutes to block secondary mediator effects. To ensure that secondary mediator signalling has been abolished completely, saturating concentrations of indomethacin (20 μM) (Figure 3.14a), cangrelor (1 μM) and MRS2179 (100 μM) (Figure 3.14b) were determined first. Given the potent inhibitory effects of these inhibitors, a higher concentration of collagen (10 μg/ml) was used to ensure 50% aggregation was still achieved in 5 minutes, following inhibition of secondary mediator signalling.

Addition of saturating concentrations of indomethacin (I) or ADP receptor antagonists (C+M) reduced collagen-mediated platelet aggregation by approximately 40%. Addition of these inhibitors together (C+M+I) resulted in an

inhibition of 65%. Incubation of platelets with SR12813 (100 μM) for 20 minutes (incubation time was increased because of the high concentration of the collagen used) along with indomethacin (I+SR) or ADP receptor antagonists (C+M+SR) or all of them together (C+M+I+SR) caused an additional reduction of approximately 20% (Figure 3.14c, d). The ability of SR12813 to further augment the existing reduction caused by indomethacin (I) or ADP receptor antagonists (C+M) or both (C+M+I) suggest that the inhibitory effects of the SR12813 on collagen-mediated platelet aggregation cannot be solely explained by its inhibitory effects on secondary mediators.

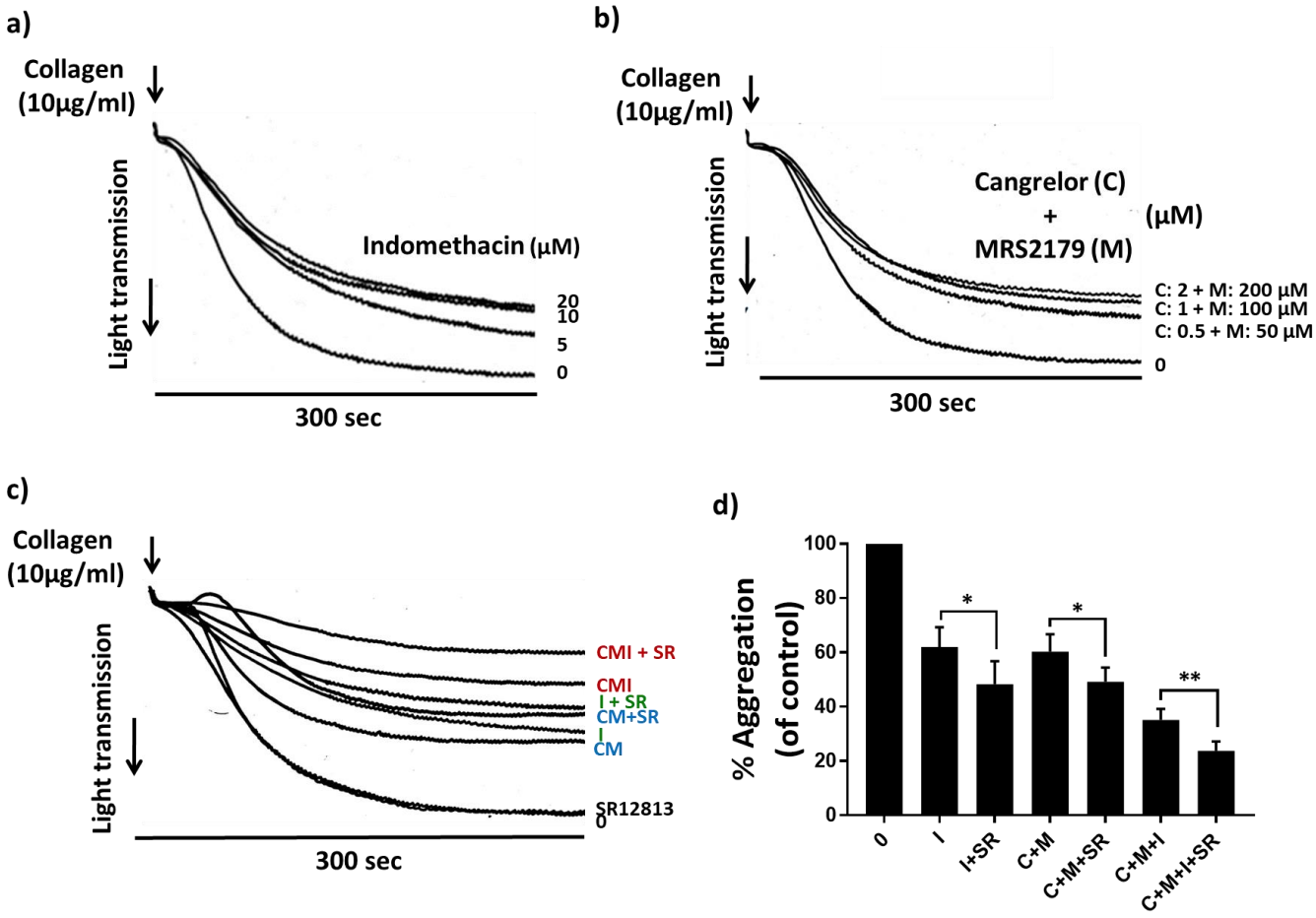


Figure 3.14: Effects of SR12813 on collagen-mediated platelet aggregation are not solely dependent on the inhibition of secretion. Washed platelets ($4x10^8$ cells/ml) pre-treated with increasing concentrations of **(a)** indomethacin (5, 10 and 20 µM) or **(b)** ADP receptor antagonists - cangrelor (0.5, 1 and 2 µM) and MRS2179 (50, 100 and 200 µM) were stimulated with collagen (10 µg/ml) and aggregations were recorded for 5 minutes to identify their saturating concentrations **(c)** Representative aggregation trace shows the levels of collagen-stimulated platelet aggregations measured for 5 minutes in the presence or absence of SR12813 (100 µM) in addition to indomethacin (20 µM) or cangrelor (1 µM) and MRS2179 (M; 100 µM) or all together. **(d)** Quantified data for collagen-stimulated platelet aggregation in the presence or absence of SR12813, along with indomethacin (I+SR) or cangrelor and MRS2179 (C+M+SR) or all of them together (C+M+I+SR). 'O' signifies the sample stimulated with collagen in the absence of SR12813 and secondary mediator signalling blockers. Data represent mean ± SEM ($n \geq 3$), *$P \leq 0.05$ and **$P \leq 0.01$ was calculated by student's t-test.

Abbreviations: I – Indomethacin, C – Cangrelor, M – MRS2179 and SR – SR12813

Similarly, secondary mediator effects in case of thrombin-stimulated platelet aggregation were blocked by treating washed platelets with indomethacin, cangrelor and MRS2179. Saturating concentrations of indomethacin (30 μM) (Figure 3.15a), cangrelor (1 μM) and MRS2179 (100 μM) (Figure 3.15b) were identified and the concentration of thrombin (0.1 U/ml) was increased to ensure 50% aggregation was still achieved in 5 minutes in the presence of all these inhibitors.

Treatment with indomethacin (I) and ADP receptor antagonists (C+M) caused aggregation to reduce by 15% and 32% respectively. Whereas, an inhibition of 53% was achieved when these inhibitors were added together (I+C+M). Interestingly, unlike collagen-mediated platelet aggregation, no further inhibition was caused by the incubation with SR12813 (100 μM) for 20 minutes in the presence of indomethacin or ADP receptor antagonists or both (I+C+M+SR12813) (Figure 3.15 c, d). These findings indicate that the inhibition of thrombin-stimulated platelet aggregation is exclusively due to the down-regulation of the TxA_2 and ADP mediated effects. These observations also fall in alignment with the initial strong inhibitions (up to 3 minutes) observed with thrombin-mediated platelet aggregation (section 3.5) in the presence of PXR ligands, which can be attributed to the previously observed attenuation of secretion (primarily ADP and TxA_2) from platelets (section 3.7, 3.8 and 3.9).

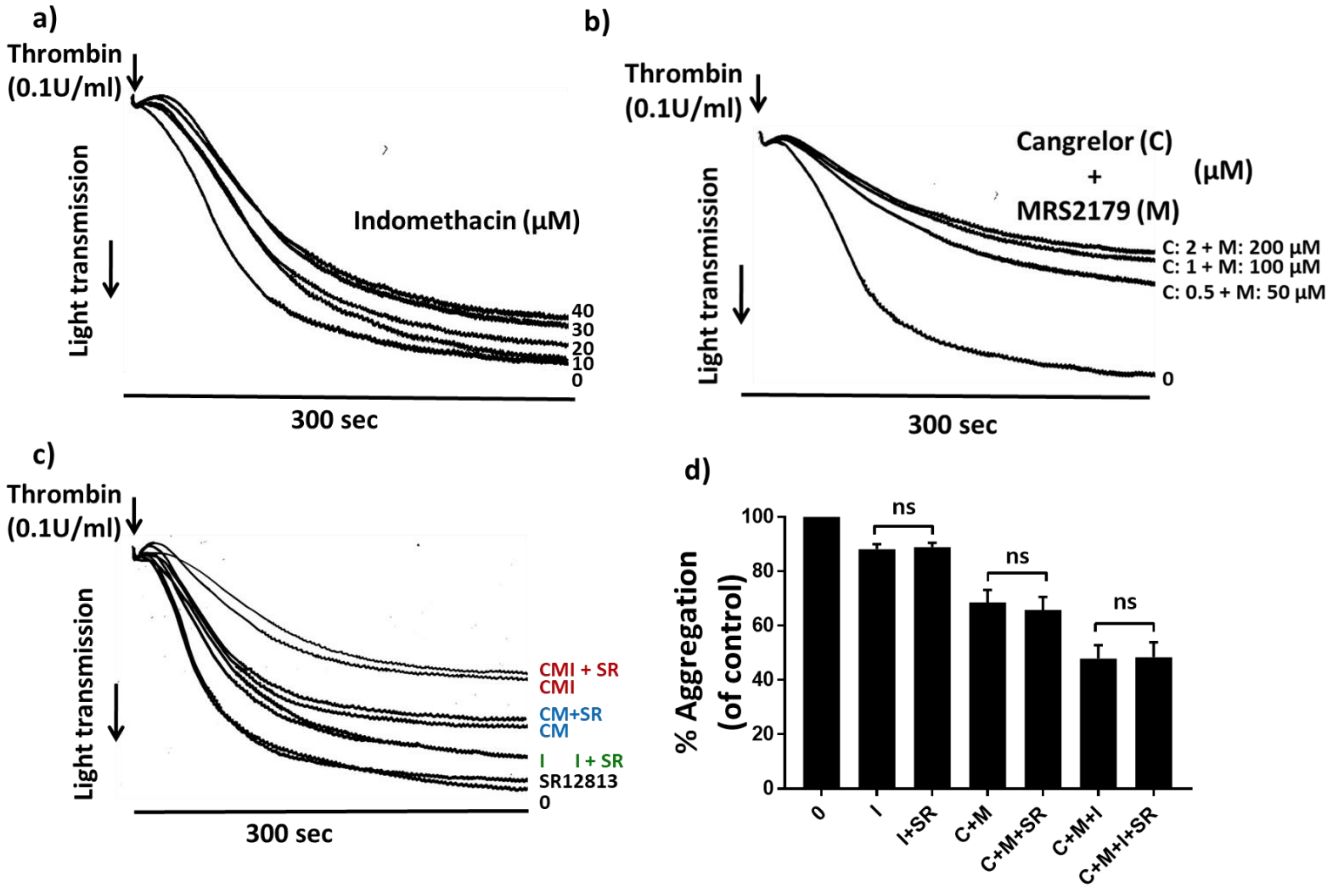


Figure 3.15: Effects of SR12813 on thrombin-evoked platelet aggregation are mediated through the inhibition of secretion. Washed platelets ($4x10^8$ cells/ml) pre-treated with increasing concentrations of **(a)** indomethacin (10, 20, 30 and 40 μM) or **(b)** ADP receptor antagonists - cangrelor (0.5, 1 and 2 μM) and MRS2179 (50, 100 and 200 μM) were stimulated with thrombin (0.1 U/ml) and aggregations were recorded for 5 minutes to identify their saturating concentrations **(c)** Representative aggregation trace shows the levels of thrombin-stimulated platelet aggregation measured for 5 minutes in the presence or absence of SR12813 (100 μM) in addition to indomethacin (30 μM) or cangrelor (1 μM) and MRS2179 (100 μM) or all together. **(d)** Quantified data for thrombin-stimulated platelet aggregation in the presence or absence of SR12813, along with indomethacin (I+SR) or cangrelor and MRS2179 (C+M+SR) or all of these together (C+M+I+SR). 'O' signifies the sample stimulated with thrombin in the absence of SR12813 and secondary mediator signalling blockers. Data represent mean ± SEM (n≥3) calculated by student's t-test.

Abbreviations: I – Indomethacin, C – Cangrelor, M – MRS2179 and SR – SR12813

3.11. Calcium-mobilisation stimulated by CRP-XL or thrombin is inhibited by PXR ligands

Elevation of intracellular calcium levels is a common feature that is shared by activation pathways downstream of all platelet agonists that signal through different platelet membrane receptors (Varga-Szabo et al., 2009). Calcium contributes towards the process of platelet activation by regulating several vital steps such as cytoskeletal rearrangement, degranulation (leading to release of secondary mediators, ADP and TXA_2) and increase in the affinity of integrin αIIbβ3 (via inside-out signalling) (Rink and Sage, 1990). As discussed in detail in chapter 1, a rise in cytosolic calcium concentration is controlled in 2 ways: (a) agonist-induced activation of platelets commonly facilitates the stimulation of PLCβ (for GPCR receptors) or PLCγ2 (for GPVI receptors), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-trisphosphate (IP3). Binding of IP3 to IP3 receptors (IP3R) on the dense tubular system (DTS) induces the release of calcium into the cytosol. (b) Following the depletion of calcium from DTS, a rapid influx of calcium occurs across the plasma membrane arbitrated by the STIM1 sensor and Orai1 channel. This process is known as store-operated calcium entry (SOCE) (Vazzana et al., 2012; Bergmeier and Stefanini, 2009). Considering the central role calcium plays to regulate all the previously discussed stages of platelet activation, it is important to understand the effects of PXR ligands on calcium mobilisation.

Calcium levels were estimated by using a ratiometric membrane permeable dye, Fura-2AM, which binds to free intracellular calcium. PRP was incubated with Fura2-AM and washed platelets ($4x10^8$ cells/ml) were prepared, which were then

incubated with PXR ligands (50 or 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes in a 96 well plate at 37°C prior to activation with either CRP-XL (0.25 μg/ml) or thrombin (0.05 U/ml). Fluorescence measurements were made using a plate reader for 5 minutes after the addition of the agonist, and $[Ca^{2+}]_i$ was estimated using the equation described in Chapter 2 (Section 2.2.8).

Treatment of Fura-2AM loaded platelets with CRP-XL (0.25 μg/ml) resulted in a rise in intracellular calcium concentration. As a characteristic feature with GPVI agonist, the rise in cytosolic calcium, post-stimulation was relatively gradual, which reached a peak concentration of approximately 420 nM in 3 minutes (Figure 3.16 ai). A concentration-dependent reduction in calcium mobilisation (peak levels) was observed in samples treated with SR12813 in comparison to vehicle-control (containing, DMSO 0.1% v/v). While the initial kinetics of calcium release associated with SR12813 treated samples were similar to vehicle treated sample, their peak cytosolic concentrations were significantly lower, as measured over a duration of 5 minutes. This observation signifies a true inhibition rather than delay in calcium mobilisation. SR12813 at 100 μM exhibited a reduction of approximately 40% in peak cytosolic calcium levels in contrast to vehicle-control (Figure 3.16 aii). In agreement with these findings, rifampicin also diminished CRP-XL induced peak calcium levels by 45% at 50 μM (Figure 3.16 bii). Due to the bright red colour of rifampicin, an interference with the emission values was observed at higher concentration. This limited its usage to lower concentrations of 20 and 50 μM only.

Stimulation with thrombin (0.05 U/ml) resulted in a rapid release of calcium which reached a peak of approximately 500 nM almost instantly after the

addition of thrombin, which is a characteristic of stimulation of platelets with GPCR agonists (Figure 3.17 ai). Peak calcium concentrations in the presence of SR12813 were found to be reduced in comparison to vehicle-treated controls. At 50 μM, a reduction of 35% was observed which increased to 55% upon incubation with 100 μM (Figure 3.17 aii). Supporting these findings, rifampicin treatment also displayed a significant degree of inhibition, with 50 μM preventing a rise in intracellular calcium levels by approximately 60% (Figure 3.17 bii).

The extent of inhibition caused in calcium mobilisation was found to be of approximately similar level as detected with fibrinogen binding, P-selectin exposure, dense granule secretion, TxB_2 production and aggregation. These observations provide an important reflection of the central role calcium release play in controlling inside-out signalling and degranulation. Also, these findings demonstrate the interdependence of several activation events on one another and how regulation of one can affect the other. As discussed earlier, calcium mobilisation is an essential contributor towards the signalling cascade instigated by all the platelet agonists. Therefore, its regulation also provides evidence that signalling events, at least upstream of calcium mobilisation might be influenced following treatment of platelets with PXR ligands.

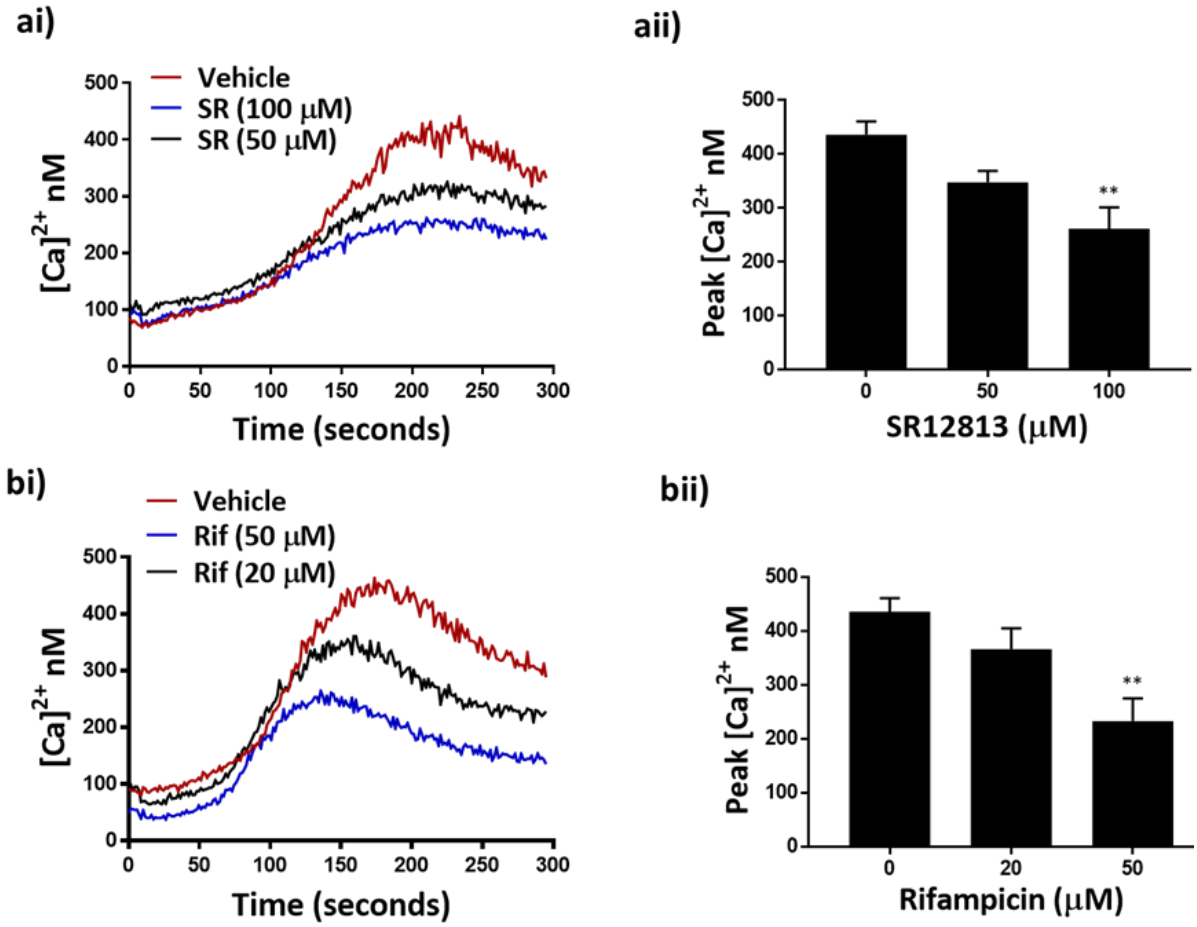


Figure 3.16: PXR ligands inhibit CRP-XL-stimulated intracellular calcium elevation. Fura-2AM loaded platelets (4x10^8 cells/ml) were incubated with **(ai, aii)** SR12813 (50 and 100 μM) or **(bi, bii)** rifampicin (20 and 50 100 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 min at 37°C prior to the addition of CRP-XL (0.25 μg/ml). Fluorescence measurements were made with excitation at 340 and 380nm and emission at 510nm using a NOVOstar plate reader. Ca^{2+} was estimated from the ratio of the 340 and 380 nm excitation signals. **(ai, bi)** Traces of calcium mobilisation over a period of 5 minutes following CRP-XL-stimulation are shown. **(aii, bii)** Cumulative data (peak calcium levels) of calcium mobilisation in the presence or absence of PXR ligands after stimulation with CRP-XL. Peak calcium levels achieved in the presence of vehicle-control defines 100%. Data represent mean ± SEM (n≥3), **P ≤ 0.01 was calculated by one-way ANOVA.
Abbreviations: SR – SR12813 and Rif - Rifampicin

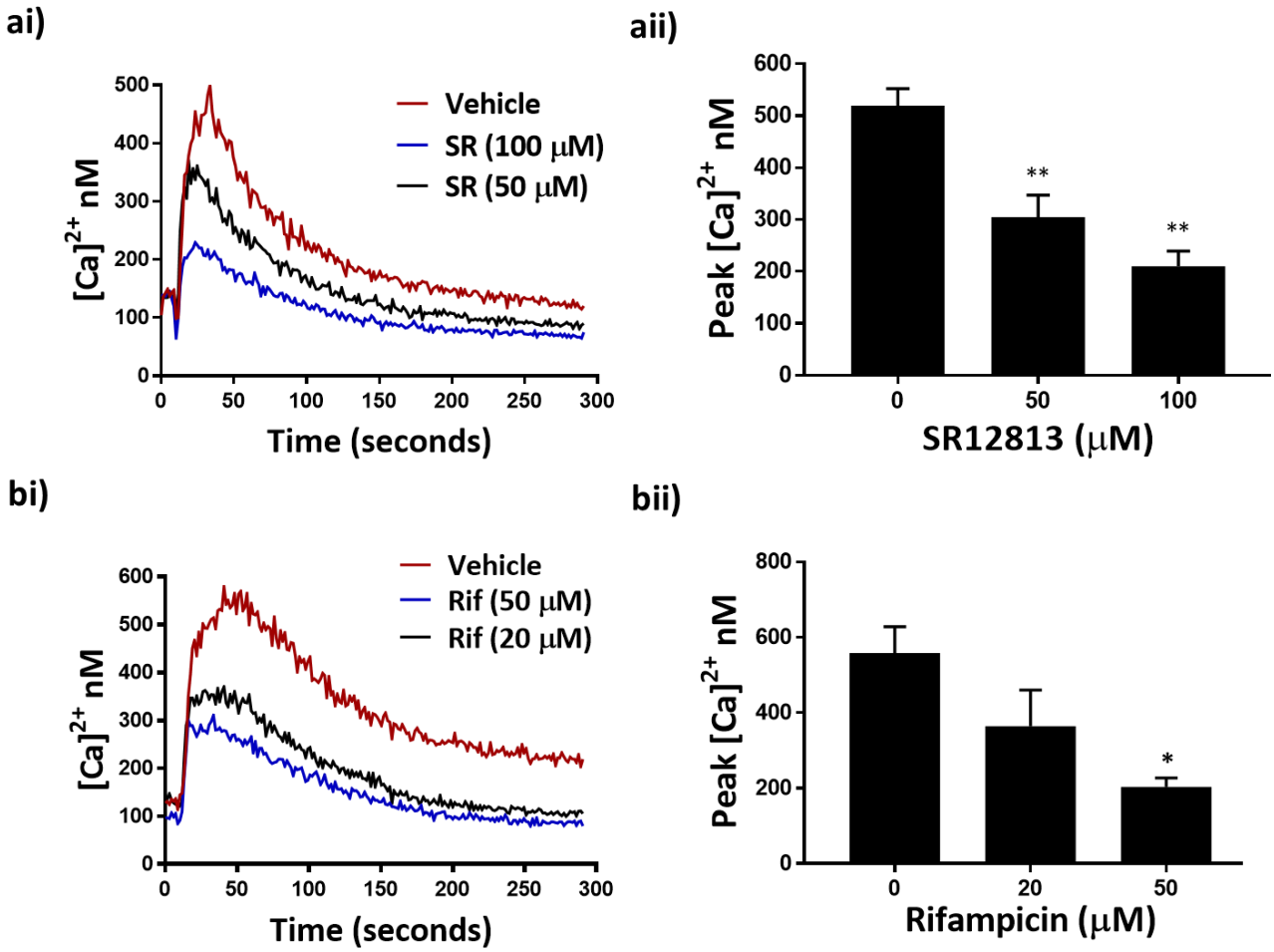


Figure 3.17: PXR ligands down-regulate thrombin-stimulated intracellular calcium elevation. Fura-2AM loaded platelets ($4x10^8$ cells/ml) were incubated with **(ai, aii)** SR12813 (50 and 100 μM) or **(bi, bii)** rifampicin (20 and 50 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 min at 37°C prior to the addition of thrombin (0.05 U/ml). Fluorescence measurements were made with excitation at 340 and 380nm and emission at 510nm using a NOVOstar plate reader. Ca^{2+} was estimated from the ratio of the 340 and 380 nm excitation signals. **(ai, bi)** Traces of calcium mobilisation over a period of 5 minutes following thrombin stimulation are shown. **(aii, bii)** Cumulative data (peak calcium levels) of calcium mobilisation in the presence or absence of PXR ligands after stimulation with thrombin. Peak calcium levels achieved in the presence of vehicle-control defines 100%. Data represent mean ± SEM (n≥3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by one-way ANOVA.

Abbreviations: SR – SR12813 and Rif - Rifampicin

3.12. PXR ligands inhibit platelet adhesion and spreading on fibrinogen

Integrin αIIbβ3 can transmit signals in a bi-directional manner across the plasma membrane. Agonist stimulation initiate 'inside-out signalling' in platelets that eventually includes the binding of talin and kindlin to the cytoplasmic domains of integrin β subunits, favouring affinity up-regulation of integrin αIIbβ3 and its subsequent binding to fibrinogen (Moser et al., 2008; Tadokoro et al., 2003). Additionally, binding of fibrinogen to integrin αIIbβ3 and consequent receptor clustering generates another signalling event referred to as 'outside-in signalling', which is propagated via the cytoplasmic domain of the integrins. These signals enable platelet spreading and clot retraction, required for a stable thrombus formation.

Platelet spreading involves an outward movement of the plasma membrane and the underlying cytoskeleton in the form of protrusions such as filopodia and lamellipodia. This process greatly increases the surface area of platelets, which enhance platelet interaction and thus provides stability to the thrombus (Shen et al., 2012). Both heteromeric G-proteins ($G\alpha_{13}$) and monomeric G-proteins (Rho family) regulate platelet spreading in the following manner; (1) Upon ligand binding, integrin αIIbβ3 undergoes a conformational change, allowing $G\alpha_{13}$ to bind directly with integrin β3 subunit and activate Src family kinases, which propagates the outside-in signal further, resulting in platelet spreading (Li et al., 2010; Gong et al., 2010). (2) Rho-family G-proteins such as cdc42 are responsible for filopodia formation, Rac regulates lamellipodia formation and Rho forms stress fibres that contract and limit spreading (Shen et al., 2012; Ridley and

Hall, 1992). Both these signalling events also cause actin polymerisation, required for the formation of filopodia and lamellipodia, essential for platelet spreading (Bearer et al., 2002).

To study the effects of PXR ligands on integrin αIIbβ3 outside-in signalling, their ability to regulate platelet spreading on immobilised fibrinogen was evaluated. In contrast to soluble fibrinogen, which only binds to high-affinity integrin αIIbβ3, immobilised fibrinogen has receptor-induced binding site (RIBS) epitopes that allow binding to low-affinity integrin αIIbβ3. This results in a conformational change in the cytoplasmic domain of αIIbβ3 that accompanies integrin clustering, resulting in outside-in signalling (Ugarova et al., 1993; Peter, 2005).

Glass coverslips were coated with fibrinogen (each 100 μg/ml) for 1 hour and incubated with BSA for 30 minutes to prevent platelet-glass attachment. Washed platelets ($2x10^7$ cells/ml) were incubated with PXR ligands (50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 minutes and added onto the coated coverslips at 37^oC. After 45 minutes of stimulation with fibrinogen, the supernatant was removed, and coverslips were washed with PBS. Samples were fixed with 0.2% (w/v) paraformaldehyde, washed with PBS, and adhered platelets were permeabilised with 0.2% (v/v) Triton X-100. After a further wash step, Alexa-Fluor 488 phalloidin that targets actin was added. Coverslips were mounted onto slides using Prolong Gold Antifade mounting media. The visualisation was performed using a confocal microscope (100X oil immersion lens). Five images were captured of each sample (taken in random locations on the slide), and from these images platelets were scored into three

categories: adhered (but not spread), filopodia formation (in the process of spreading) or lamellipodia formation (fully spread), with the percentage of each population under different experimental treatments calculated.

Figure 3.18a and 3.19a shows representative images of platelet adhesion/ spreading on fibrinogen in the presence or absence of SR12813 or rifampicin respectively. Incubation with SR12813 (Figure 3.18b) or rifampicin (Figure 3.19b) inhibited adhesion of platelets to fibrinogen-coated coverslips at both the concentrations tested (50 and 100 μM). Approximately 60% inhibition was demonstrated by both PXR ligands in comparison to vehicle-control (containing, DMSO 0.1% v/v). This signifies the ability of PXR ligands to negatively regulate integrin αIIbβ3 outside-in signalling. This further adds to the evidence of reduced fibrinogen binding (and aggregation) observed previously in samples treated with PXR ligands and stimulated with CRP-XL or thrombin.

As displayed in figure 3.18a and 3.19a, platelets that were treated with SR12813 or rifampicin respectively, and were found adhered to fibrinogen, demonstrated incapacity to spread fully in comparison to vehicle-control (containing, DMSO 0.1% v/v). Vehicle-treated samples displayed a large population of fully spread platelets, displaying lamellipodia. On the contrary, a significant number of platelets were found suspended in early stages of spreading, i.e. adhered or filopodial, with an equivalent decrease in lamellipodial extensions in samples treated with SR12813 or rifampicin. In vehicle-treated samples, approximately 76% of the platelets were fully spread, while treatment with SR12813 reduced it to 45% and 30% at 50 μM and 100 μM respectively (figure 3.18c). Similarly, 35% and 45% of platelets were observed as having filopodial

extensions at 50 μM and 100 μM of SR12813 respectively, in contrast to 18% in vehicle-control. Lastly, in SR12813 treated samples, a significant rise by 20% (at 50 μM) and 25% (at 100 μM) was noticed in the population of adhered platelets (but not spread) to fibrinogen, when compared with vehicle-control, which was just 3% (figure 3.18c). In alignment with these observations, a reduction in spreading was also displayed by platelets treated with rifampicin (figure 3.19c).

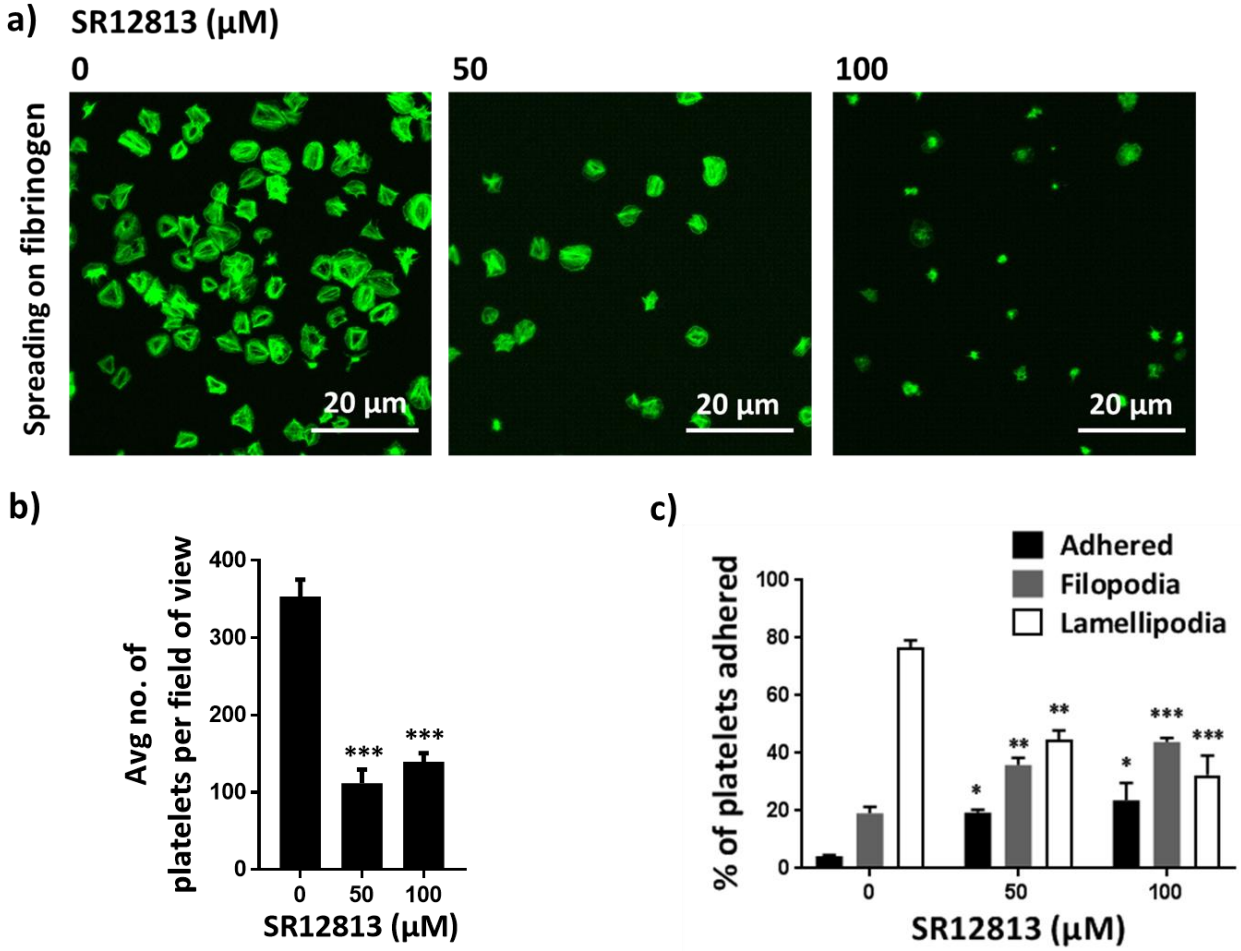


Figure 3.18: SR12813 prevent adhesion and spreading of platelets on fibrinogen. Human washed platelets ($2x10^7$ cells/ml) were treated with SR12813 (50 and 100 µM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 min and then added onto fibrinogen (100 µg/ml) coated coverslips for 45 mins at 37°C. Samples were fixed with 0.2% (w/v) paraformaldehyde and permeabilised with 0.2% (v/v) Triton X-100. Alexa-Fluor 488 phalloidin was then added for 1 hour and coverslips were mounted onto slides using Prolong Gold Antifade mounting media. The visualisation was performed using a Nikon A1-R confocal microscope with a 100X oil immersion lens. Five images were captured of each sample at random locations on the slide. **(a)** Representative image of platelet adhesion and spreading on fibrinogen is shown. **(b)** An average number of platelets adhered in each sample are shown. **(c)** Spreading platelets were divided into 3 classes: (adhered but not spread; filopodia: platelets in the process of spreading and lamellipodia: fully spread). Results expressed (as relative frequency) as the percentage of the total number of platelets adhered. Data represent mean ± SEM (n=3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

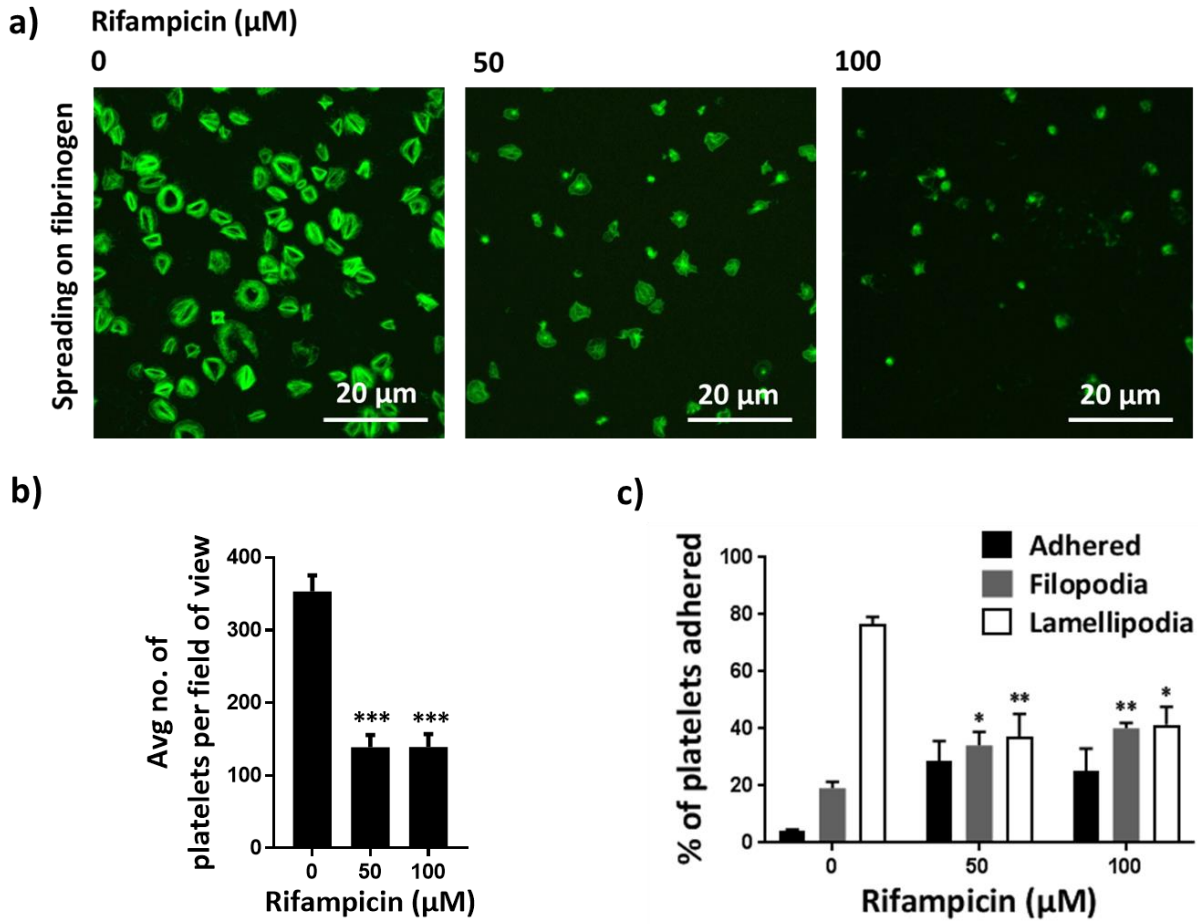


Figure 3.19: Rifampicin treatment inhibits platelet adhesion and spreading on fibrinogen. Human washed platelets ($2x10^7$ cells/ml) were treated with rifampicin (50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 min and then added onto fibrinogen (100 μg/ml) coated coverslips for 45 mins at 37°C. Samples were fixed with 0.2% (w/v) paraformaldehyde and permeabilised with 0.2% (v/v) Triton X-100. Alexa-Fluor 488 phalloidin was then added for 1 hour and coverslips were mounted onto slides using Prolong Gold Antifade mounting media. The visualisation was performed using a Nikon A1-R confocal microscope with a 100X oil immersion lens. Five images were captured of each sample at random locations on the slide. **(a)** Representative image of platelet adhesion and spreading on fibrinogen is shown. **(b)** An average number of platelets adhered in each sample are shown. **(c)** Spreading platelets were divided into 3 classes: (adhered but not spread; filopodia: platelets in the process of spreading and lamellipodia: fully spread). Results expressed (as relative frequency) as the percentage of the total number of platelets adhered. Data represent mean ± SEM (n=3), *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 was calculated by one-way ANOVA.

3.13. PXR ligands reduce platelet adhesion and spreading on collagen

Spreading of platelets on collagen involves contribution by adhesion receptor α2β1 and collagen receptor GPVI. Inoue *et al.* (2003) demonstrated α2β1-mediated spreading on collagen through a Src kinase-dependent pathway, which involves Syk, SLP-76, PLCγ2 and Ca^{2+} release. A direct role of GPVI and its signalling components (Syk, SLP-76, PLCγ2 and PI3K) on platelet spreading on CRP-XL has also been identified (Falet et al., 2000).

As can be seen in figure 3.20a and figure 3.21a, treatment with both PXR ligands significantly reduced platelet-adhesion to collagen-coated coverslips (100 μg/ml) in a concentration-dependent manner. An inhibition of 55% was observed with 50 μM of SR12813, while 100 μM caused inhibition of 62%, in comparison to vehicle-control (containing, DMSO 0.1% v/v) (Figure 3.20b). Similarly, rifampicin treatment also resulted in an equivalent level of inhibition (Figure 3.21b).

Platelet spreading on collagen was also substantially down-regulated in samples incubated with SR12813 (Figure 3.20a) or rifampicin (Figure 3.21a). A considerable difference in the appearance of platelets spreading on fibrinogen and collagen was noticed. While platelets spread on fibrinogen displayed well-defined lamellipodia with actin skeleton protruding outwards, giving them a ring-like structure. In the case of collagen, the actin cytoskeleton appeared to be more localised towards the platelet interior with thin projections of lamellipodia extending outwards, lacking a definite shape. The extent of spreading on collagen was inhibited strongly by PXR ligands in comparison to fibrinogen. For instance, the proportion of platelets becoming adhered but not spread were approximately

four times (52%) higher in 100 μM SR12813 treated samples, in comparison to vehicle-control (13%) (Figure 3.20c). Moreover, the proportion of platelets that were found fully spread in SR12813 treated groups were approximately 60% (at 50 μM) and 70% (at 100 μM) lower, in comparison to vehicle-control (Figure 3.20c). Spreading on collagen in rifampicin-treated samples were also attenuated to a similar extent (Figure 3.21c).

These findings clearly demonstrate that PXR ligands can down-regulate attachment of platelets with collagen. Since both integrin α2β1 and GPVI receptors are involved in the adhesion of platelets to collagen, these reductions might be an outcome of the effects of PXR ligands on either of the receptors or both and require further investigation. Moreover, this lack of adhesion and spreading on collagen is likely to have an impact on the initial build-up and stability of thrombus *in vivo*.

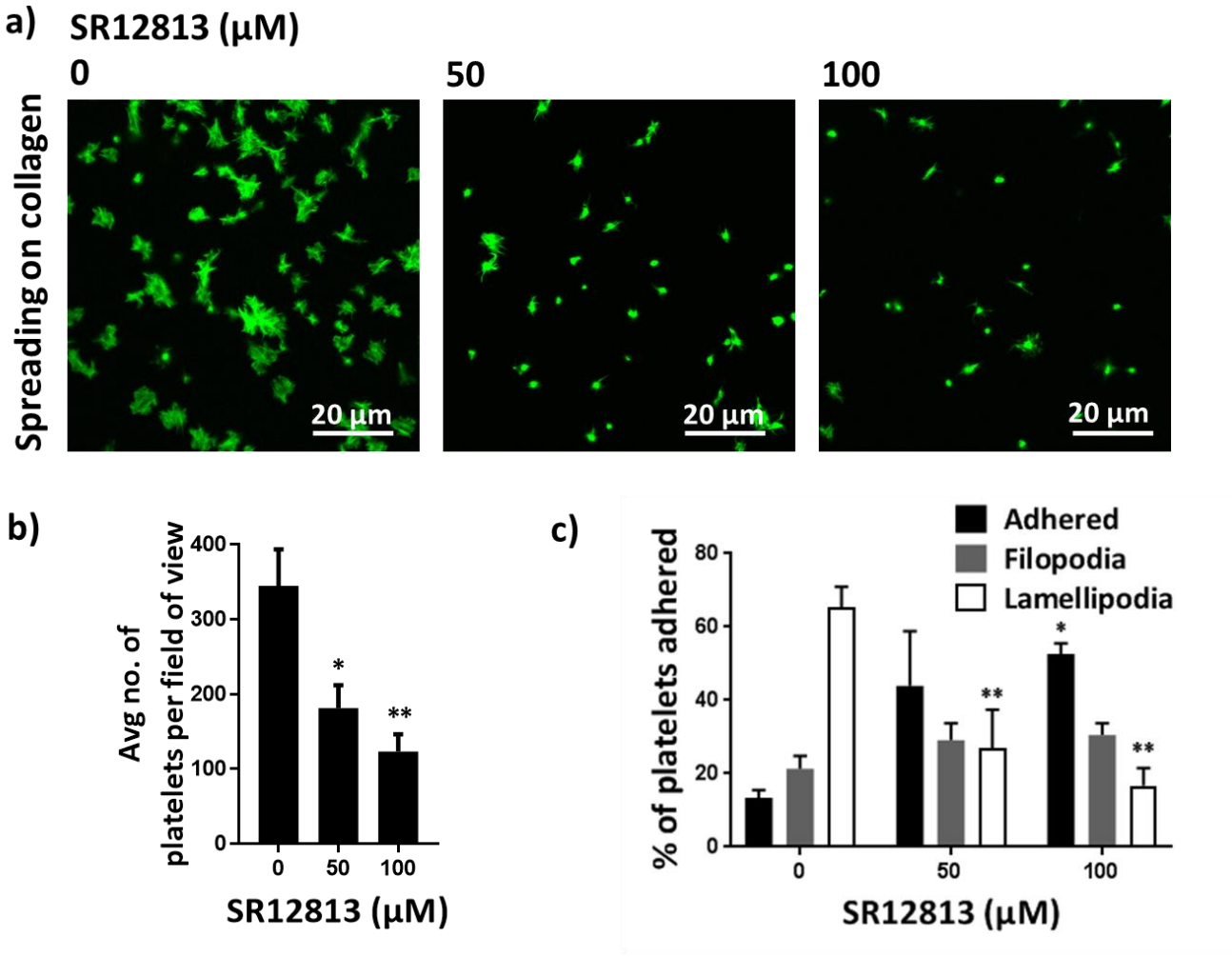


Figure 3.20: Negative-regulation of platelet adhesion and spreading on collagen following SR12813 treatment. Human washed platelets ($2x10^7$ cells/ml) were treated with SR12813 (50 and 100 µM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 min and then added onto collagen (100 µg/ml) coated coverslips for 45 mins at 37°C. Samples were fixed with 0.2% (w/v) paraformaldehyde and permeabilised with 0.2% (v/v) Triton-X-100. Alexa-Fluor 488 phalloidin was then added for 1 hour and coverslips were mounted onto slides using Prolong Gold Antifade mounting media. The visualisation was performed using a Nikon A1-R confocal microscope with a 100X oil immersion lens. Five images were captured of each sample at random locations on the slide. **(a)** Representative image of platelet adhesion and spreading on collagen is shown. **(b)** An average number of platelets adhered in each sample are shown. **(c)** Spreading platelets were divided into 3 classes: (adhered but not spread; filopodia: platelets in the process of spreading and lamellipodia: fully spread). Results expressed (as relative frequency) as the percentage of the total number of platelets adhered. Data represent mean±SEM (n=3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by one-way ANOVA.

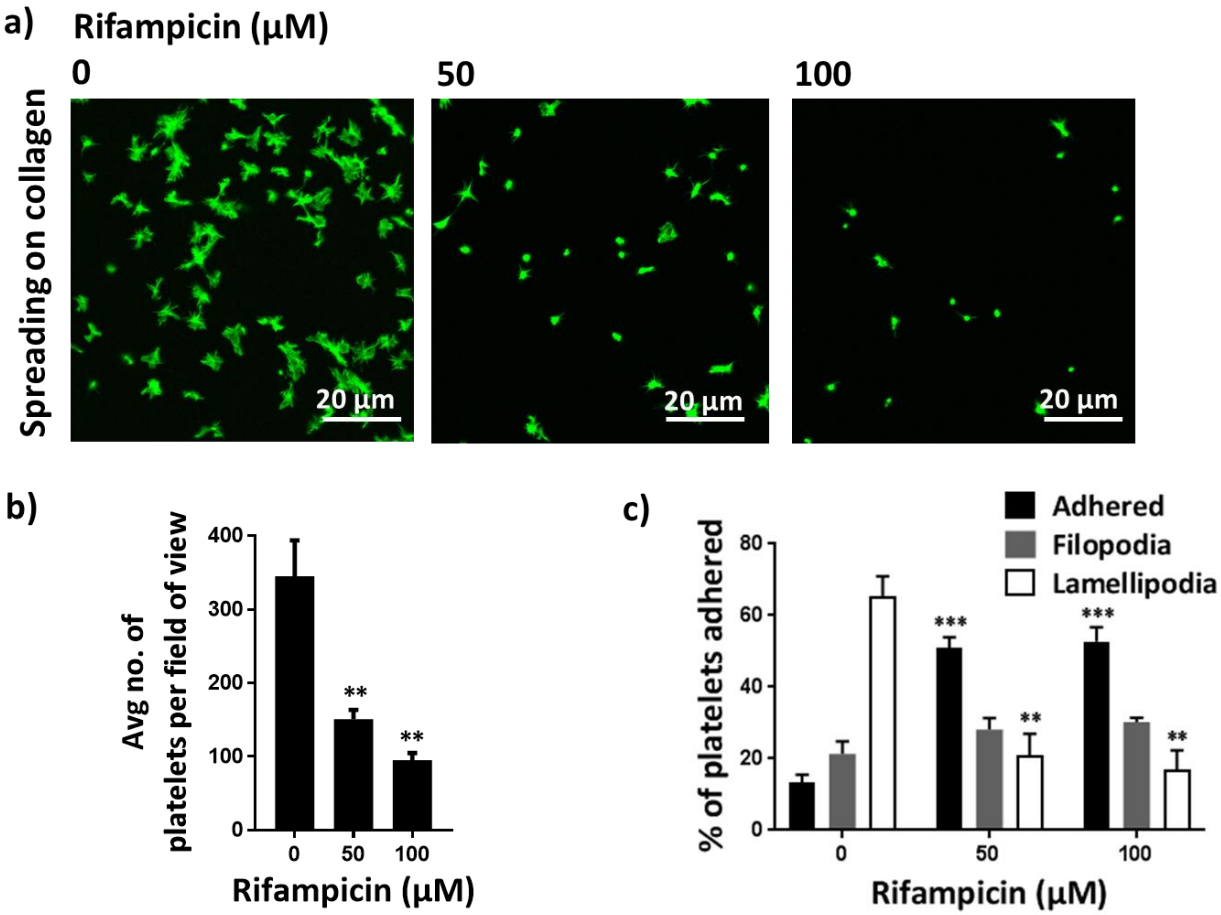


Figure 3.21: Platelet adhesion and spreading on collagen is attenuated following rifampicin treatment. Human washed platelets ($2x10^7$ cells/ml) were treated with rifampicin (50 and 100 µM) or vehicle-control (containing, DMSO 0.1%) for 20 min and then added onto collagen (100 µg/ml) coated coverslips for 45 mins at 37°C. Samples were fixed with 0.2% (w/v) paraformaldehyde and permeabilised with 0.2% (v/v) Triton-X-100. Alexa-Fluor 488 phalloidin was then added for 1 hour and coverslips were mounted onto slides using Prolong Gold Antifade mounting media. The visualisation was performed using a Nikon A1-R confocal microscope with a 100X oil immersion lens. Five images were captured of each sample at random locations on the slide. **(a)** Representative image of platelet adhesion and spreading on collagen is shown. **(b)** An average number of platelets adhered in each sample are shown. **(c)** Spreading platelets were divided into 3 classes: (adhered but not spread; filopodia: platelets in the process of spreading and lamellipodia: fully spread). Results expressed (as relative frequency) as the percentage of the total number of platelets adhered. Data represent mean ± SEM (n=3), **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

3.14. PXR ligands negatively regulate clot retraction

Clot retraction is a phenomenon that occurs within minutes or hours after a clot has been formed. It mainly involves pulling off the injured edges of the vessel close together with the expulsion of serum. This reduces the size of the injured area and stabilises the clot for better healing of the wound. Clot retraction is mediated by the release of several coagulation factors from platelets trapped in the fibrin clot. However, it depends primarily on the engagement of integrin αIIbβ3 present on the platelet surface and is regulated by the integrin-mediated outside-in signalling (Osdoit and Rosa, 2001). The physiological relevance of clot retraction lies in the clearance of the obstructed vessel for renewal of the blood flow. The evidence of direct involvement of integrin αIIbβ3 towards the regulation of clot retraction can be observed in patients suffering from Glanzmann thrombasthenia, which is due to the defects in αIIbβ3. Characterised by excessive bleeding, this disorder displays inability of platelets to aggregate and retract a fibrin clot (Nurden, 1999). Following the development of a clot, its retraction begins with platelets exerting a contractile force on their actin-myosin cytoskeleton, which are coupled to the cytoplasmic domain of integrin $\alpha_{IIb}\beta_3$. The external domain of these receptors, in turn, exist in a close association with fibrin network of the clot, which starts shrinking amidst the influence of contractile forces generated by the actin-myosin cytoskeleton of platelets (Li et al., 2010; Shattil et al., 1998; Shattil and Newman, 2004). A lack of clot retraction results in the formation of a layer of loosely packed platelets, which display less resistance against the mechanical forces of blood flow, resulting in reduced stability of the thrombus (Bye et al., 2017; Wohner, 2008).

Given the negative regulation of platelet spreading, studying clot retraction in the presence of PXR ligands would provide additional insights regarding their effects on outside-in signalling. Moreover, such effects would be investigated in a microenvironment that comprises of most of the components that participate in coagulation and haemostasis; thus, it would also help understanding the efficacy of PXR ligands in a slightly more physiological system.

Human PRP (supplemented with RBCs to visualise clot retraction) was incubated with PXR ligands (20, 50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 minutes. The reaction was initiated by adding thrombin (1 U/ml final concentration) and terminated after 60 minutes, at which the clot in the vehicle-treated sample was seen to have retracted completely. Following this, the clots were weighed to compare the extent of clot retraction. There exists an inverse relationship between clot weight and clot retraction with increased mass of clot corresponding to reduced contraction and extrusion of serum.

SR12813 treatment inhibited clot retraction at all the concentrations tested, however, the maximum retraction was noted at 100 μM (Figure 3.22 ai). Vehicle-treated samples displayed a mean clot weight of 73 mg, whereas in SR12813 (100 μM) treated samples this was 147mg, exhibiting a 101% increase in mean clot weight. Lower concentrations of 20 μM and 50 μM also resulted in a trend towards higher clot weights, in comparison to the vehicle but did not achieve significance (Figure 3.22 aii). Comparable to SR12813, rifampicin treatment also resulted in reduced clot retraction (Figure 3.22 bi). 100 μM of rifampicin exhibited a mean clot weight of 142.5 mg in comparison to vehicle-control (77.5 mg), demonstrating an increment of 84% (Figure 3.22 bii).

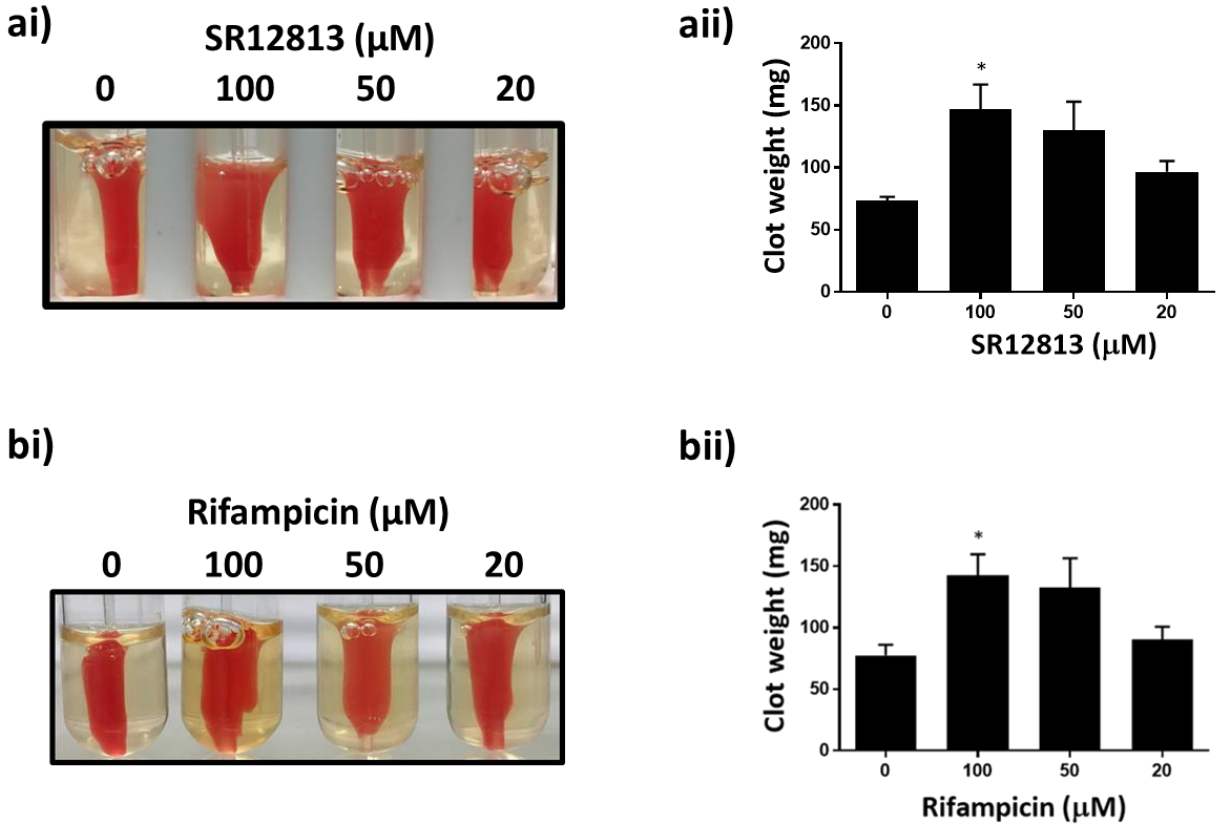


Figure 3.22: PXR ligands negatively regulate clot retraction. Human PRP was incubated with SR12813 or rifampicin (20, 50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 minutes. Thereafter, PRP was transferred into test tubes containing Tyrodes (supplemented with RBCs) to study and visualise the rate of clot retraction. The reaction was initiated by adding thrombin (1 U/ml final concentration) and a glass pipette was placed immediately into the centre of each test tube, around which the clot would form. Samples were placed in an incubator at 37°C. Clots were photographed every 15 minutes, and the assay was terminated after 60 minutes. **(ai, bi)** Representative images of clot retraction after the end of the assay in the presence of SR12813 or rifampicin. **(aii, bii)** Cumulative data represent clot weight (in mg) of samples treated with SR12813 or rifampicin and compared with vehicle-control. Data represent mean ± SEM (n=4), *P ≤ 0.05 was calculated by one-way ANOVA.

3.15. Chapter discussion

Besides thrombosis, platelets are important contributors towards the progression of atherosclerosis. Platelets via α2β1 and GPVI receptors (and vWF-GPIb interaction) become immobilised (and activates) on the collagen-cap exposed on the atherosclerotic endothelium. The cell adhesion molecules expressed on platelets favour recruitment of leukocytes (mostly monocytes), which promote inflammation (the primary cause of atherosclerosis) (Massberg et al., 2002). Immobilised platelets also interact with leukocytes via their surface P-selectin resulting in the formation of platelet-leukocyte aggregates that are pro-inflammatory in nature. Furthermore, activated platelets secrete chemokines that attract more inflammatory cells (macrophages, dendritic cells and lymphocytes) at the site of atherosclerotic lesion, promoting atherosclerosis (Galkina and Ley, 2009).

Dysregulation in the activity of PXR has been associated with the development of atherosclerosis while administration of PXR ligands have been proposed to upregulate the synthesis of HDL and its major constituent apolipoprotein A-I (ApoA-I), which promotes cholesterol efflux and reduce atherosclerosis (Bachmann et al., 2004; de Haan et al., 2009; Li et al., 2007; Masson et al., 2005). The presence of PXR, recently reported in the human vasculature, where it provides protection against oxidative stress represents a direct link between PXR and the cardiovascular system (Swales et al., 2012). Based on this and the presence of several other NRs in platelets, we investigated the presence and potential impact of PXR ligands on platelet functions. The major findings of this chapter include:

I. Trafficking of PXR following activation of platelets

Having established the expression of PXR in human and mouse platelets, immunofluorescence studies displayed a punctate arrangement of PXR in the cytosol of resting and permeabilised human platelets. Upon stimulation by U46619, they appeared to translocate towards the surface followed by their release. While super-resolution microscopy studies will be required to explore these findings completely, following two speculations can be made based on these observations: (i) A bulk of platelet secretions arise from granules and released via the OCS. Given the secretion of PXR from activated platelets, it might be possible that the location of PXR is associated with any of these organelles. It has been previously reported that RXR and PPARγ are secreted from activated platelets in the form of microparticles (derived from the plasma membrane) (Ray et al., 2008). This finding is interesting and relevant in the context of PXR as the shedding of PXR in the form of microparticles could be one of the possibilities. (ii) Signalling molecules such as $G\alpha_q$, Syk, Btk and PLCγ2 become translocated on the plasma membrane alongside several platelet receptors (GPIb-V-IX, GPVI, αIIbβ3 etc.) upon platelet activation to initiate signalling (Pula et al., 2005; Sarkar, 1998; Berger et al., 1996; Bobe et al., 2001). Interaction of NRs such as RXR, LXR and PPARγ with these signalling molecules has been reported previously (Moraes et al., 2010b; Moraes et al., 2007; Spyridon et al., 2011). Based on these observations, it can therefore be anticipated that NRs (seemingly PXR as well) migrate towards the membrane in an association with these proteins, which is also consistent with their potential involvement in signalling that will be discussed in chapter 4.

II. The potential role of PXR-RXR heterodimers in platelets

Based on the coimmunoprecipitation and immunofluorescence studies, the existence of PXR-RXR heterodimers in platelets was established. Previously, PPARγ has also been identified to exist in a bound state with RXR (Ray et al., 2008). While the binding of RXR to other NRs is vital for transcription under genomic regulation, the role of such heterodimers in the absence of DNA is unclear. Platelets are known to possess mRNA (derived from megakaryocytes), which can undergo translation (Zimmerman and Weyrich, 2008; Rowley et al., 2012). Recently Schwertz *et al.* (2017) proposed that RARα can bind to a subset of mRNA in human platelets and regulate protein synthesis by blocking translation. It is, therefore, possible that other NRs including PXR (in a bound or unbound state with RXR) can interact with mRNA in platelets and regulate translation as mediated by some of the NRs genomically (Xu and Koenig, 2004; Ottaviani et al., 2014). It would also be interesting to consider if NR carrying microparticles possess the ability to become internalised by other cell types after their release from platelets and whether they can instigate genomic (or non-genomic) regulation in these cells. Since releasates from platelets affect a multitude of cells (Lam et al., 2015), there could be numerous cell types that can act as potential recipients of the released PXR and RXR (or heterodimers). Ray *et al.* (2008) have reported that PPARγ bearing microparticles after release from activated platelets were internalised by a monocytic cell line THP-1, where they regulated cell function.

III. PXR ligands negatively regulate a range of platelet functions

Treatment with PXR ligands was observed to demonstrate inhibitory effects on several aspects of platelet activation. Since, the isolation of platelets from blood for an *in vitro* laboratory analysis greatly diminishes their activity over time, investigating chronic effects of a compound, which require prolonged incubation periods, becomes a limitation. Therefore, we explored the acute effects of PXR ligands, which require shorter incubation periods with higher concentrations of ligands (ranged between 10-100 μM in this study). A lack of high-affinity PXR ligands has been reported, resulting in an *in vitro* usage of PXR ligands at higher concentrations, generally two to three orders of magnitude higher than concentrations found circulating in plasma (Iyer et al., 2006; Navaratnarajah et al., 2012), which was an additional reason for choosing higher concentrations of PXR ligands for this study.

Both SR12813 and rifampicin were observed to inhibit platelet aggregation, fibrinogen binding to integrin αIIbβ3, degranulation and TxB_2 synthesis instigated by GPVI and GPCR agonists with significant reductions obtained mostly at 50 and 100 μM. A trend of inhibition (non-significant) was also exhibited at lower concentrations (10-20 μM) of PXR ligands. This is relevant considering the fact that the administration of rifampicin (600 mg) to patients for the treatment tuberculosis can achieve peak plasma levels up to 20 μM (Seth et al., 1993; van Ingen et al., 2011; Ruslami et al., 2007; Acocella, 1978). Therefore, it is probable that chronic exposure of PXR ligands at such concentrations might substantially inhibit platelet activation. It is also worth noting that a few case studies have reported thrombocytopenia as a rare side-effect in patients taking high-dose of rifampicin (Dixit et al., 2012; Zargar et

al., 1990; Verma et al., 2010; Hadfield, 1980; Ferguson, 1971). This is due to impaired platelet production or through the generation of anti-rifampicin antibodies that fix complement on the platelets, causing platelet destruction.

Besides the attenuation of platelet functions, a negative-regulation in CRP-XL or thrombin-stimulated calcium mobilisation was also exhibited by PXR ligands. Formerly, the ligands of RXR, LXR, FXR and PPARs have been reported to inhibit calcium mobilisation in platelets, which along with the present findings suggest a potentially fundamental role of NR ligands in regulating calcium homeostasis (Moraes et al., 2010b; Moraes et al., 2007; Moraes et al., 2016; Spyridon et al., 2011; Unsworth et al., 2017c; Ali et al., 2006; Ali et al., 2009a). Besides this, these results also add more evidence to the previously reported non-genomic modulation of calcium signalling by NR ligands in a range of cell types, which is one of the most consistent features of known non-genomic effects of NRs (Ordonez-Moran and Munoz, 2009). Since calcium plays a central role in platelet signalling, an alteration of platelet signalling by PXR ligands is seemingly plausible, with isoforms of phospholipase C (PLCγ2 and PLCβ) and protein kinase C, operative in GPVI and GPCR signalling being the prospective regulatory targets due to their close interaction with calcium mobilisation.

IV. Regulation of outside-in signalling by PXR ligands

Platelets treated with PXR ligands displayed a weaker fibrin clot retraction along with an attenuation of platelet spreading on fibrinogen-coated coverslips, indicative of reduced outside-in signalling. Lack of adhesion on fibrinogen specifies that only a small population of integrin αIIbβ3 exhibited an open confirmation

following treatment with PXR ligands. This could partly explain the observations on reduced spreading and clot retraction, since binding of fibrinogen to integrin αIIbβ3 is a prerequisite for the initiation of outside-in signalling, which is mediated by Gα13 and Rho GTPase (Arthur et al., 2000; Flevaris et al., 2007). These PXR ligands induced defects in outside-in signalling along with the inability of integrin αIIbβ3 to bind with fibrinogen following CRP-XL or thrombin stimulation (mediated by inside-out signalling) demonstrate the capability of PXR ligands to modulate bidirectional signalling of integrin αIIbβ3.

Similar to these observations, adhesion and spreading of platelets on collagen was found to be attenuated following treatment with PXR ligands. The inhibitory effects of PXR ligands on collagen-mediated spreading were stronger in contrast to fibrinogen. This can be explained based on similarities in the proteins participating in integrin α2β1 outside-in and GPVI-mediated signalling (Src family kinases, syk and PLCγ2). Inhibition of any (or all) of these signalling proteins would potentially affect signalling downstream of these two prominent receptors responsible for platelet adhesion/spreading on collagen and thereby causing profound inhibitions (Inoue et al., 2003). In addition to this, the dimeric form of GPVI is known to have approximately 100-fold higher affinity for collagen (Jung et al., 2009; Jung et al., 2012). It is therefore possible that PXR ligands may prevent dimerisation of GPVI, which reduces its adhesion to collagen. Recently, Poulter *et al.* (2017) reported a mechanism where clustering of GPVI receptors enhanced its avidity for collagens. The clustering was also proposed to enhance the proximity of GPVI-associated signalling molecules, which could assist in initiating and amplifying GPVI-mediated signalling (Poulter et al., 2017). Having mentioned this, it would be

interesting to explore whether PXR ligands regulate GPVI dimerisation and subsequent clustering to influence adhesion and spreading on collagen.

The findings presented in this chapter demonstrate that PXR ligands can negatively regulate numerous vital aspects of platelet activation, including adhesion to collagen, calcium mobilisation, degranulation, fibrinogen binding to integrin αIIbβ3, and integrin outside-in signalling. Based on these observations, it can be speculated that such inhibitory effects of PXR ligands are likely to have an impact on the development and stability of the thrombus under *in vitro* arterial flow conditions or *in vivo*. This could potentially regulate haemostasis as well. In addition to this, it would also be interesting to explore whether the inhibitory effects of PXR ligands are an outcome of their influence on the molecular mechanisms that control platelet activation via different signalling pathways.

Chapter-4

Species-specific effects and the inhibition of collagen-mediated platelet signalling by PXR ligands

4.1. Introduction

The PXR is the only NR in the superfamily which displays a significant sequence divergence amongst species. On an average, less than 80% sequence-identity exists between numerous mammalian PXR isoforms (Jones et al., 2000). For example, human and rodent ligand binding domain (LBD) sequence display only 74% similarity in their amino acid residues (Figure 4.1), which reduces further down to 50% between human and chicken or fish (Zhang et al., 2008). In contrast to PXR, the corresponding sequence identities between other members of the NR superfamily is 15-20% higher. Furthermore, even the PXR DNA binding domain, which is more conserved across species than the LBD, displays more cross-species diversity than other NRs (Ekins et al., 2008). Apart from dietary dissimilarities, differences in the composition of bile acids are proposed to be the most important physiological reasons driving the evolution of unexpectedly large sequence variation in the ligand binding pocket amongst different species (Ekins and Schuetz, 2002; Krasowski et al., 2005).

This sequence-variation in the LBD has consequently resulted in species-specific activation of PXR by species-specific ligands. For instance, the antibiotic rifampicin and SR12813 (candidate cholesterol-lowering drug) are potent activators of human PXR, while, they do not influence the activity of mouse PXR (Jones et al., 2000). Similarly, pregnenolone-16α-carbonitrile (PCN) is specific to mouse PXR and does not affect human PXR (Watkins et al., 2001; Iyer et al., 2006). Since, rodents are widely used as model-species to evaluate the efficacy and toxicity-profile of any candidate human drug, species-specific nature of PXR ligands severely limits the evaluation of human PXR ligands in mouse models. This

flagged the way for the development of 'humanised' PXR mice (hPXR) in which the endogenous gene is replaced with human PXR gene. Xie *et al*. (2000) generated a conditional or whole body humanised PXR mice on the mouse PXR-null background via homologous recombination, where the mouse PXR was removed, and a human PXR cDNA was introduced into the mouse liver through a liver-specific transgene (Xie et al., 2000). Besides this a few other groups have also developed whole body hPXR expressing mice through different approaches such as insertion of the complete human PXR coding sequence contained within a bacterial artificial chromosome (Ma et al., 2007a) or insertion of the human PXR coding region into wild-type mice by using the flipase recombinase system (Scheer et al., 2008; Scheer et al., 2010). The response profile of PXR in these mice was found to be positive towards rifampicin while no response towards mouse PXR ligands was observed (Ma et al., 2007a; Xie et al., 2000).

Given the inhibitory effects of human PXR ligands observed over a range of platelet functions in the previous chapter, the aims of this chapter were:

1. To explore whether human and mouse PXR ligands display species-specific effects on platelets as reported in other cell types.
2. To investigate the effects of human and mouse PXR ligands on thrombus formation *in vitro* in whole-blood at an arterial flow rate.
3. To evaluate the influence of human PXR ligand SR12813 on thrombosis and haemostasis in transgenic humanised PXR mice (Taconic Biosciences).
4. To study the effects of human PXR ligands on platelet signalling

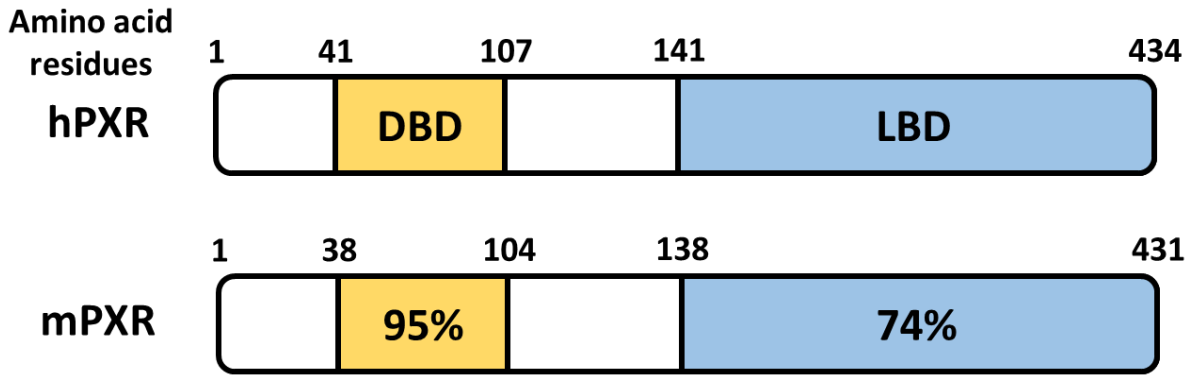


Figure 4.1: Comparison of amino acid sequence similarity between human and mouse PXR genes. The percentage amino acid sequence identity between the DNA binding domain (DBD) and ligand binding domain (LBD) of human PXR (hPXR) and mouse PXR (mPXR) is indicated. (Adapted from Xie *et al*. 2002)

4.2. Human and mouse PXR ligands exhibit species-specific effects on platelet function

As mentioned earlier, due to dissimilarity in the sequence of the LBD, there exist a high degree of inter-species differences in ligands that activate PXR (Zhang et al., 2008). This feature of PXR was investigated using both human and mouse platelets. The effects of human (SR12813 or rifampicin) or mouse PXR ligands (PCN) on CRP-XL stimulated fibrinogen binding were investigated to study species-specific effects of PXR ligands.

Human or mouse PRP was incubated with human PXR ligands - SR12813 or rifampicin (50 and 100 μM) or mouse PXR ligand - PCN (50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v for SR12813 or rifampicin and 0.5% v/v for PCN treated samples). After an incubation period of 10 minutes with PXR ligands FITC-conjugated anti-human fibrinogen antibody was added. PRP was stimulated with the GPVI-specific agonist, CRP-XL (0.25 μg/ml for human PRP and 0.5 μg/ml for mouse PRP) for 20 minutes at room temperature, with occasional gentle mixing. Samples were then fixed with 0.2% (w/v) formyl saline, and fluorescence was measured for 10,000 events by flow cytometry.

It was not possible to directly compare the responses of human and mouse platelets as their activation profile is quite different towards the similar concentrations of platelet agonists. For instance, a concentration of 0.25 μg/ml of CRP-XL was sufficient to activate human platelet samples and study the effects of human PXR ligands, whereas, this produced a much more modest effect on mouse platelets. For this reason, the CRP-XL concentration was enhanced to 0.5 μg/ml in experiments using mouse PRP.

In comparison to vehicle-control (containing, DMSO 0.1% v/v), 100 μM of SR12813 reduced CRP-XL-stimulated (0.25 μg/ml) fibrinogen binding in human platelets by 50% (Figure 4.2 ai). However, SR12813 did not cause any change in mouse platelet responses stimulated with CRP-XL (0.5 μg/ml) (Figure 4.2 aii). Furthermore, incubation of human PRP with rifampicin resulted in an inhibition of fibrinogen binding by 55% (Figure 4.2 bi), whereas, a modest reduction of 15% was exhibited with mouse platelets (Figure 4.2 bii). Similarly, mouse PXR ligand, PCN (100 μM), inhibited CRP-XL evoked fibrinogen binding in mouse platelets by 25% in comparison to vehicle-control (containing, DMSO 0.5% v/v) (Figure 4.2 cii), whereas, no effect was observed on human platelets (Figure 4.2 ci). Consistent with the species-specific activation of PXR by its ligands reported in other cell types, these results demonstrate the ability of human and mouse PXR ligands to selectively target PXR in platelets in a species-specific manner (Ostberg et al., 2002; Wang et al., 2012).

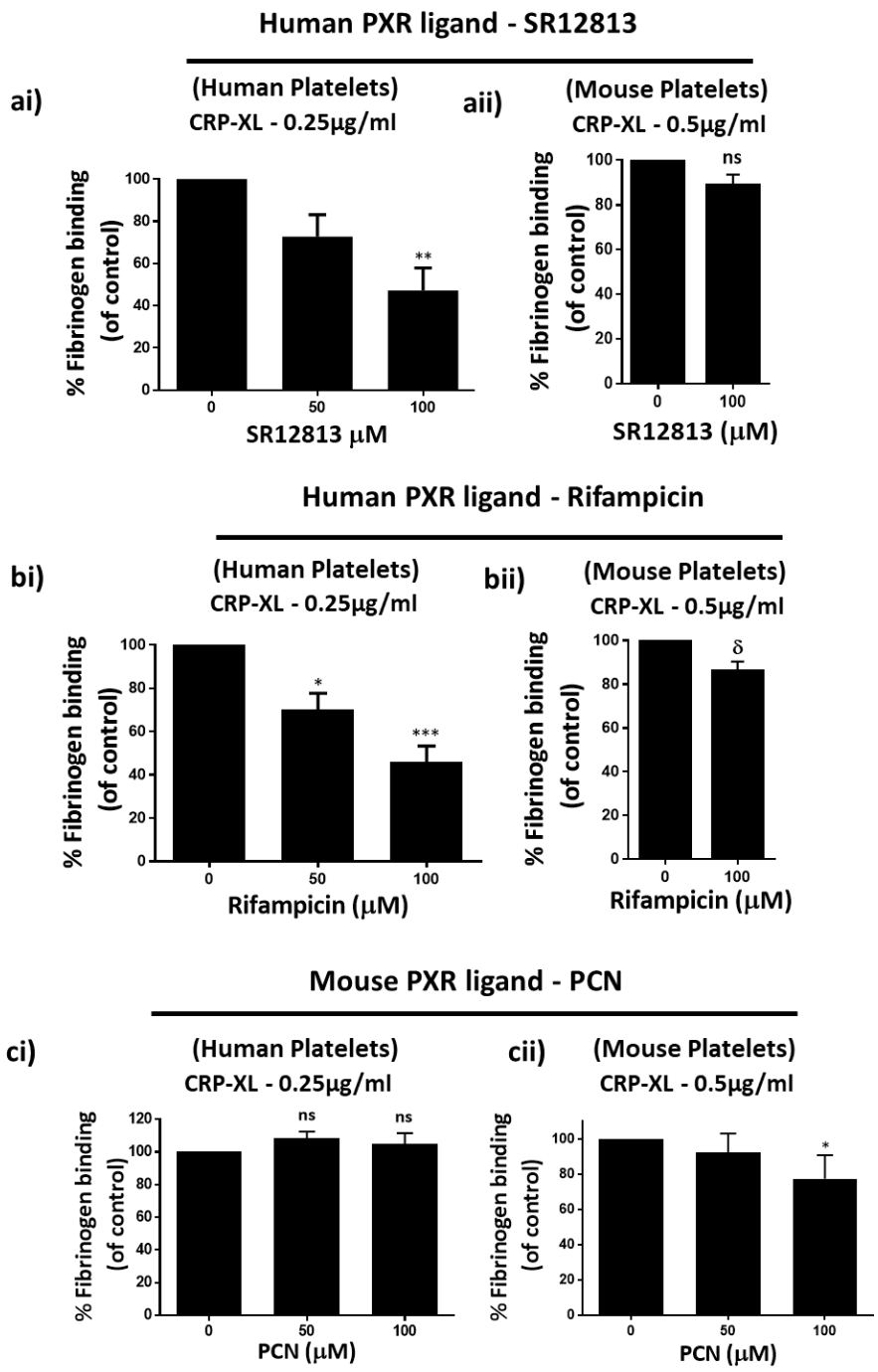


Figure 4.2: Human and mouse PXR ligands inhibit fibrinogen binding in a species-specific manner. Human and mouse PRP was incubated with **(ai, aii)** SR12813 or **(bi, bii)** rifampicin (50 and 100 µM) or **(ci, cii)** PCN (50 and 100 µM) or vehicle (containing, DMSO 0.1% v/v for human PXR ligands or 0.5% v/v for PCN) for 10 minutes. This was followed with the addition of FITC-labelled rabbit anti-fibrinogen antibody and stimulation of samples with CRP-XL (0.25 µg/mL for human PRP and 0.5 µg/mL for mouse PRP) for 20 minutes with occasional gentle mixing. Post-stimulation, samples were fixed with 0.2% (v/v) formyl saline and analysed by flow cytometry. Data represent percentage fibrinogen binding compared to vehicle-treated control, which is defined as 100% fibrinogen binding. Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by by one-way ANOVA. $^{\delta}$P ≤ 0.05 was calculated by by student t-test.

4.3. Human and mouse PXR ligands inhibit thrombus formation *in vitro* in a species-specific manner

Having observed the ability of PXR ligands to inhibit platelet activation induced by GPVI agonists, collagen and CRP-XL (discussed in chapter-3), their effects on thrombus formation on collagen *in vitro* under arterial flow condition was examined. Conventional *in vitro* assays such as aggregation are performed on isolated platelets that are stirred in the presence of an agonist and does not consider numerous factors that can influence thrombus development. For instance, at the site of injury i*n vivo*, platelets become exposed to collagens, only to form a monolayer over it. After that, it is the mainly the role of prothrombotic molecules that are secreted from platelets (and thrombin which is a product of coagulation cascades) to recruit more platelets and amplify thrombus formation. Moreover, platelets circulate physiologically under the constant presence of other blood cells, plasma proteins and varying shear rates (largely depends on the part of circulation), which regulate their function and modulate the stability of the thrombus. Additionally, some of the ligands/drugs have a tendency to bind with plasma proteins (such as albumin, immunoglobulins, lipoproteins and glycoproteins), which can considerably affect availability towards the target cell and thereby reducing their efficacy (Trainor, 2007). We therefore sought to explore the effects of PXR ligands on thrombus formation in whole blood using flow assay, which would help in elucidating a bit more accurate effects of PXR ligands on platelets.

Vena8 Biochips were coated with type-I collagen (100 μg/ml) to perform the assay and citrated human blood was incubated with lipophilic dye DiOC6 (5 μM) for an hour at 30^{o}C. Excess collagen was washed away with modified Tyrodes-HEPES buffer. Whole blood was incubated with the human PXR ligands, rifampicin or SR12813 (100

μM) or vehicle-control (containing, DMSO 0.1% v/v) for 20 minutes at 30^{o}C before perfusion through the collagen-coated microfluidic channels under arterial flow conditions (shear stress: 20 dyne/cm^{2} or shear rate: 500 s^{-1}). Fluorescence was excited at 488nm with an argon laser and emission was detected at 500-520nm. The thrombus formation was observed using a Nikon A1-R confocal microscope with a 20X objective and images were captured (focused on a single section) every 1 second for 600 seconds. Mean thrombus fluorescence intensity was calculated using NIS elements software (Nikon) and normalised to the level of fluorescence at the end of the assay in the vehicle-treated sample.

Representative images from the end of the assay display vehicle-treated (containing, DMSO 0.1% v/v) samples forming big, bright and stable thrombi in 10 minutes (figure 4.3ai). Treatment with human PXR ligand SR12813 significantly reduced the development of thrombus where thrombi appeared to be smaller and unstable, causing them to break under the influence of flow. Consequently, as shown in figure 4.3aii, a consistent significant reduction in thrombus formation right from the early stages of the assay was observed. Overall, SR12813 reduced the formation of thrombus by 60% at the 10-minute endpoint (Figure 4.3 aii). A similar inhibitory profile exhibited in rifampicin-treated samples (figure 4.3bi), caused 65% inhibition in thrombus growth in comparison to vehicle-control (Figure 4.3 bii). These observations demonstrate the potential of PXR ligands to down-regulate platelet activation even in the presence of plasma proteins and other blood cells.

Reduction in thrombus formation from the beginning of the assay (Figure 4.3 aii, bii) suggests that human PXR ligands may affect the ability of platelets to adhere to collagen, which subsequently hinders the initiation of thrombus development. A level of

significance with reduced thrombus formation was detected as early as the third minute and the first minute for the SR12813 and rifampicin treated samples respectively (Figure 4.3 aii, bii). To test this hypothesis, a similar flow assay was performed in the presence of integrillin (4 μM, an integrin αIIbβ3 antagonist) to block platelet-platelet interactions. A lack of interaction between platelets would prevent thrombus formation, allowing the effects of PXR ligands to be studied on platelet adhesion specifically. As shown in figure 4.3ci, in vehicle treated sample (containing, DMSO 0.1% v/v), the presence of integrillin substantially reduced the formation of thrombus, with platelets only adhering to collagen. Further treatment of blood (in addition to integrillin) with SR12813 (100μM) reduced platelet adhesion to collagen by 30%, in comparison to vehicle-control. This finding is in accordance with the previous observation that PXR ligands reduce adhesion of platelets to collagen-coated coverslips, as discussed in chapter-3 (section 3.13). Based on these results, the overall inhibition of thrombus growth in the presence of PXR ligands can be explained partly due to the inability of platelets to adhere to collagen. Since both GPIb and GPVI receptors control the adhesion of platelets to collagen (Nieswandt and Watson, 2003), the observed decrease in adhesion might be a consequence of reduced attachment of GPIb or GPVI (or both) to collagen. Moreover, the involvement of PXR ligands to regulate integrin α2β1-dependent attachment to collagen cannot be ruled out (Surin et al., 2008).

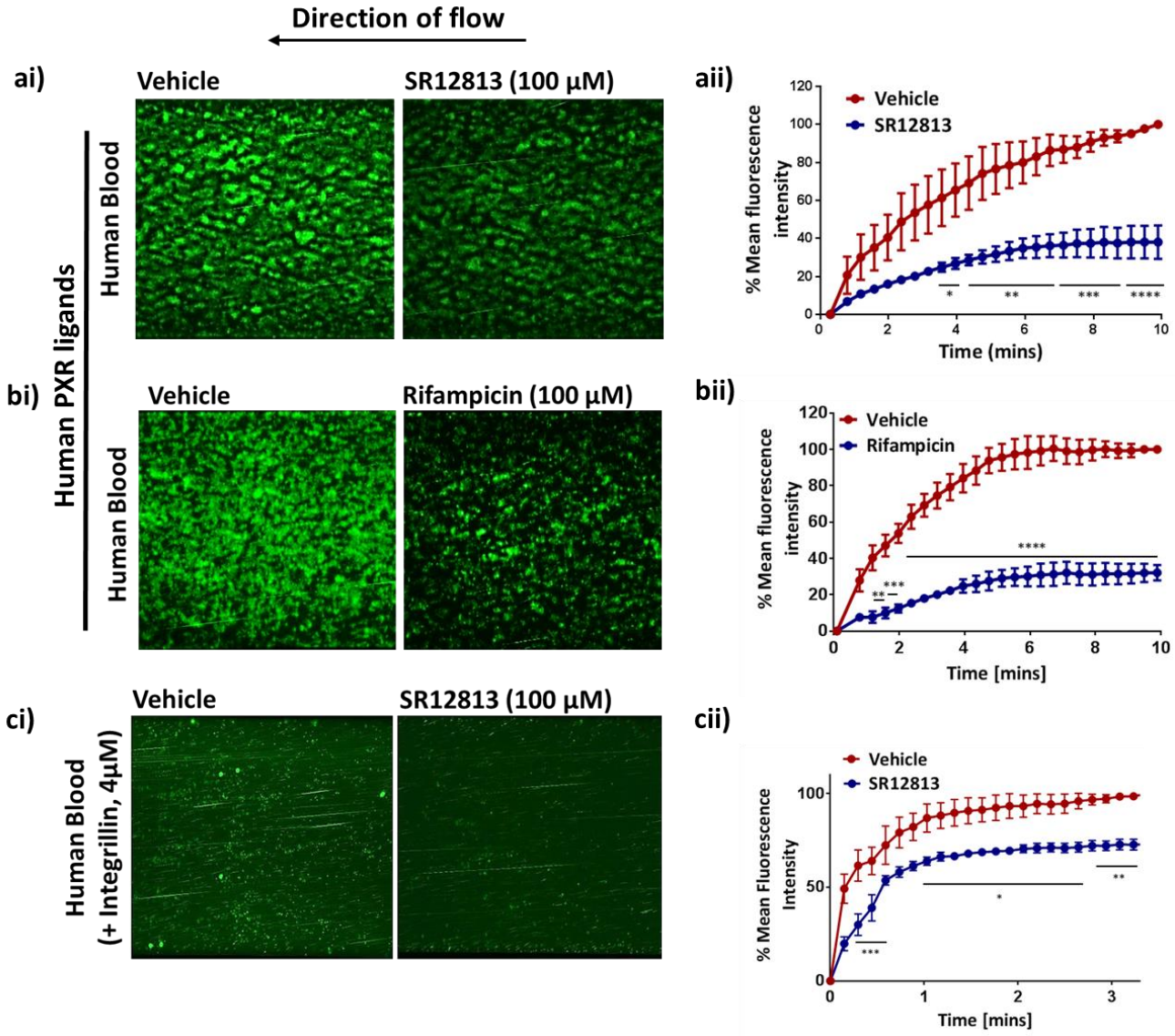


Figure 4.3: Human PXR ligands attenuate thrombus formation *in vitro*. Citrated human blood, incubated with DiOC6 (5 µM) for an hour at 30°C was perfused through collagen-coated (100 µg/ml) Vena8 microfluidic chips under arterial flow condition (shear stress: 20 dyne/cm^2 or shear rate: 500 s^{-1}) after treatment with vehicle (containing, DMSO 0.1% v/v) or PXR ligands (100µM) for 20 minutes. Representative images of thrombus formation (10-minute endpoint) in samples treated with vehicle or **(ai)** SR12813 or **(bi)** rifampicin are shown. **(ci)** Level of platelet adhesion to collagen was also measured using blood treated with integrillin (4 µM) and incubated with vehicle or SR12813 for 20 minutes. Fluorescence was excited at 488nm with an argon laser and emission was detected at 500-520nm. The thrombus formation was observed using a Nikon A1-R confocal microscope (20X objective), and images were captured every 1 second for 600 seconds (focused on a single section). Quantified data represent mean thrombus fluorescence intensity for **(aii)** SR12813, **(bii)** rifampicin and **(cii)** SR12813 (+integrillin) treated samples calculated using NIS elements software (Nikon) and normalised to the level of fluorescence at the end of the assay in the vehicle-treated sample. Data represent mean ± SEM (n=3), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by two-way ANOVA.

As discussed in section 4.2, PCN exerted a modest reduction (25%) in CRP-XL stimulated fibrinogen binding in mouse platelets. It is observed that such subtle effects on platelet functions might become much more pronounced and evident when investigated using an *in vitro* flow assay on whole blood. Based on this understanding and to further study whether PCN retains its inhibitory effects in mouse blood, its ability to regulate thrombus formation *in vitro* was investigated. Additionally, this assay would also be helpful in exploring the species-specific effects of the PXR ligands with an improved and different perspective. The conditions of the assay were similar for both human and mouse blood (in contrast to the fibrinogen binding assay) as the same concentration of collagen (100 μg/ml) is used to coat the channels of the microfluidic chip. Since only a limited amount of blood was obtained after bleeding the mice, the time of the assay was reduced from 10 minutes to 8 minutes.

In comparison to vehicle-treated control (containing, DMSO 0.5% v/v), treatment of mouse blood with PCN (100 μM) for 20 minutes significantly inhibited thrombus formation, as shown in figure 4.4ai. A 50% reduction in the level of thrombus formation was achieved in 8 minutes (Figure 4.4aii). This extent of reduction was found to be double that of the level with CRP-XL-mediated fibrinogen binding in PCN treated mouse PRP samples, discussed in section 4.2. The profile of thrombus formation in PCN treated mouse blood samples also followed a similar pattern as observed with human blood treated with human PXR ligands. In comparison to the vehicle control, the thrombi formed in PCN treated mouse blood samples displayed instability causing them to break apart from the thrombus, under the influence of flow. Although, the early kinetics of thrombus formation were identical to the vehicle-treated control for up to first 2 minutes (Figure 4.4aii). This variation in the initial kinetics between human and

mouse platelets is unclear. A possible reason might be related to the variation in the sequence and structure between human and mouse PXR ligand binding domain, which can affect the manner and extent to which their corresponding ligands bind and thus affect their potency.

In contrast to the effects of mouse PXR ligand studied on mouse blood, treatment of human blood with PCN (100 μM) exhibited no significant difference in thrombus formation in comparison to vehicle-control (containing, DMSO 0.5% v/v) as shown in figure 4.4bi. Both vehicle and PCN treated samples exhibited the same level of thrombus growth during 8 minutes with a subtle inhibition of only 10% achieved at the end point of the assay (Figure 4.4bii). To further investigate the species-specific nature of the PXR ligands, the effect of human PXR ligand SR12813 was evaluated on mouse blood. It was previously shown in figure 4.3ai that human blood post-treatment with SR12813 (100 μM) exhibited a substantial reduction in the thrombus growth. However, the initial and late kinetics of thrombus growth in mouse blood treated with SR12813 were similar to the vehicle-treated control (containing, DMSO 0.1% v/v) as shown in figure 4.4ci with no significant change in the thrombus formation at the endpoint of the assay (figure 4.4cii).

These findings clearly demonstrate that both human and mouse PXR ligands can down-regulate thrombus formation *in vitro* in human and mouse blood respectively. The inability of human and mouse PXR ligands to affect thrombus formation in mouse and human blood samples, respectively, demonstrate the species-specific action of these ligands.

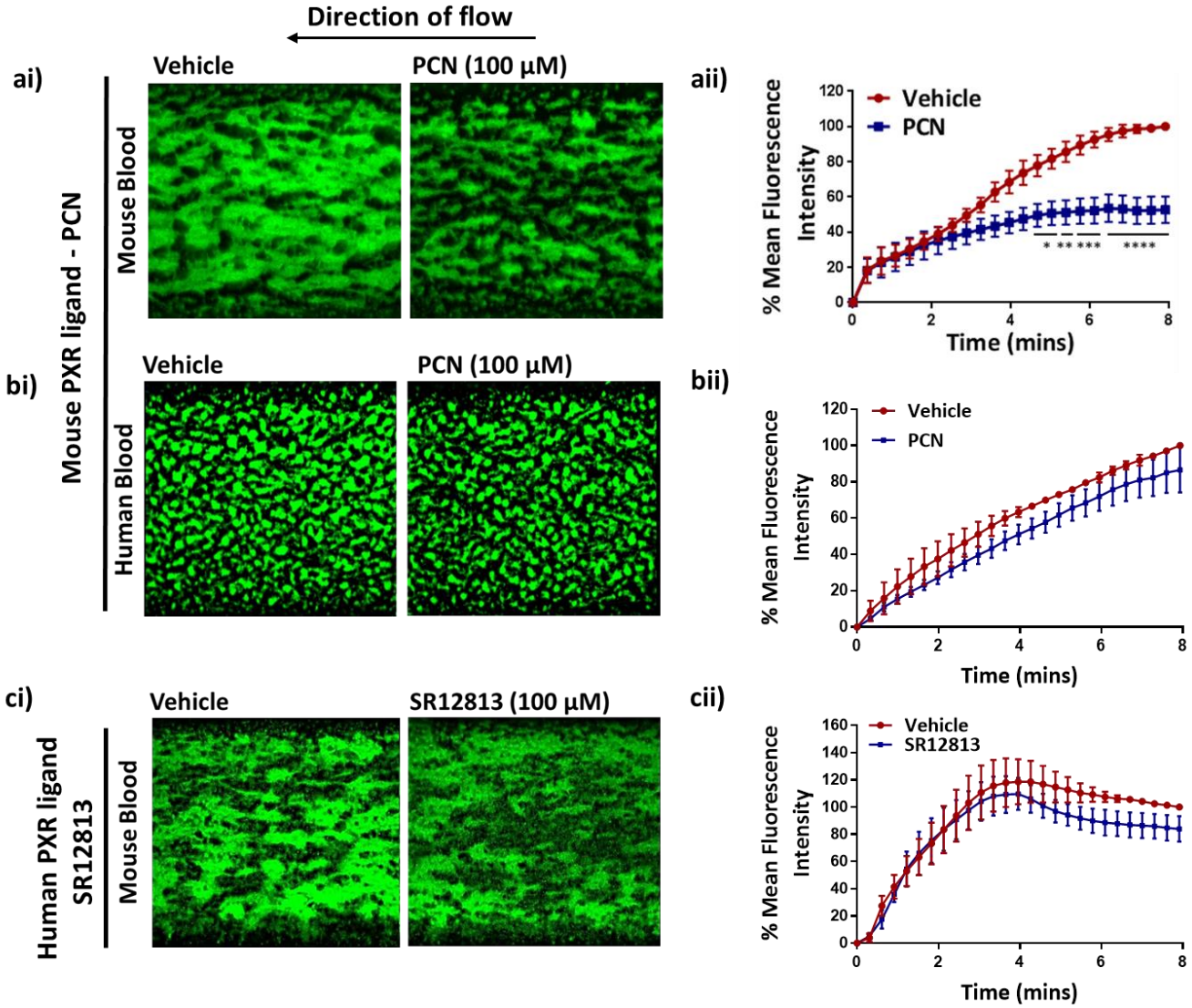


Figure 4.4: Mouse and human PXR ligands inhibit thrombus formation *in vitro* in a species-specific manner. Citrated mouse and human blood incubated with DiOC6 (5 µM) for an hour at 30°C were perfused through collagen-coated (100 µg/ml) Vena8 microfluidic chips under arterial flow conditions (shear stress: 20 dyne/cm^2 or shear rate: 500 s^{-1}) after treatment with vehicle or PXR ligands for 20 minutes. Representative images display thrombus formation (8-minute endpoint) in **(ai)** mouse blood sample treated with vehicle (containing, DMSO 0.5% v/v) or mouse PXR ligand PCN (100 µM), **(bi)** human blood sample treated with vehicle (0.5% v/v DMSO) or PCN (100 µM) and **(ci)** mouse blood sample treated with vehicle (containing, DMSO 0.1% v/v) or human PXR ligand SR12813 (100 µM). Fluorescence was excited at 488nm with an argon laser and emission was detected at 500-520nm. The thrombus formation was observed using a Nikon A1-R confocal microscope (20X objective), and images were captured every 1 second for 480 seconds (focused on a single section). Quantified data represents mean thrombus fluorescence intensity for **(aii)** PCN (mouse blood sample), **(bii)** PCN (human blood sample) and **(cii)** SR12813 (mouse blood sample) treated samples calculated using NIS elements software (Nikon) and normalised to the level of fluorescence at the end of the assay in the vehicle-treated sample. Data represent mean ± SEM (n=4), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by two-way ANOVA.

4.4. Characterisation of the humanised PXR mice for *in vivo* experiments

As discussed previously, owing to a variation in the LBD sequence of PXR amongst different species, humanised models of mice (hPXR) expressing human PXR gene were developed (Scheer et al., 2008; Ma et al., 2007a; Xie et al., 2000). Considering the species-specific effects of human and mouse PXR ligands observed in human and mouse platelets, we used a hPXR transgenic mouse (purchased from Taconic Biosciences and bred at the bioresource unit of the University of Reading, 2017-18) to investigate the effects of human PXR ligand SR12813 *in vivo*. The mice were developed through a knock-in of a human PXR cDNA/genomic construct (fusion of exons 2 through 4, exons 4 and 8, and exons 8 and 9) onto the ATG sequence of murine PXR in C57BL/6NTac-derived embryonic stem cells (Scheer et al., 2010; Scheer et al., 2008).

Each hPXR mouse was genotyped to evaluate the presence of hPXR gene as per the manufacturer's protocol prior to their use in experiments. The protocol followed is described in detail in chapter 2 (section 2.2.14). An example of genotype data is shown in figure 4.5a, demonstrating PCR analysis of genomic DNA isolated from four different ear-clip samples of hPXR mice and wild-type C57BL/6 mouse. The band was found at the expected size of 364bp for the hPXR and 733bp for WT mouse.

Prior to the investigation, the expression levels of platelet receptors: integrin α2β1 (figure 4.5b), integrin αIIbβ3 (figure 4.5c), GPIb (figure 4.5d) and GPVI (figure 4.5e) on hPXR and its background C57BL/6 wild-type mice were evaluated and were found to be similar in resting and activated platelets.

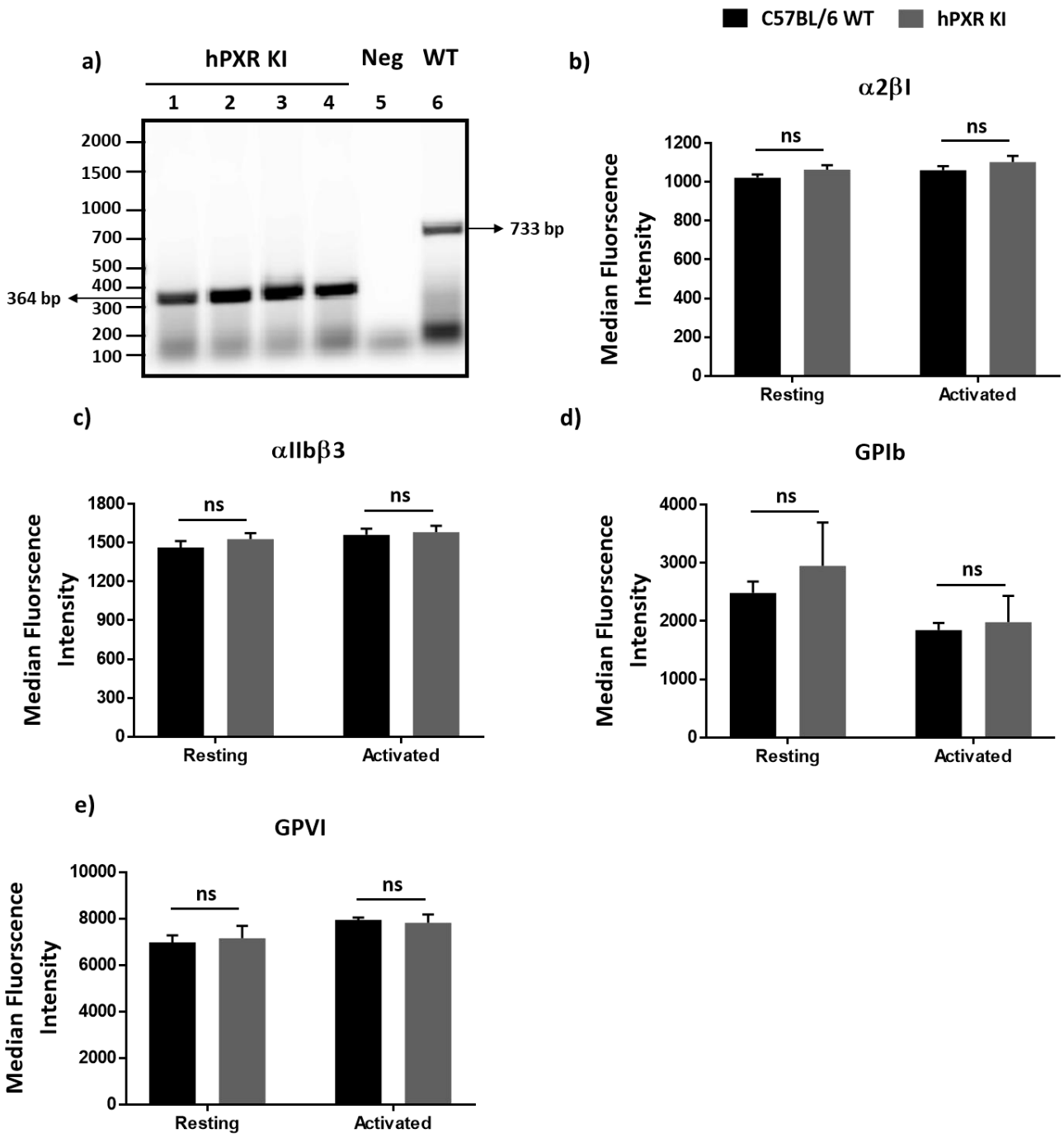


Figure 4.5: Insertion of human PXR gene does not affect platelet receptor levels in hPXR mice. (a) A representative image of genotyping performed on ear tissue samples. Lane 1-4 represents a band size of 364bp for human PXR insert in hPXR mouse; Lane 5 shows negative control and Lane 6 shows a band of 733bp of WT PXR in C57BL/6 mouse. The expression levels of **(b)** α2β1, **(c)** αIIbβ3, **(d)** GPIb, and **(e)** GPVI were analysed on resting and activated (CRP-XL 1µg/ml) platelets from hPXR and C57BL/6 wild-type mice by flow cytometry. Data represent median fluorescence intensity as mean ± SEM (n=4). Student t-test was used to evaluate statistical significance.

Abbreviations: hPXR- humanised PXR, KI- Knock-in, WT- Wild type, Neg- Negative control

4.5. The human PXR ligand SR12813 inhibits thrombosis and haemostasis in humanised PXR mice

Having established that PXR ligands negatively regulate thrombus formation *in vitro*. We further sought to examine their potential implications on thrombosis and haemostasis *in vivo* in hPXR mice. In comparison to the *in vitro* thrombus formation assay, this assay offers the benefit of being more physiological in nature as it considers the influence of factors such as endothelial cells, blood flow, blood pressure and metabolism on the growth of the thrombus and efficacy of the ligand being tested.

The potential effects of the human PXR ligand SR12813 on thrombosis was evaluated using an *in vivo* thrombosis assay performed on hPXR mice as described by Falati *et al.* (2002). This assay measures the ability of fluorescently labelled platelets to form thrombi following a laser-induced injury to an arteriole of the cremaster muscle. Briefly, to perform the assay, mice were anesthetised, and the cremaster muscle of the testicle exteriorised. Connective tissue was removed, an incision was made, and the muscle was affixed over a glass slide as a single sheet, hydrated throughout with buffer. Platelets were labelled with DyLight 649 anti-GPIbα antibody, after which the cremaster arteriole wall was injured using a Micropoint ablation laser unit. Thrombus formation was then observed for 5 minutes, with images captured both prior to and after injury using a digital camera; multiple thrombi were formed in vehicle and SR12813 treated mice (20 minutes). Data analysis was performed by analysing images using Slidebook software (version 6). Median fluorescence of all thrombi was integrated and displayed as a line graph. Procedures on these mice require micro-surgery expertise and were therefore performed in collaboration with Dr P. Sasikumar.

As shown in figure 4.6 ai, large and stable thrombi were formed in vehicle-treated mice (containing, DMSO 0.1% v/v), whereas, treatment with SR12813 (100 μM) was associated with reduced size and stability of thrombi, which was characterised by continuous thrombus embolisation and reformation. However, the initiation of thrombus formation in both SR12813 and vehicle-treated mice was very similar for the first 30 seconds after the induction of injury, but thrombi receded much more rapidly in SR12813 treated mice, as displayed in the image (figure 4.6 ai) and line graph (figure 4.6 aii), which represents integrated median fluorescence intensity of all the thrombi.

Any discontinuity in the endothelial layer stimulates endothelial cells to express and release a variety of molecules that increase platelet adhesion to the site of injury. For instance, Weibel-Palade bodies stored inside the endothelial cells contain vWF, P-selectin, angiopoietin-2, tissue plasminogen activator, and endothelin-1, which are all active participants of platelet adhesion (Yau et al., 2015). Their release following laser-induced vascular injury might be one of the reasons for the initial rapid kinetics of thrombus formation in both SR12813 and vehicle-treated mice. Also, it was observed in mice treated with SR12813 that the surface of the thrombi in contact with the endothelial layer was much smaller at all time points after the first 30 seconds. This can explain the quick initial elimination of the thrombi, which might be due to an unstable adhesion of platelets on collagen. Similar observations were made in thrombus formation assays *in vitro* in the presence of human PXR ligands (figure 4.3). The average size of the thrombus was evaluated by calculating the area under the median fluorescence intensity curve of each thrombi and was found to be reduced substantially by approximately 80% in mice treated with SR12813 in comparison to vehicle-control (figure 4.6 aiii). Furthermore, maximum or peak fluorescence intensity was also noted

to decrease by approximately 45% in SR12813 treated mice against mice treated with vehicle (figure 4.6 aiv). The extent of inhibition was of a comparable magnitude as that observed *in vitro* in the presence of PXR ligands. These observations are indicative of a reduction in the number of platelets accumulating to form thrombi after treatment with SR12813 and consequent inhibition in thrombus growth.

The effect of SR12813 on haemostasis was measured using a tail-bleeding assay on hPXR mice. SR12813 (0.8 µl per gram weight of the mice) or vehicle-control was injected into the femoral vein of mice based on its weight and blood volume. The volume of SR12813 (10 mM stock) or DMSO (10% v/v stock) injected was expected to give a concentration of 100 µM and 0.1% v/v respectively in the blood assuming 2 ml of blood is present in 25 gms of mouse. 20 minutes post injection of vehicle-control or SR12813, the tip of the tail was removed using a sharp razor blade and placed in sterile saline (37°C) and time to cessation of bleeding (secs) was measured. This assay was performed in collaboration with Dr L. Holbrook.

Vehicle-treated mice bled for approximately 275 seconds, whereas mean time to cessation of bleeding was prolonged to approximately 500 seconds in mice treated with SR12813 (figure 4.6b). A consistent and substantial effect of the PXR ligand was observed across all the SR12813 treated mice with each mouse exhibiting a longer bleeding time than the vehicle-treated mice. The shortest time to cessation of bleeding in SR12813 treated mice was 486 seconds, which was still 121 seconds higher than the vehicle-treated mouse that exhibited the longest time to cessation (365 seconds). Thus, it was concluded that human PXR ligand SR12813 impairs haemostasis *in vivo*.

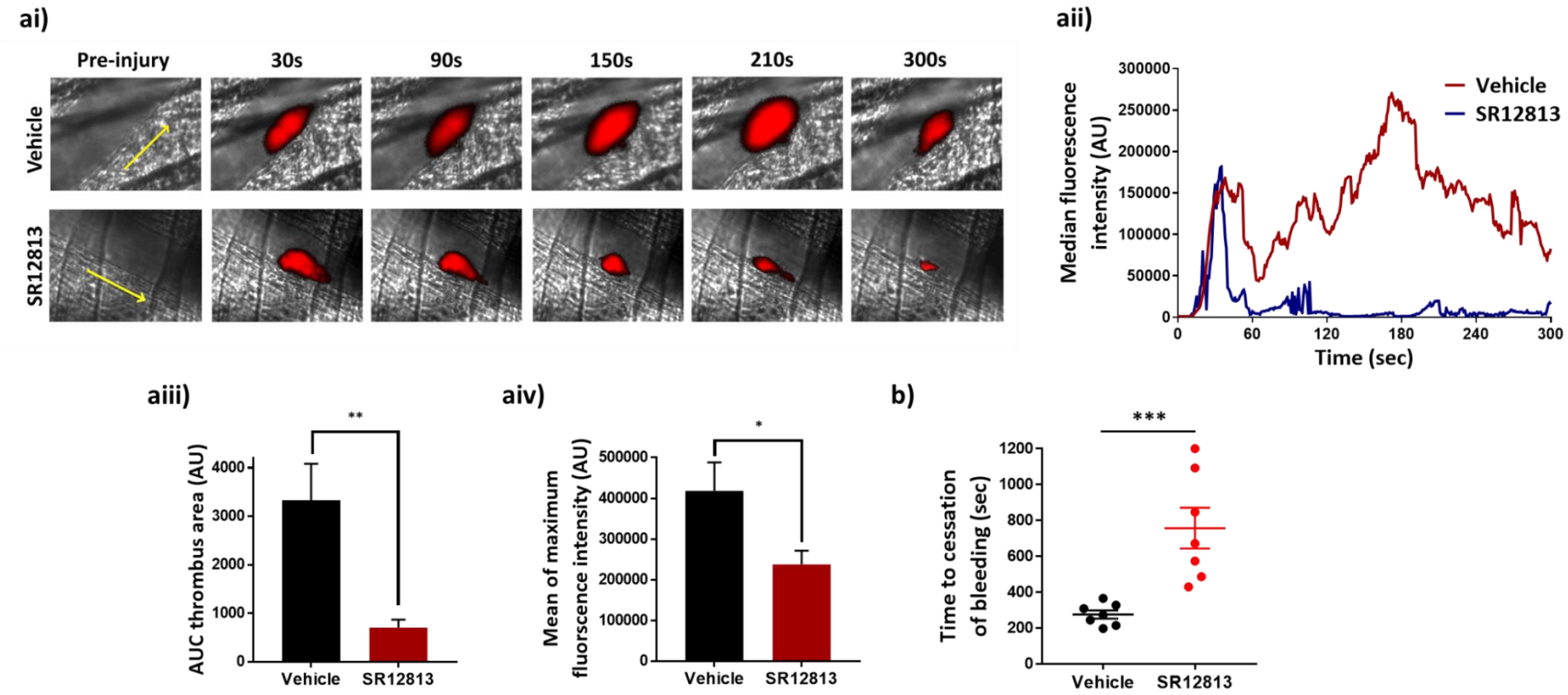


Figure 4.6: SR12813 inhibit thrombus formation and increase bleeding time in hPXR mice. *In vivo* thrombosis was assayed in hPXR mice through intravital microscopy using the laser-induced injury model. Vehicle-control (DMSO 10% v/v for 0.1% v/v final concentration) or SR12813 (10 mM for 100 µM final concentration) was administered intravenously to mice and incubated for 20 minutes. Platelets were fluorescently labelled with DyLight 649 conjugated anti-GPIbα antibody. Following laser-induced injury, thrombus formation was monitored by intravital microscopy. **(ai)** Representative images of thrombi obtained at different time intervals are shown. Arrow indicates the direction of blood flow. Data represent **(aii)** median fluorescence intensity measured for 8 to 10 thrombi from 3 mice each of control and treated groups, **(aiii)** thrombus-size was determined by calculating the area under the median fluorescence intensity curve of each thrombi **(aiv)** mean of maximum fluorescence intensity of the thrombus. **(b)** Tail bleeding was performed on hPXR mice pre-treated with vehicle DMSO (DMSO 10% v/v for 0.1% v/v final concentration) or SR12813 (10 mM for 100 µM final concentration) for 20 min (n=7) to determine the time to cessation of bleeding after tail-tip excision. Results are mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 was calculated by the nonparametric Mann–Whitney U test.

4.6. Human PXR ligands does not affect inhibitory signalling in platelets

The work presented so far demonstrate the potential ability of PXR ligands to down-regulate a range of platelet functions, thrombus formation (both *in vitro* and *in vivo*) and haemostasis. In addition to these findings, calcium mobilisation, which is directly regulated by all the activatory signalling pathways operative in platelets, was also reduced upon treatment with PXR ligands (Chapter-3, section 3.11). Therefore, we extended our investigation and examined whether these implications of PXR ligands on platelets are due to their ability to modulate different platelet activation signalling pathways. The examination of this hypothesis is both relevant and important since various signalling mechanisms in platelets have been reported to be altered by ligands that target different NRs, discussed in detail in chapter-1.

Physiologically, platelets maintain a quiescent state by the action of inhibitory molecules such as prostacyclin (PGI_2) and nitric oxide (NO), released from the endothelial lining of the blood vessels, which prevent their untimely activation. PGI_2 binds to IP receptor on platelets and stimulate adenylyl cyclase to synthesise cAMP, which further activates protein kinase A (PKA) (Yan et al., 2009). Similarly, NO induce guanyl cyclase to synthesise cGMP, which activates protein kinase G (PKG) (Du, 2007). Both PKA and PKG subsequently inhibit platelet activation through several mechanisms, most important being the suppression of IP3 receptors to prevent the release of calcium (Noe et al., 2010; Walter and Gambaryan, 2009).

Several NRs such as PPARα, PPARβ, PPARγ, FXR and RXR have been identified to exhibit their inhibitory effects through the upregulation of PKA and PKG activity, in cAMP or cGMP dependent or independent manners (Ali et al., 2009a; Unsworth et al., 2017d; Ali et al., 2009b; Moraes et al., 2016; Unsworth et al., 2017c). Consequently, studying the effects of PXR ligands on inhibitory mechanisms in platelets was considered a good starting point for investigating their influence on platelet signalling.

The effects of human PXR ligands - SR12813 and rifampicin on the extent of VASP (Vasodilator-stimulated phosphoprotein) S157 and S239 phosphorylation (PKA and PKG selective phosphorylation sites respectively) were evaluated in resting human platelets. The samples treated with positive controls, PGI_2 (1 μg/ml) and PAPANOATE (100 μM; a pure NO donor) exhibited increased phosphorylation levels of VASP S157 (figure 4.7ai, aii) and S239 (figure 4.7bi, bii) respectively. However, treatment with vehicle (containing, DMSO 0.1% v/v), SR12813 (figure 4.7ai, bi) or rifampicin (figure 4.7aii, bii) for 20 minutes was not associated with increased phosphorylation of VASP S157 and S239.

These observations suggest that inhibitory actions of PXR ligands on platelet activation are not mediated through the upregulation of PKA/PKG activity or inhibitory cyclic nucleotide signalling, and additional signalling mechanisms acting through the GPCRs or GPVI receptors require exploration to determine the mechanisms of action of PXR ligands in platelets.

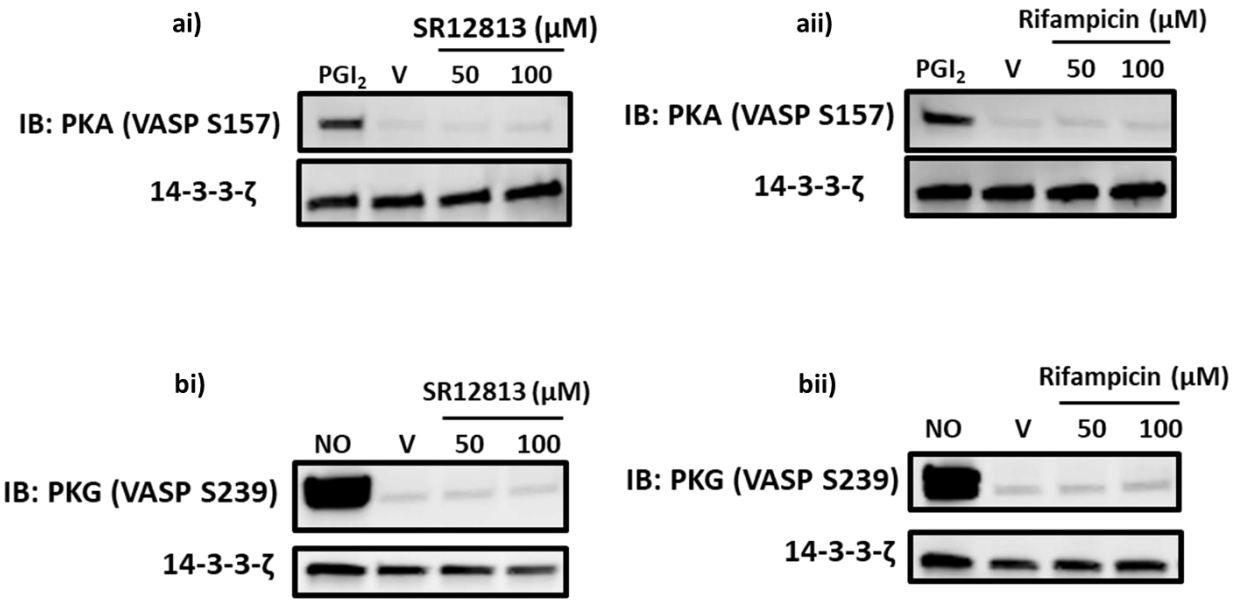


Figure 4.7: Human PXR ligands does not regulate PKA or PKG activity in resting platelets. Resting human washed platelets ($4x10^8$ cells/ml) were treated with vehicle (containing, DMSO 0.1% v/v) or SR12813 or rifampicin (50 and 100 μM) for 20 min, and samples were immunoblotted to detect VASP S157 and S239 phosphorylation, a marker of PKA and PKG activity respectively. PGI_2 (1 μg/ml) and PAPANOATE (100 μM), which upregulates the activity of PKA and PKG respectively through the activation of adenylyl cyclase and guanyl cyclase respectively, were included as positive controls. Blotting samples were lysed in Laemmli sample buffer before separation by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. 14-3-3-ζ was used as a loading control. Representative blots from 3 different experiments show the effects of **(ai, bi)** SR12183 or **(aii, bii)** rifampicin on the phosphorylation levels of VASP S157 and VASP S239.

4.7. PXR ligands does not modulate GPCR-mediated signalling events

Since PXR ligands were found not to modulate cyclic nucleotide-mediated signalling in platelets, their effects on platelets may be due to the regulation of GPCR or GPVI-mediated activation pathways in platelets. The effects of PXR ligands on platelet signalling evoked by thrombin or U46619 (a TxA_2 mimetic peptide) were therefore examined. Both thrombin and TxA_2 receptors in platelets are coupled to $G\alpha_q$, which upon agonist stimulation, initiates the phosphorylation of PLCβ leading to the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) to form diacylglycerol (DAG) and inositol trisphosphate (IP3). This facilitates degranulation, calcium mobilisation and affinity upregulation of αIIbβ3 (Stalker et al., 2012; Zhang et al., 2013; Joo, 2012).

Washed platelets ($4x10^8$ cells/ml) were prepared under non-aggregation conditions in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM) to block signalling stimulated via ADP receptors, TxA_2 production and aggregation respectively. The concentration of thrombin (0.1 U/ml) and U46619 (1 μM) was increased to enable detection of tyrosine phosphorylation by western blotting. Since the concentrations of agonists were increased, the incubation period of platelets with PXR ligands was extended to 20 minutes. The initial examination of the effects of PXR ligands on thrombin and U46619 mediated phosphorylation (90 seconds) of signalling components was performed by evaluating total tyrosine phosphorylation levels.

As shown in Figure 4.8, in comparison to the untreated sample, pre-treatment of platelets with SR12813 or rifampicin (0, 50 and 100 μM) for 20

minutes did not alter thrombin (0.1 U/ml) (figure 4.8 ai, aii) or U46619 (1 μM) (figure 4.8 bi, bii) instigated total tyrosine phosphorylation levels.

The secondary messengers IP3 and DAG synthesised in the $G\alpha_q$ mediated activation pathway facilitate a simultaneous mobilisation of calcium and activation of protein kinase C (PKC) respectively. Given the reduction of thrombin-evoked calcium mobilisation (Chapter-3, section 3.11) by PXR ligands, their effects on the extent of serine/threonine PKC substrate phosphorylation were evaluated. Incubation with SR12813 or rifampicin did not result in inhibition of thrombin (figure 4.9 ai, aii) or U46619 (figure 4.9 bi, bii) stimulated phosphorylation of PKC, in comparison to the untreated sample.

No effects of PXR ligands on GPCR induced signalling might be because of the reason that the effects of PXR ligands on platelet functions were observed only at low GPCR agonist concentrations. For instance, aggregation stimulated by thrombin or U46619 was found to be reduced only at a concentration range between 0.03-0.04 U/ml and 0.2 μM respectively (Chapter-3, section 3.5). Even a modest rise in the agonist concentration was found to overcome the effects of PXR ligands. Due to the challenges of detecting phosphorylation levels at such low agonist concentrations using western blotting, the effects of PXR ligands were studied only at high agonist concentrations, which may explain this observed lack of effect of PXR ligands on GPCR signalling.

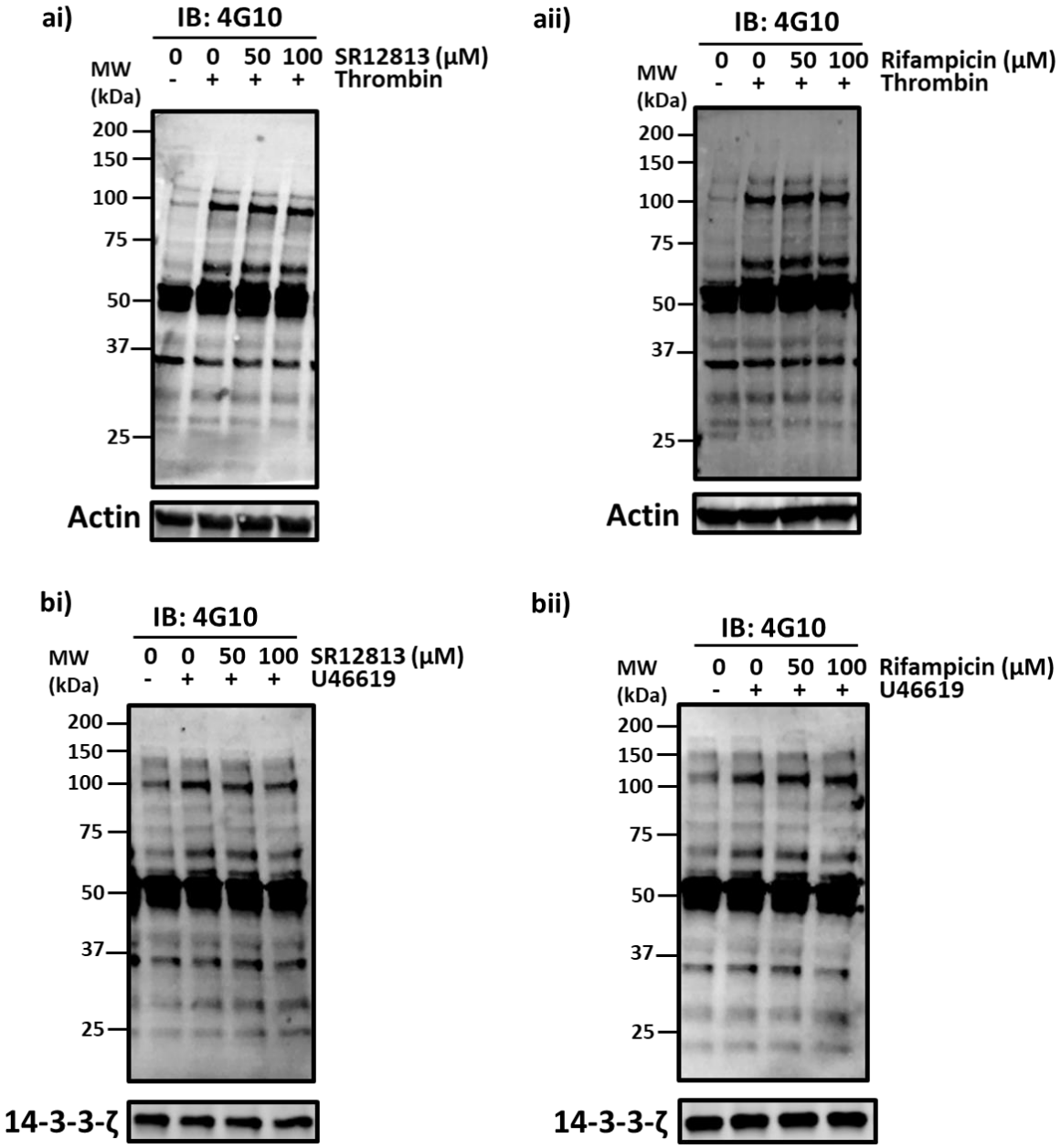


Figure 4.8: Human PXR ligands does not modify thrombin or U46619-mediated total tyrosine phosphorylation. Human washed platelets ($4x10^8$ cells/ml) under non-aggregation conditions [in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM)] were treated with SR12813 or rifampicin (0, 50 and 100 μM) for 20 min and samples were tested for total tyrosine phosphorylation after a 90 seconds stimulation with **(ai, aii)** thrombin (0.1 U/ml) or **(bi, bii)** U46619 (1 μM). Blotting samples were lysed in Laemmli sample buffer before separation by SDS–PAGE and transferred onto PVDF membranes. Actin or 14-3-3-ζ was used as a loading control. Representative blots from 3 different experiments are shown.

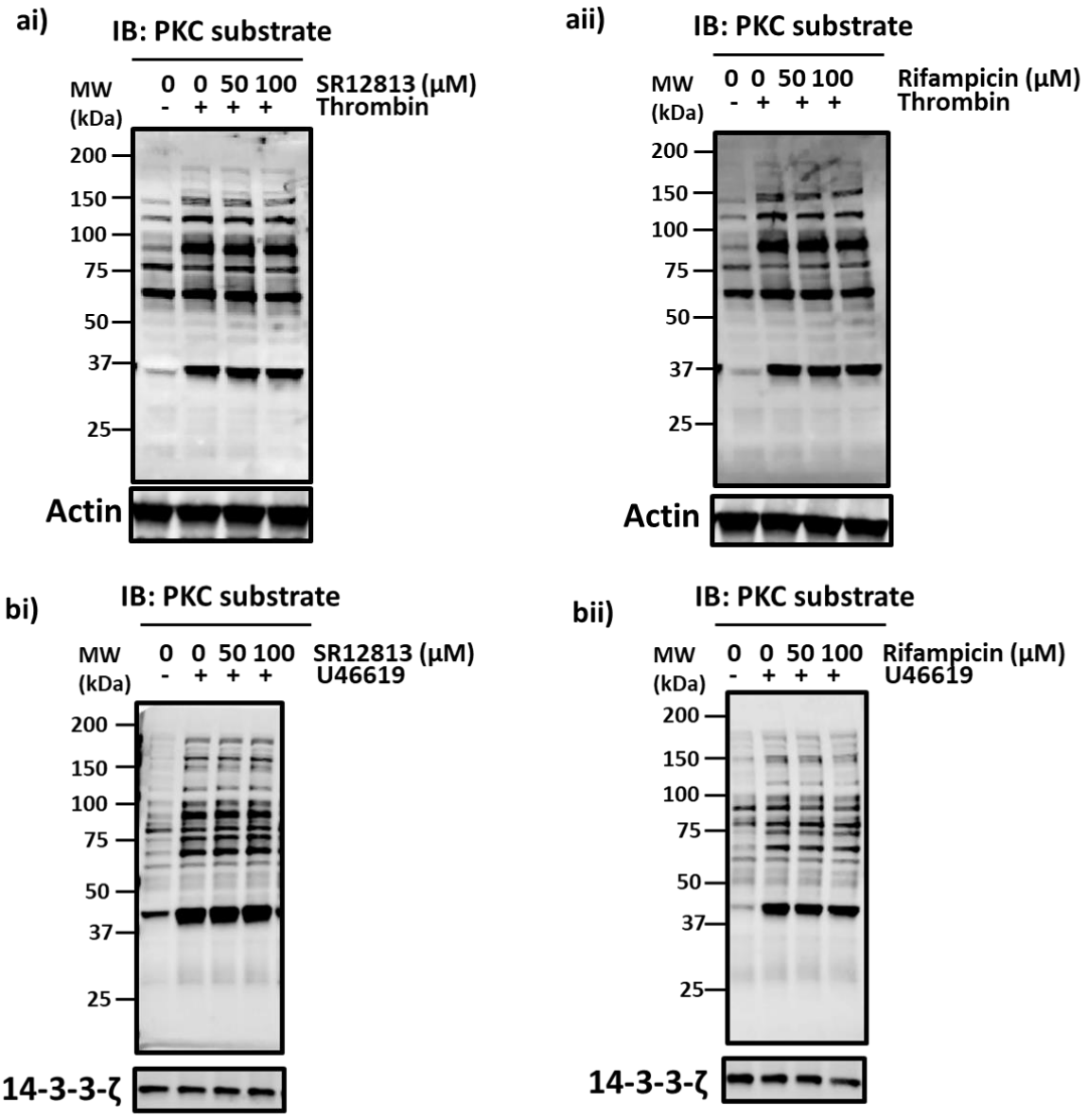


Figure 4.9: Human PXR ligands does not inhibit thrombin or U46619-mediated PKC substrate phosphorylation. Human washed platelets ($4x10^8$ cells/ml) under non-aggregation conditions [in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM)] were treated with SR12813 or rifampicin (0, 50 and 100 μM) for 20 min and samples were tested for PKC substrate phosphorylation after a 90 seconds stimulation with **(ai, aii)** thrombin (0.1 U/ml) or **(bi, bii)** U46619 (1 μM). Blotting samples were lysed in Laemmli sample buffer before separation by SDS–PAGE and transferred onto PVDF membranes. Actin or 14-3-3-ζ was used as a loading control. Representative blots from 3 different experiments are shown.

4.8. PXR ligands negatively regulate GPVI-mediated signalling in platelets

In chapter-3 it was observed that PXR ligands significantly down-regulated platelet aggregation stimulated by collagen. Unlike thrombin stimulation, the effects of PXR ligands at higher incubation period (20 minutes) were noted to be substantially robust. Besides this, PXR ligands were also associated with inhibition of CRP-XL stimulated degranulation and calcium mobilisation. These findings suggest that the inhibitory effects of PXR ligands are more likely to be an outcome of the regulation of signalling downstream of GPVI. Therefore, the effects of PXR ligands on the tyrosine phosphorylation of key signalling components involved in GPVI signalling pathway were studied.

The signalling proximal to GPVI receptor is mainly characterised by tyrosine phosphorylation cascades. Broadly, collagen-induced GPVI clustering induces auto-phosphorylation of Src family kinases (SFKs), which phosphorylate the ITAM-containing FcRγ-chain and subsequently, spleen tyrosine kinase (Syk) undergoes recruitment and auto-phosphorylation. The phosphorylation of linker for activation of T cells (LAT) by Syk results in the formation of LAT-signalosome, which is responsible for the recruitment of phosphoinositide 3-kinase (PI3K) and conversion of PIP2 into PIP3. This is followed by the phosphorylation and activation of phospholipase Cγ2 (PLCγ2), responsible for cleavage of PIP2, resulting in the generation of IP3 and DAG, which promotes calcium mobilisation and activation of PKC respectively. This leads to degranulation and affinity upregulation of integrin αIIbβ3, resulting in platelet aggregation (Stalker et al., 2012; Li et al., 2010; Bye et al., 2016).

To investigate the effects of PXR ligands on GPVI signalling, washed platelets ($4x10^8$ cell/ml) under non-aggregation conditions [indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM)] were treated with SR12813 or rifampicin (0, 50 and 100 μM) or vehicle control (containing, DMSO 0.1% v/v) for 20 minutes prior to their stimulation with CRP-XL (1 μg/ml) for 90 seconds. A high concentration of CRP-XL was selected to enable observation of tyrosine phosphorylation of GPVI signalling components under non-aggregation conditions by western blotting (Unsworth et al., 2017c) and therefore the incubation time of PXR ligands was subsequently increased.

The role of PXR ligands in regulating GPVI signalling was investigated by firstly examining their effects on the total tyrosine phosphorylation. Pre-treatment with SR12813 (0, 50 and 100 μM) caused a significant inhibition of the CRP-XL stimulated total tyrosine phosphorylation levels, in comparison to untreated (vehicle-control, containing DMSO 0.1% v/v) sample. 50 μM of SR12813 exhibited a reduction of 32%, while approximately 45% inhibition was attained using 100 μM (figure 4.10 ai, aii). Similarly, treatment with 50 μM of rifampicin attenuated total tyrosine phosphorylation by 25%, whereas, an inhibition of 30% was observed with 100 μM in comparison to vehicle-control (figure 4.10 bi, bii).

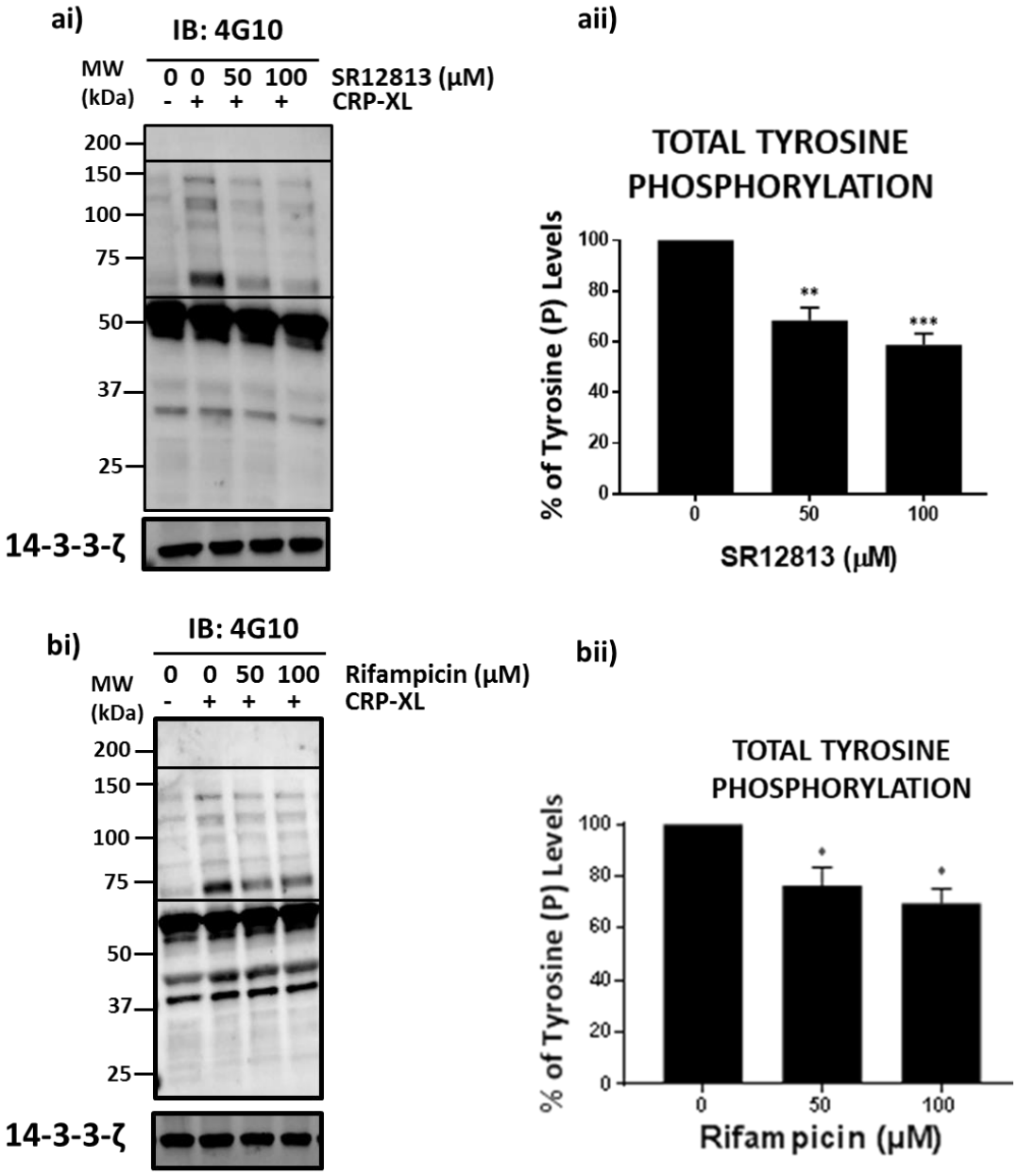


Figure 4.10: PXR ligands inhibit CRP-XL-stimulated total tyrosine phosphorylation. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. Total tyrosine phosphorylation levels were detected with 4G10 anti-phosphotyrosine antibody. Representative blots for **(ai)** SR12813 and **(bi)** rifampicin are shown. Levels of total tyrosine phosphorylation for **(aii)** SR12813 and **(bii)** rifampicin were quantified (for the bands present in the box) and expressed as a percentage of untreated (vehicle) controls. 14-3-3-ζ was used as a loading control. Results are mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

Since PXR ligands inhibited CRP-XL-mediated total tyrosine phosphorylation; their effects were further examined on the early GPVI signalling events that are primarily controlled by Syk, LAT and PLCγ2 tyrosine phosphorylation. Consistent with their inhibitory effects on the total tyrosine phosphorylation, SR12813 and rifampicin attenuated phosphorylation of Syk at its auto-phosphorylation site pY525/526 (Sada et al., 2001). Pre-treatment with 50 μM and 100 μM of SR12813 (20 minutes) reduced the CRP-XL stimulated tyrosine phosphorylation of Syk by 27% and 40% respectively in comparison to vehicle-control (figure 4.11 ai, aii). Whereas, rifampicin treatment displayed inhibition of 35% and 40% at 50 μM and 100 μM respectively in comparison to untreated sample (figure 4.11 bi, bii).

The phosphorylated form of Syk proceeds to phosphorylate the transmembrane protein LAT, which establishes a signalosome complex that coordinates downstream signalling (Gibbins et al., 1998). The tyrosine phosphorylation of LAT at pY200 (which is the equivalent of Y171 and phosphorylated by SFKs and Syk) (Jiang and Cheng, 2007; Paz et al., 2001) was down-regulated by SR12813, with an inhibition of 35% and 50% demonstrated at 50 μM and 100 μM respectively in comparison to untreated (vehicle-control) sample (figure 4.11 ci, cii). Likewise, 50 μM and 100 μM of rifampicin treatment also reduced CRP-XL-mediated LAT phosphorylation by approximately 40% and 45% respectively (figure 4.11 di, dii).

The LAT signalosome facilitates the recruitment and phosphorylation of PLCγ2. It signifies an important event in GPVI signalling considering its role in the generation of second messengers, IP3 and DAG, that directly regulate crucial platelet activation events such as calcium mobilisation and degranulation (Watson et al., 2005). PXR ligands reduced the extent of PLCγ2 phosphorylation at pY1217, which is a Bruton's

tyrosine kinase (Btk) phosphorylation site (Watanabe et al., 2001; Wu et al., 2017). SR12813 inhibited PLCγ2 phosphorylation by approximately 23% at 50 μM and by 32% at 100 μM in comparison with the untreated (vehicle-control) sample (figure 4.12 ai, aii). Whereas, rifampicin caused a reduction by 25% and 30% at 50 μM and 100 μM respectively (figure 4.12 bi, bii).

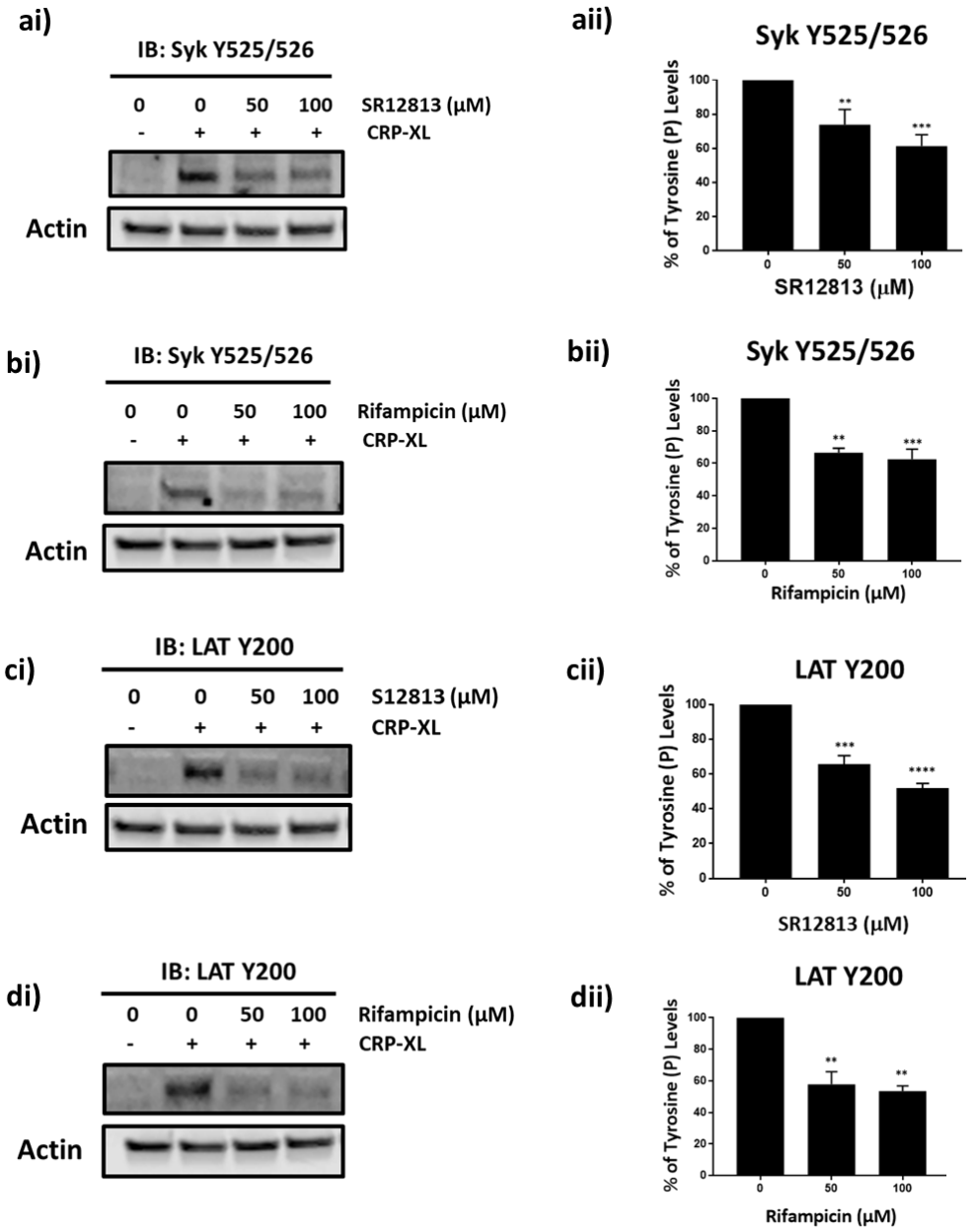


Figure 4.11: PXR ligands down-regulate CRP-XL-stimulated Syk and LAT tyrosine phosphorylation. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with site-specific phospho-antibodies for Syk (Y525/526) and LAT (Y200). Representative blots for the phosphorylation levels of **(ai, bi)** Syk and **(ci, di)** LAT after treatment with SR12813 and rifampicin are shown. The phosphorylation levels of **(aii, bii)** syk and **(cii, dii)** LAT after treatment with SR12813 and rifampicin were quantified and expressed as a percentage of untreated (vehicle) controls. Actin was used as a loading control. Results are mean ± SEM (n≥3), **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

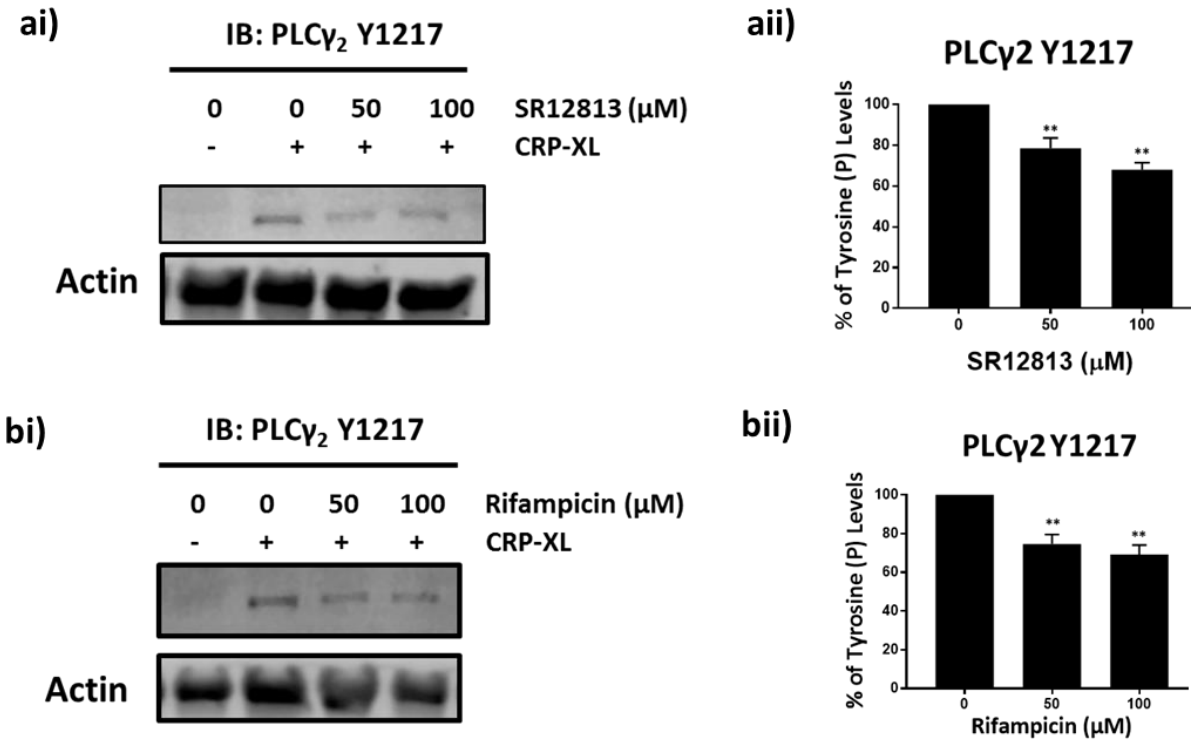


Figure 4.12: PXR ligands inhibit CRP-XL-mediated PLCγ2 tyrosine phosphorylation. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with site-specific phospho-antibody for PLCγ2 (Y1217). Representative blots for the phosphorylation levels of PLCγ2 after treatment with **(ai)** SR12813 and (**bi)** rifampicin are shown. The phosphorylation levels of PLCγ2 after treatment with **(aii)** SR12813 and **(bii)** rifampicin were quantified and expressed as a percentage of untreated (vehicle) controls. Actin was used as a loading control. Results are mean ± SEM (n≥3), **P ≤ 0.01 was calculated by one-way ANOVA.

Besides the negative regulation of PLCγ2, inhibition of CRP-XL-evoked calcium mobilisation (chapter-3, section 11) provides some evidence that the effects of PXR ligands might not be restricted to the modulation of just the early GPVI signalling and might follow further downstream. The IP3 stimulated calcium mobilisation, and DAG-mediated activation of serine/threonine PKC family (Yacoub et al., 2006) are events that follow the phosphorylation of PLCγ2. Therefore, we investigated the influence of PXR ligands on PKC activity using an antibody raised against the phosphorylated PKC substrate recognition sequence. SR12813 exhibited inhibition of PKC substrate phosphorylation by 40% and 50% at 50 μM and 100 μM, respectively, in comparison with the untreated (vehicle-control) sample (figure 4.13 ai, aii). A reduction of 50% and 60% was observed with 50 μM and 100 μM rifampicin, respectively (figure 4.13 bi, bii).

The functions of myosin light chain (MLC) are modulated in a calcium and PKC substrate-dependent manner. Its phosphorylation on serine 19 (S19) is a critical step enabling interaction of myosin with actin filaments that control shape change and secretion (Unsworth et al., 2017d; Bye et al., 2016). The phosphorylation level of MLC at S19 (catalysed by Ca^{+2}/calmodulin dependent MLC kinase) (Getz et al., 2010) was therefore investigated. An incubation with SR12813 was associated with inhibition of 40% and 60% at 50 μM and 100 μM respectively in comparison with the untreated (vehicle control) sample (figure 4.14 ai, aii). A similar degree of reduction was also exhibited following rifampicin treatment (figure 4.14 bi, bii). Altogether, these findings suggest a potential role of PXR ligands in regulating both early and late phases of signalling proximal to the GPVI receptor.

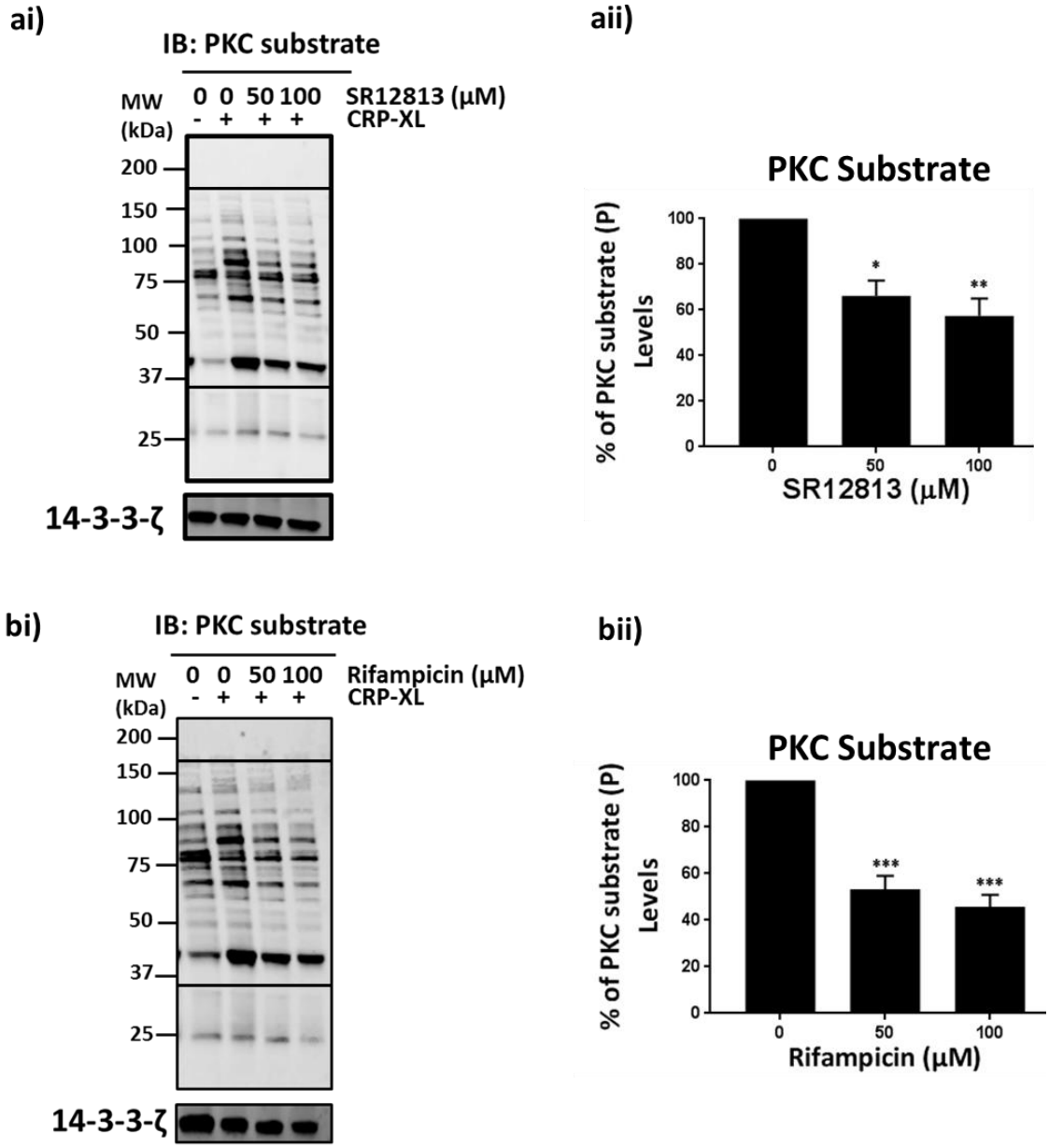


Figure 4.13: PXR ligands attenuate CRP-XL-stimulated PKC substrate phosphorylation. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with an antibody raised against the phosphorylated PKC substrate recognition sequence. Representative blots for **(ai)** SR12813 and **(bi)** rifampicin are shown. Levels of PKC substrate phosphorylation for **(aii)** SR12813 and **(bii)** rifampicin were quantified (for the bands present in the box) and expressed as a percentage of untreated (vehicle) controls. 14-3-3-ζ was used as a loading control. Results are mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

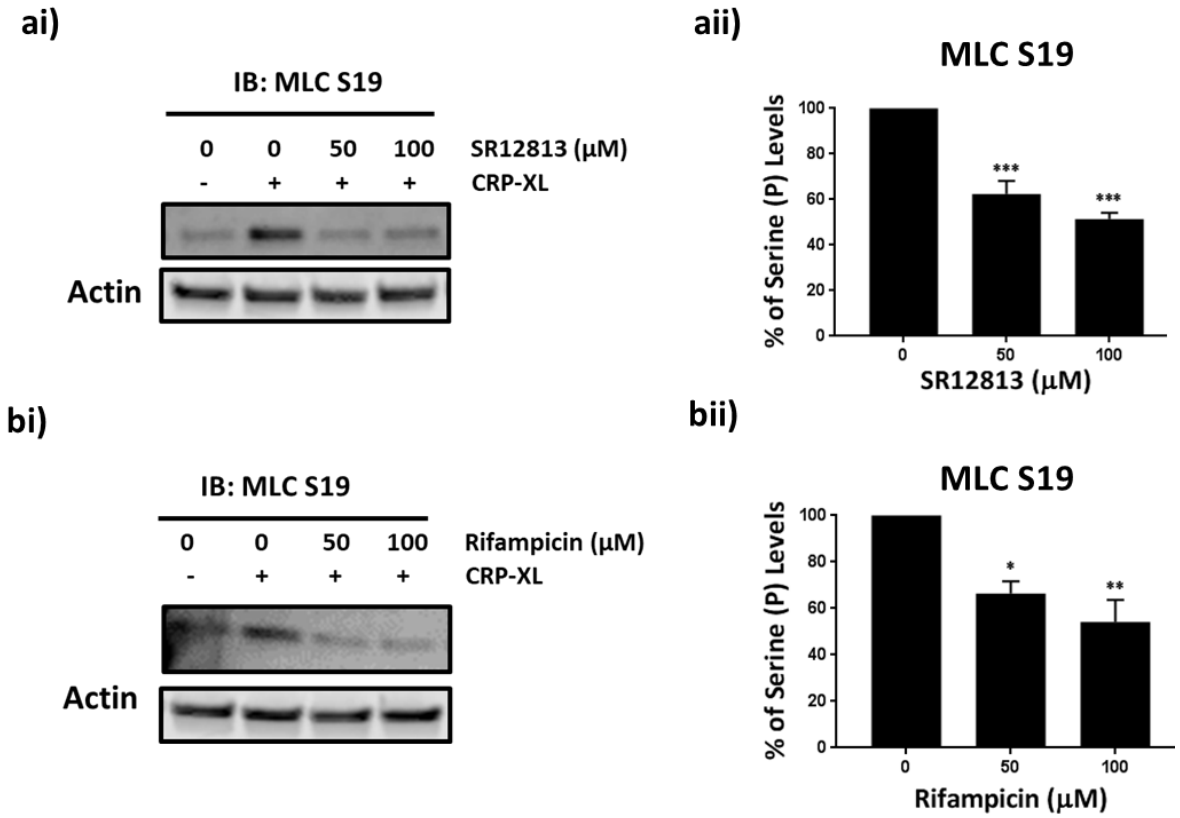


Figure 4.14: PXR ligands negatively-regulate MLC phosphorylation. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with site-specific phospho-antibody for MLC (S19). Representative blots for the phosphorylation levels of MLC after treatment with **(ai)** SR12813 and **(bi)** rifampicin are shown. The phosphorylation of MLC after treatment with **(aii)** SR12813 and **(bii)** rifampicin were quantified and expressed as a percentage of untreated (vehicle) controls. Actin was used as a loading control. Results are mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

4.9. Inhibition of Src family kinases as a general mechanism by which the PXR ligands function

Given the broad range of regulation instigated by PXR ligands on numerous signalling components functioning in the early and late stages of the GPVI pathway. It can be recognised that the modulations observed are largely an outcome of a cascade effect, where modulation in the phosphorylation of one component is likely to influence the downstream regulation of its interacting partner. Therefore, identification of specific target elements of PXR ligands that may contribute towards the initiation of these effects would be helpful in understanding the mechanisms governed by PXR ligands in platelets. Consequently, we studied the events participating in the onset of GPVI signalling, upstream of Syk tyrosine phosphorylation.

4.9.1. PXR ligands negatively regulate CRP-XL stimulated phosphorylation of SFKs

The collagen-mediated clustering of GPVI receptor induces trans-auto-phosphorylation of the Src family kinases (SFKs) such as Src, Lyn and Fyn, associated with a proline-rich juxtamembrane region of the GPVI receptor (Senis et al., 2014). This is followed by SFKs dependent phosphorylation of tandem tyrosine residues in the ITAM containing FcRγ-chain, which further recruits and phosphorylate tyrosine kinase Syk (Ellison et al., 2010). Given the inhibition observed in the tyrosine phosphorylation level of Syk and downstream components, the effects of PXR ligands on CRP-XL stimulated regulation of SFKs were examined.

Pre-treatment with SR12813 for 20 minutes caused a significant reduction in the CRP-XL-stimulated (90 seconds) tyrosine phosphorylation of Src at pY418, which is its auto-phosphorylation site (Bye et al., 2017). Incubation with 50 μM of SR12813 attenuated phosphorylation by 30%, whereas 100 μM exhibited inhibition of 45% (figure 4.15 ai, aii). Consistent with this, 50 μM and 100 μM of rifampicin were associated with inhibition of Src phosphorylation by 35% and 45% respectively (figure 4.15 bi, bii). To ascertain whether the effects of PXR ligands are restricted only to Src or they extend to other members of the SFKs, the effects of PXR ligands were evaluated on Lyn phosphorylation. Similar to Src, incubation with SR12813 inhibited phosphorylation level of Lyn at its auto-phosphorylation site pY396 (Futami et al., 2011) by approximately 30% at both the concentrations tested (figure 4.15 ci, cii). Similarly, rifampicin treatment down-regulated Lyn phosphorylation by 25% and 37% at 50 μM and 100 μM respectively (figure 4.15 di, dii). These findings provide evidence that the inhibitory effects of PXR ligands are mediated during the beginning of the GPVI signalling, which are communicated downstream of the SFKs causing an overall inhibition of the GPVI signalling pathway. To assess whether the regulation of SFKs marks a general mechanism through which PXR ligands arbitrate their inhibitory actions, signalling pathways initiated by other platelet receptors (such as CLEC-2 and integrin αIIbβ3) were evaluated. These receptors are reported to work independently of GPVI but are characterised by the activity of SFKs as a common feature.

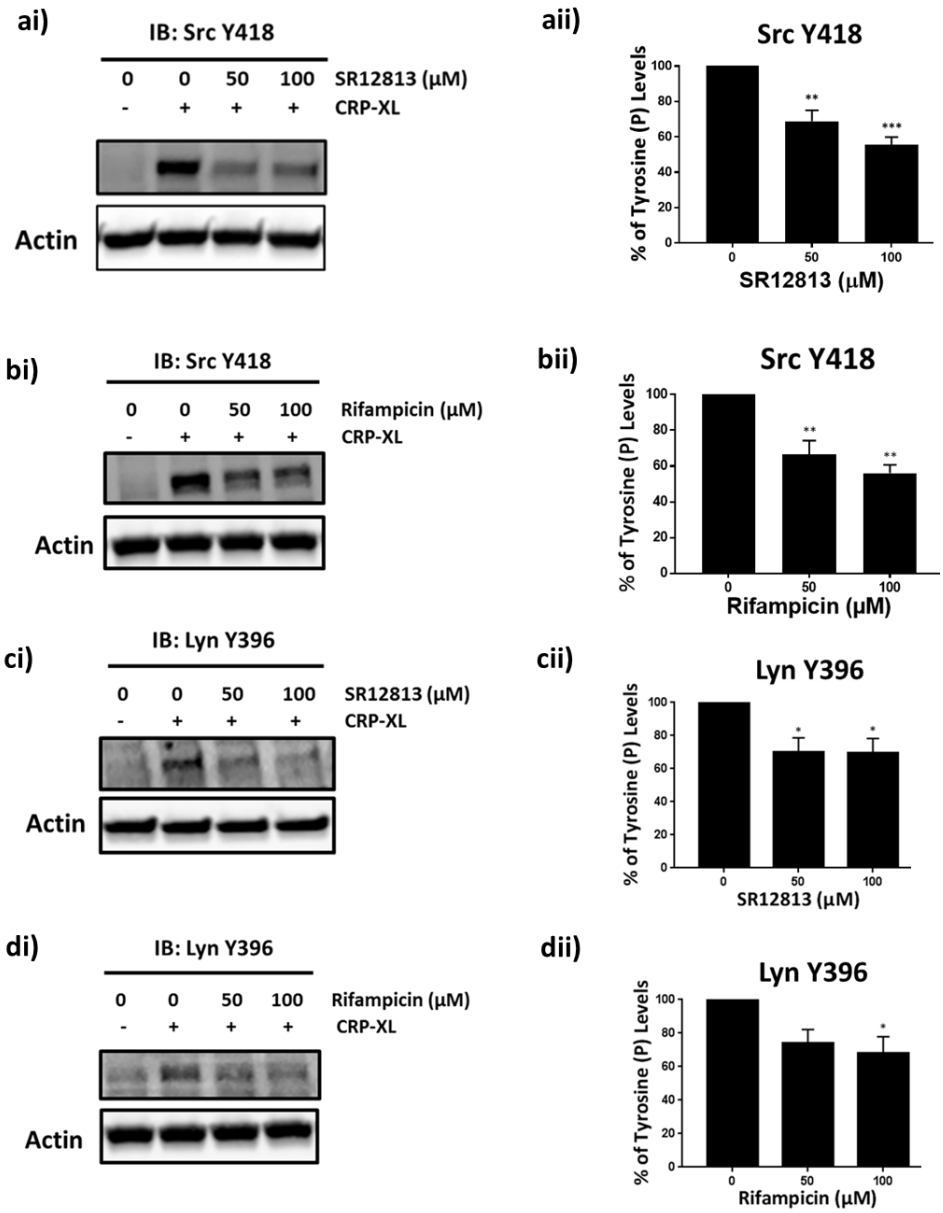


Figure 4.15: PXR ligands attenuate CRP-XL stimulated tyrosine phosphorylation of SFKs. Platelets ($4x10^8$ cells/ml) were pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 μM) for 20 minutes and stimulated with CRP-XL (1 μg/ml) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with site-specific phospho-antibodies for Src (Y418) and Lyn (Y396). Representative blots for the phosphorylation levels of **(ai, bi)** Src and **(ci, di)** Lyn after treatment with SR12813 and rifampicin are shown. The phosphorylation levels of **(aii, bii)** Src and **(cii, dii)** Lyn after treatment with SR12813 and rifampicin were quantified and expressed as a percentage of untreated (vehicle) controls. Actin was used as a loading control. Results are mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

4.9.2. PXR ligands inhibit SFKs phosphorylation downstream of the CLEC-2 receptor

C-type lectin-like type II (CLEC-2) is another platelet receptor that signals via Src- and PLCγ2-dependent tyrosine kinases pathway. Its endogenous agonist includes podoplanin, while isolated from the venom of *Calloselasma rhodostoma*, rhodocytin is also able to activate CLEC-2. The CLEC-2 receptor is characterised by a single YxxL motif in the cytosolic tail of its ITAM (HemITAM), in contrast to the tandem ITAM $(YxxL)_{6-12}(YxxL)$ sequence in FcRγ chain of the GPVI receptor (Gibbins et al., 1996; Watson et al., 2010). To explore whether PXR ligands regulate SFKs in CLEC-2 signalling, we firstly investigated their broad effects on rhodocytin-evoked platelet aggregation.

Platelets were pre-treated with PXR ligands for 20 minutes prior to stimulation with rhodocytin (100 nM). As a characteristic feature of the CLEC-2 receptor-mediated aggregation, a lag time of approximately 60 to 90 seconds was observed in the initiation of aggregation with rhodocytin (figure 4.16 ai, bi) (Suzuki-Inoue et al., 2001a; Shin and Morita, 1998). The extent of inhibition observed with SR12813 was observed to be stronger at the 2-minute interval from the initiation of aggregation in comparison to 5 minutes interval. SR12813 inhibited aggregation by 40% and 70% at 50 μM and 100 μM respectively at 2 minutes after the initiation of aggregation in comparison to vehicle-treated control (containing, DMSO 0.1% v/v) (figure 4.16 aii). The level of inhibition was reduced to 30% at 100 μM after 5 minutes (figure 4.16 aiii). Similar observations were made following rifampicin treatment. (figure 4.16 bii, biii).

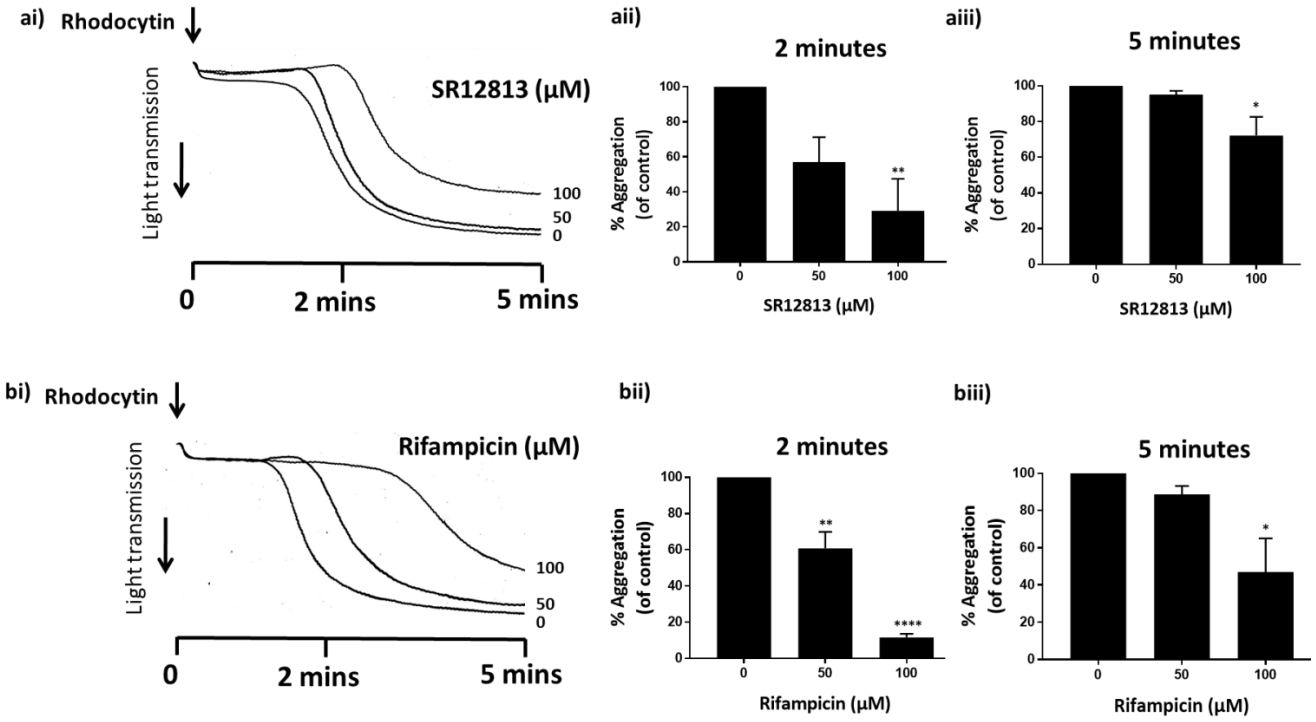


Figure 4.16: PXR ligands inhibit rhodocytin-mediated platelet aggregation. Washed human platelets (4×10^8 cells/mL) were incubated with SR12813, rifampicin (50 and 100 μM) or vehicle (containing, DMSO 0.1% v/v) for 20 minutes prior to stimulation with rhodocytin (100 nM) and aggregation was measured for 300 seconds. Representative aggregation traces of platelets treated with **(ai)** SR12813 or **(bi)** rifampicin are shown. Quantified data displays the percentage of aggregation attained by **(aii, aiii)** SR12813 or **(bii, biii)** rifampicin treated samples in 2 mins and 5 mins respectively (vehicle-treated samples represents 100% aggregation). Results are mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

Following these observations, we examined the role of PXR ligands in modulating the phosphorylation levels of SFKs downstream of the CLEC-2 receptor. Washed platelet ($4x10^8$ cells/ml) under non-aggregation conditions (as described previously) were treated with PXR ligands for 20 minutes prior to their stimulation with rhodocytin (100 nM). Stimulation time with rhodocytin was enhanced to 120 seconds to detect phosphorylation, considering the long lag phase associated with the initiation of platelet activation by rhodocytin. Treatment with SR12813 diminished the extent of rhodocytin-stimulated Src tyrosine phosphorylation at pY418 by 35% and 45% at 50 μM and 100 μM respectively in comparison to the untreated (vehicle-control) samples (figure 4.17 ai, aii). Likewise, incubation with rifampicin also attenuated the level of Src phosphorylation by approximately 42% and 35% at 50 μM and 100 μM respectively (figure 4.17 bi, bii).

In addition to the inhibition of SFKs by PXR ligands in CRP-XL-mediated platelet signalling, these additional findings suggest the role of PXR ligands in regulating SFKs in CLEC-2 signalling as well. This provides further evidence that potentially suggests that SFKs are the general target of PXR ligands through which they elicit their inhibitory effects.

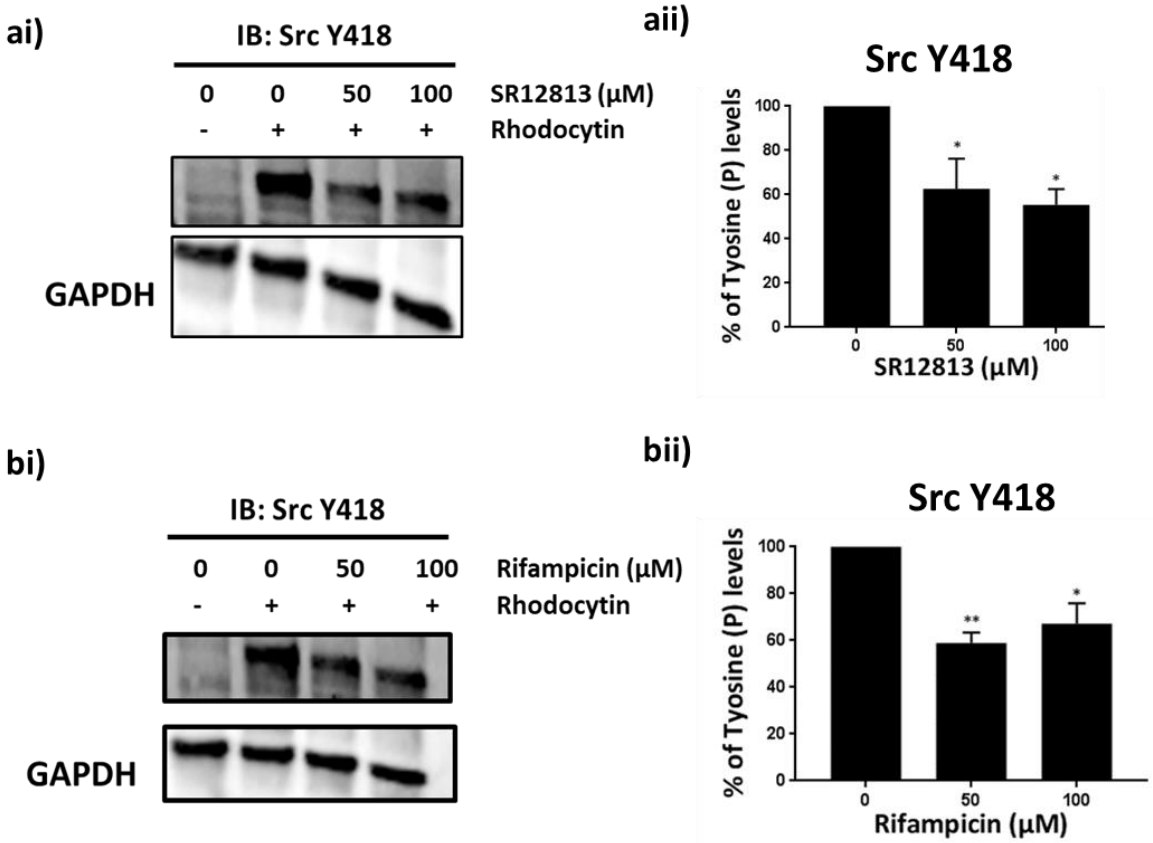


Figure 4.17: PXR ligands negatively-regulate rhodocytin-mediated SFKs phosphorylation. Platelets ($4x10^8$ cells/ml) pre-treated with vehicle (containing, DMSO 0.1% v/v), SR12813 or rifampicin (0, 50 and 100 µM) for 20 minutes were stimulated with rhodocytin (100 nM) for 120 seconds under non-aggregation conditions [indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM)]. Samples were lysed in Laemmli sample buffer, separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with Src (Y418) site-specific phospho-antibody. Representative blots show the phosphorylation of Src after treatment with **(ai)** SR12813 and **(bi)** rifampicin. The phosphorylation levels of Src after treatment with **(aii)** SR12813 and **(bii)** rifampicin were quantified and expressed as a percentage of untreated (vehicle) controls. GAPDH was used as a loading control Results are mean ± SEM (n≥3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by one-way ANOVA.

4.9.3. SR12813 attenuate phosphorylation of SFKs downstream of integrin αIIbβ3

SFKs play a vital role to initiate and propagate signals from integrin αIIbβ3 and exist in an association with the β3 domain. In resting platelets, SFKs are maintained in an inactive state by Csk, which form a complex with Src and β3. Following fibrinogen binding stimulated clustering of integrin αIIbβ3, Src undergoes auto-phosphorylation at pY418, which subsequently phosphorylates the β3 subunit. The phosphorylated form of β3 provides a docking site for adaptor proteins, cytoskeleton proteins (MLC and actinin), tyrosine kinases (Syk and FAK), lipid kinases (PI3K) and guanine nucleotide exchange factors. Each of these molecules ultimately participates in the initiation of outside-in signalling that facilitates spreading, secretion, stable adhesion and clot retraction (Li et al., 2010; Senis et al., 2014; Shattil et al., 1998).

Previously it was shown that the events emanating from outside-in signalling such as spreading (chapter-3, section 3.12) and clot retraction (chapter-3, section 3.14) were attenuated following treatment with PXR ligands. To investigate whether PXR ligands regulate integrin signal and evaluate additional evidence that can further confirm the effects of PXR ligands are mediated through their action on SFKs, the phosphorylation levels of Src were evaluated in platelets stimulated with fibrinogen, in the presence or absence of PXR ligands. Washed platelets ($4x10^8$ cells/ml), pre-treated with PXR ligands (50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v), were exposed to fibrinogen-coated wells (100 μg/ml) of a tissue culture plate and allowed to adhere at 37°C. Samples were lysed in Laemmli sample buffer after 30 minutes and adhered cells were scraped off using a glass rod. Treatment with SR12813 inhibited tyrosine

phosphorylation of Src at pY418 by approximately 30% and 40% at 50 μM and 100 μM, respectively, in comparison to vehicle-control (figure 4.18 ai, aii). Interestingly, no alteration in Src phosphorylation was observed in rifampicin-treated samples (figure 4.18 b). This might be attributed to the challenges of studying signalling stimulated by fibrinogen under static conditions. Stimulation time with fibrinogen plays a crucial role in detecting the phosphorylation levels. For instance, it was observed that at stimulation periods for up to 20 minutes, extremely low or no phosphorylation of Src was observed. Whereas, at increased stimulation periods of 45 or 60 minutes, no effects of PXR ligands were observed. Sufficient levels of phosphorylation were, however, observed only at 30 minutes of stimulation with fibrinogen to study the effects of PXR ligands. Therefore, this assay requires a fine balance with the stimulation time of fibrinogen, as shorter periods would not provide sufficient extent of phosphorylation to study, while longer stimulation periods might overcome the effects of PXR ligands.

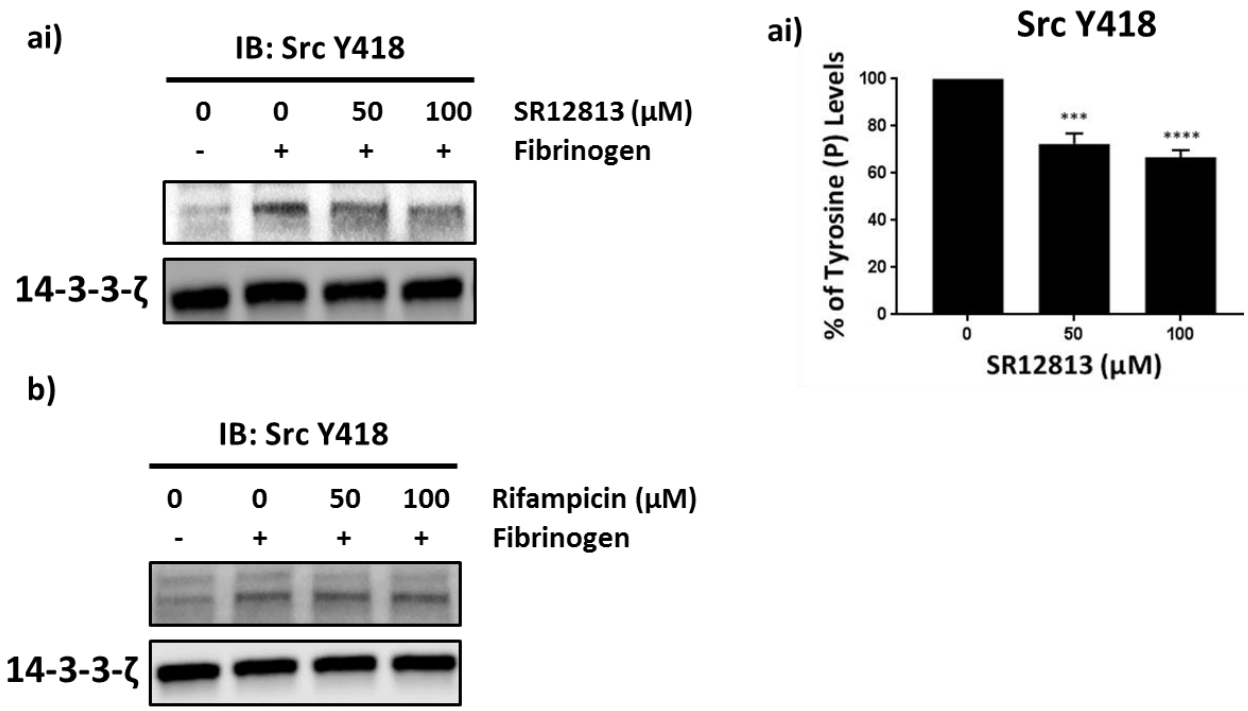


Figure 4.18: SR12813 negatively-regulate fibrinogen-stimulated tyrosine phosphorylation of Src. Washed platelets ($4x10^8$ cells/ml), pre-treated with PXR ligands (0, 50 and 100 μM) or vehicle-control (containing, DMSO 0.1% v/v) were exposed to fibrinogen-coated wells (100 μg/ml) of a tissue culture plate and allowed to adhere at 37°C. Samples were lysed in Laemmli sample buffer after 30 minutes and adhered cells were scraped off using a glass rod. The lysed samples in Laemmli sample buffer were separated by SDS PAGE and transferred to PVDF membranes. The phosphorylation levels were detected with Src (Y418) site-specific phospho-antibody. Representative blots for the phosphorylation levels of Src after treatment with **(ai)** SR12813 and **(b)** rifampicin are shown. The phosphorylation of Src after treatment with **(aii)** SR12813 was quantified and expressed as a percentage of untreated (vehicle) controls. 14-3-3-ζ was used as a loading control. Results are mean ± SEM (n=4), ***P ≤ 0.001 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

4.10. Chapter discussion

In the previous chapter, PXR ligands were proposed to exhibit anti-platelet effects in humans. Although the findings that were presented were mostly based on the effects of PXR ligands on washed platelets or platelet rich plasma, they provided an important basis for the evaluation of their effects on thrombosis and haemostasis in blood under *in vitro* and *in vivo* conditions. Besides this, PXR displays unusually low sequence conservation in the LBD across species, causing its ligands to exhibit species-specific activation of PXR in several cell types under genomic regulation. This feature was scrutinised with an aim to assess the existence of such species-specific effects of PXR ligands in human and mouse platelets. Additionally, the plausible influence of PXR ligands on the regulation of molecular mechanisms that govern platelet activation were explored in this chapter. The important outcomes of this chapter will now be discussed:

I. Structural basis of PXR promiscuity and its species-specific nature

PXR has evolved with numerous exclusive structural features that enable it to function as a sensor for the detection of structurally distinct compounds (Timsit and Negishi, 2007). Although such diverse interactions clearly define promiscuity, PXR also exhibits specificity. It has been reported that PXR activators differ from non-activators in only a few atoms, suggesting that PXR binds to a diverse but precise array of compounds, a feature that is implied as "directed promiscuity" (Ngan et al., 2009; Watkins et al., 2001). This promiscuous yet selective recognition of ligands by PXR can be best recognised by studying significant differences in pharmacological activation of PXR across

species. While human PXR is activated by rifampicin and SR12813, mouse PXR is not. Likewise, mouse PXR is activated by the synthetic steroid pregnenolone-16α-carbonitrile, whereas the human receptor is not.

Similar to the species-specific activation of PXR reported in other cell types, the effects of PXR ligands in human and mouse platelets were also observed in a species-specific manner. While human blood treated with SR12813 exhibited a significant inhibition in thrombus formation *in vitro*, no effect was observed on mouse platelets. Supporting this, mouse PXR ligands inhibited thrombus formation in mouse blood by 50%, whereas no influence was demonstrated in human blood. These findings are important as they not only identify the species-specific response of PXR ligands in human and mouse platelets but also indirectly demonstrate that the effects of PXR ligands in platelets are mediated through PXR.

The promiscuity and species-specific nature of PXR can be explained by studying the nature of amino acid residues that comprise the ligand binding site of PXR. Out of 28 amino acid residues that constitute the LBD of human PXR, 20 are hydrophobic in nature while rest are polar or charged (4 each) (Ngan et al., 2009). The structure of LBD reflects the characteristic features of most of the known PXR ligands that are generally hydrophobic and possess a small number of polar groups capable of forming a hydrogen bond (Watkins et al., 2002). This not only favours the binding of PXR to a wide range of compounds but also permit a ligand to dock inside the LBD in multiple orientations, a feature which is highly unique to PXR, in contrast to other NRs that display high ligand specificity. For instance, SR12813 has been reported to bind LBD of human PXR in three

distinct orientations with the interaction of Phe^{288} being the only common feature of all the three ligand conformations (Watkins et al., 2001). It was also observed that alteration in a few polar amino acid residues lining the LBD of PXR could markedly alter its response profile towards its ligands. For example, Watkins *et al.* (2001) reported that the mutation of four mouse PXR amino acid residues in the LBD to corresponding human PXR amino acids (Arg^{203}→Leu, Pro^{205}→Ser, Gln^{404}→His and Gln^{407}→Arg) substantially affected its activation by PCN, while making it more responsive to SR12813 (Watkins et al., 2001). This explains the reason underlying the species-specific pharmacological activation of PXR. Furthermore, the existence of a flexible loop in the LBD of human PXR is an additional factor that contributes towards the promiscuity of PXR. The loop constituted from amino acids 309-321 is linked to the binding cavity by a non-solvent accessible pore, which can open or close to offer structural flexibility for the binding of both large (such as rifampicin) and small PXR ligands (Ngan et al., 2009; Watkins et al., 2001).

II. Inhibition of thrombus formation and haemostasis by PXR ligands

The inhibition of numerous features associated with platelet activation, by PXR ligands, signifies their plausible role in modulating thrombus formation and haemostasis. As described previously, *in vitro* thrombus formation (on collagen) in the presence of human PXR ligands was not only identified to be reduced, it was also noted that this inhibition was partly due to (i) instability of the growing thrombus and (ii) defects in the adhesion of platelets to collagen. The PXR ligands, as discussed in chapter-3 (section 3.6 and 3.12) demonstrated their potential to

down-regulate binding of integrin αIIbβ3 receptors to fibrinogen. Therefore, it was not surprising to observe attenuated platelet-platelet interaction in samples treated with PXR ligands, resulting in constant breakage of the thrombus, which significantly reduced its growth, in comparison to vehicle-treated controls. Indeed, the potential lack of attachment of platelet GPVI or GPIb receptors (or both) to collagen, as witnessed in samples treated with integrillin and PXR ligands, could be one the primary reasons for the diminished growth during initial and late stages of thrombus formation. Since von Willebrand factor (vWF) play a crucial role in the attachment of platelet GPIb receptor with collagen, it would be interesting to study whether PXR ligands can also directly regulate these interactions and thus cause inhibition of thrombus formation. Since PXR ligands can modulate integrin signalling in platelets, the potential involvement of integrin α2β1 towards the observed reduction in platelet adhesion in the presence of PXR ligands is also worth exploring.

Given the species-specific inhibition of human and mouse platelet activation displayed by PXR ligands, we employed a hPXR mouse model (Taconic Biosciences) to investigate the effects of human PXR ligand – SR12813 on thrombosis and haemostasis. Treatment with SR12813 reduced substantially arterial thrombosis induced by laser-injury. The tail-bleeding time of hPXR mice, post-treatment with SR12813 was also identified to be significantly augmented, which is indicative of a dysregulated haemostatic response. These observations clearly demonstrate the role of PXR ligands as important regulators of platelet activation in response to vascular injury. The apparent role of PXR ligands in modulating the progression of atherosclerosis (de Haan et al., 2009; Li et al., 2007;

Zhou et al., 2009a; Masson et al., 2005) make these finding even more relevant. The rupture of atherosclerotic plaques in arterial circulation causes collagen to get exposed, leading to adhesion of platelets, their consequent activation and thrombus formation (Adiguzel et al., 2009). The PXR ligands can potentially avert the adhesion and activation of platelets on collagen exposed at the extracellular matrix of the ruptured atherosclerotic plaque and might play a therapeutic role to prevent thrombus formation that can possibly occlude the artery, triggering a heart attack or stroke.

III. Modulation of GPVI signalling by PXR ligands

Given the potent inhibitory effects of PXR ligands on a variety of platelet functions, thrombosis and haemostasis, their role to regulate underlying platelet signalling pathways were studied. While PXR ligands did not alter cyclic nucleotide or GPCR (thrombin or U46619) evoked platelet signalling, they demonstrated profound effects exclusively on the signalling downstream of the GPVI receptor. These observations were found to be interesting contemplating the fact that majority of the NRs with known mechanisms of action in platelets (such as FXR, RXR, PPARα, PPARβ and PPARγ) have been found to regulate one or more signalling pathways with modulation of inhibitory platelet signalling being the most common feature (Ali et al., 2009a; Unsworth et al., 2017d; Ali et al., 2009b; Moraes et al., 2016; Unsworth et al., 2017c).

PXR ligands were found to inhibit CRP-XL stimulated total-tyrosine phosphorylation and calcium mobilisation from intracellular stores. Since both of these processes are vital for ensuring a sustained activation of platelets, their

inhibition by PXR ligands provided valuable insights that enabled the study of their effects on specific molecules functioning in the GPVI signalling pathway.

Treatment with PXR ligands inhibited tyrosine phosphorylation of SFKs (Src and Lyn), suggesting their influence on the earliest stages of the GPVI pathway. These findings are novel in a sense that none of the NRs that have been reported can regulate signalling at such early stages of the GPVI signalling cascade. NRs such as LXR and PPARγ have been proposed to regulate collagen-mediated signalling in platelets by modulating phosphorylation of Syk and LAT, which function of downstream of SFKs (Moraes et al., 2010b; Spyridon et al., 2011). Indeed, exposure to PXR ligands was observed to attenuate the tyrosine phosphorylation of both molecules substantially. However, this can be well anticipated considering the kinase activity of Src family that regulates phosphorylation of Syk, which in turn controls the phosphorylation profile of LAT. The tyrosine phosphorylation of LAT is followed by phosphorylation of tyrosine residues in PLCγ2, which was also found to be reduced following treatment with PXR ligands.

The SFKs play a fundamental role to regulate platelet activation because of their participation in activatory platelet signalling generated from a range of platelet surface receptors that include GPVI, vWF/GPIb-IX-V receptor complex, integrin αIIbβ3, integrin α2β1, FcRγIIA and CLEC-2 receptor (Senis et al., 2014). Based on this, we further evaluated whether the regulation of SFKs is a general mechanism by which PXR ligands elicit their functions in platelets. Binding of fibrinogen to integrin αIIbβ3 stimulates outside-in signalling, which aims to stabilise the thrombus and is characterised by tyrosine phosphorylation of SFKs (also Syk and PLCγ2) (Durrant et al., 2017). Treatment with SR12813 caused negative-regulation in the

phosphorylation of Src at Y418, indicating its influence on the activity of SFKs. Besides this, the participation of SFKs also induces inside-out signalling downstream of the vWF/GPIb-IX-V complex, causing activation of integrin αIIbβ3. This further ensures firm adhesion of platelets to the sites of vascular injury and development of a stable thrombus (Ozaki et al., 2005; Senis et al., 2014). In support of this, the stability of the growing thrombus, both *in vitro* and *in vivo*, was found to be considerably reduced, which might be an outcome of reduced activity of SFKs downstream of GPVI, GPIb-IX-V and integrin αIIbβ3. Furthermore, inhibition in the phosphorylation of Src was also observed downstream of the podoplanin or rhodocytin receptor CLEC-2, which provides additional evidence that PXR ligands broadly affect the activity of SFKs in multiple signalling pathways and thus elicit their effects. Besides an alteration in the early events of the GPVI stimulated signalling, PXR ligands were also able to markedly reduce phosphorylation levels of PKC and MLC, demonstrating their ability to alter both early and late events associated with GPVI receptor signalling.

Throughout the evaluation of the GPVI-mediated signalling, it was noticed that the effects of both 50 and 100 μM of PXR ligands were quite similar. There can be two reasons for this: (1) Use of non-aggregation conditions block ADP and TxA_2 stimulated effects, which might substantially increase the efficacy of the PXR ligands at a lower concentration as well. (2) An incubation time of 20 minutes with PXR ligands (50 and 100 μM) was used for studying GPVI signalling, which has been previously identified to demonstrate stronger inhibitory responses in reducing collagen-mediated aggregation (chapter-3, section 3.5) potentially due to their differential ability to cross the plasma membrane.

Previously, the inhibition of GPVI signalling by LXR was proposed to be mediated through a direct interaction between LXR-Syk and LXR-PLCγ2. Also, PPARγ-Syk and PPARγ-LAT interactions were also reported as a potential mechanism of action by which PPARγ down-regulate GPVI signalling. Based on these findings, the plausible interactions between PXR and GPVI signalling molecules such as Src, Syk, LAT and PLCγ2 were evaluated. However, no such interactions were observed. Therefore, how PXR facilitates inhibition of numerous components of the GPVI signalling pathway is still unclear.

Future work should involve the identification of potential interacting partners of PXR in the GPVI signalling pathway in the presence and absence of PXR ligands using mass spectrometry. This would enable us to elucidate better and understand the underlying mechanism by which PXR regulates GPVI signalling.

Chapter-5

RXR ligands negatively regulate thrombosis and haemostasis

5.1. Introduction

The retinoid X receptors belong to the NR superfamily and are widely distributed in skin, lungs, brain, kidney, liver, eyes, pituitary and adrenal gland (Mangelsdorf et al., 1990; Mangelsdorf et al., 1992; Chambon, 1996). Three isoforms of RXR (α, β and γ) are expressed by NR2B1, NR2B2 and NR2B3 genes, respectively, that are activated by retinoids and vitamin A derivatives (Dawson and Xia, 2012). RXRs control and regulate the functions of a quarter of the known human NRs (including PXR) by interacting and forming a heterodimer, which suggests their importance in human physiology (Evans and Mangelsdorf, 2014). RXR can also form homodimers to facilitate their own action (Sato et al., 2010). Thus, RXR either alone or in the form of heterodimers can regulate transcription of specific genes that control a diverse range of biological processes such as cellular proliferation, differentiation, lipid metabolism, bone development, haematopoiesis and embryogenesis (Mangelsdorf and Evans, 1995; Nagy et al., 1998). Like other NRs, RXR consists of up to 5 domains: (i) the N-terminal domain represents the transcriptional activation domain and varies in sequence and length; (ii) the DNA-binding domain (DBD) containing a zinc finger promote interaction of DNA with hormone response element; (iii) the hinge region links DBD to (iv) the ligand-binding domain, which facilitates attachement of the ligand; (v) the C terminal domain is another highly variable region that has not yet been functionally characterised in RXRs (Lv et al., 2013).

Endogenous ligands of RXR include 9-*cis*-retinoic acid (9-*cis*-RA) and docosahexaenoic acid while several synthetic ligands such as methoprene acid, Bexarotene and LG100268 exist (Dawson and Xia, 2012). RXR ligands have been

reported to exert cardioprotective effects by reducing atherosclerosis in apolipoprotein E knockout mice (Claudel et al., 2001). Additionally, anti-diabetic effects of RXR ligands were also proposed in type-2 diabetes mellitus mouse models (Leibowitz et al., 2006).

Human platelets (and megakaryocytes) were reported to express RXRα and RXRβ and treatment with 9-*cis*-RA or methoprene acid inhibited U46619 or ADP-mediated platelet aggregation (Moraes et al., 2007). This negative regulation was observed to be an outcome of an interaction between RXR and Gq in the presence of 9-*cis*-RA, which resulted in reduced activation of Rac protein and release of calcium from intracellular stores (Moraes et al., 2007). The effects of RXR ligands towards low concentrations of GPVI receptor agonist (CRP-XL and collagen) and thrombin were, however, unclear. Our lab has previously identified the ability of NRs such as LXR and PPARγ (and through this study PXR) to modulate collagen mediated signalling in platelets (Moraes et al., 2010b; Spyridon et al., 2011). Given the recent findings which suggests RXR as an active dimer partner of PXR, LXR and PPARγ in platelets (Unsworth et al., 2017c), the effects of RXR ligands on collagen mediated platelet activation were revisited.

In the present study, we aimed to evaluate the effects of RXR ligands on platelet activation stimulated by GPVI receptor agonists (collagen and CRP-XL) and thrombin. Their implications on haemostasis and thrombus formation both *in vitro* and *in vivo* were also explored.

5.2. Expression and localisation of RXR in platelets

An immunoblot analysis using mouse monoclonal anti-RXR antibody (SantaCruz; sc-46659) raised against amino acid residues 198-462 of human origin and capable of identifying all the three isoforms of RXR confirmed the expression of RXR in megakaryocyte cell line Meg-01, human and mouse platelets (figure 5.1a). These findings are in alignment with the previous study that reported the presence of RXRα and RXRβ (but not RXRγ) in Meg01, human and mouse platelets (Moraes et al., 2007).

In chapter-3 (section 3.4), the interaction between RXR and PXR was identified using coimmunoprecipitation (Co-IP) assay and immunofluorescence microscopy. However, RXR in addition to PXR also interacts with numerous other NRs that are also expressed in platelets. Therefore, we extended our investigation to evaluate the potential existence of additional RXR heterodimers in platelets. Co-IP assays were performed, where RXR was isolated from resting human platelets using an anti-RXR mouse monoclonal antibody (SantaCruz; sc46659). Following this, western blot analysis was performed using an anti-LXR (Abcam; ab28479) or anti-PPARα (SantaCruz; sc-9000) rabbit polyclonal antibody or anti-PPARγ (SantaCruz; sc-1984) goat polyclonal antibody. An equivalent amount of anti-RXR antibody was used a negative control. The primary antibodies were recognised using a secondary antibody (Abcam; ab131366) that prevent identification of the heavy chain of denatured IgGs. LXR, PPARα and PPARγ were found to co-immunoprecipitate with RXR, which indicates the ability of RXR to interact with different NRs that exist in platelets (Figure 5.1 b).

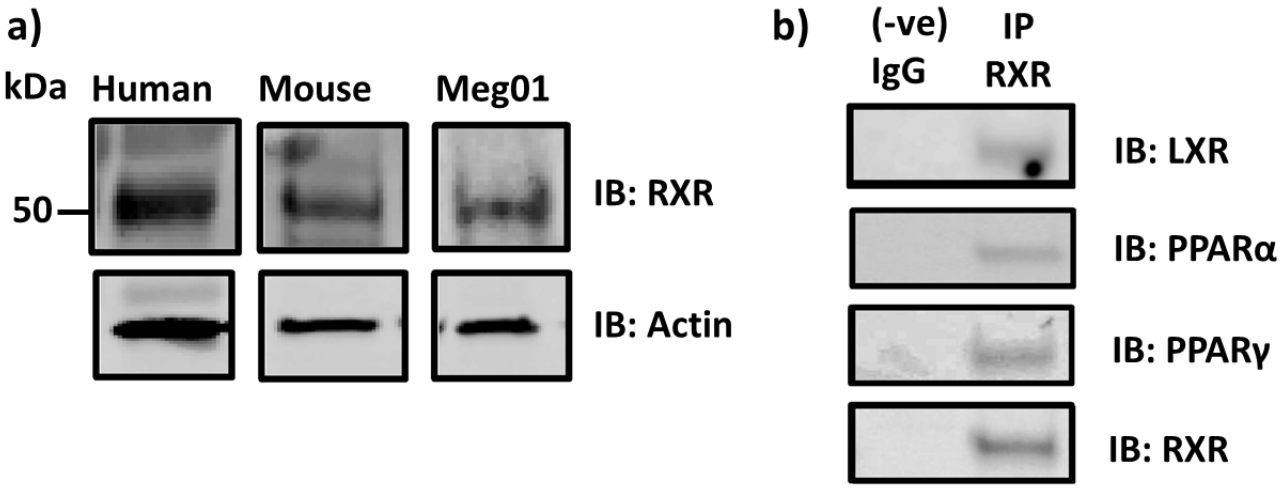


Figure 5.1: RXR is expressed in human and mouse platelets and forms heterodimer with different NRs in human platelets. (a) The expression of RXR was evaluated by western blot analysis of human and mouse whole platelet lysates and Meg01 cells using a mouse monoclonal anti-RXRα/β/γ antibody (targeting amino acids 198-462). Actin was used as a loading control. **(b)** Presence of RXR-LXR, RXR-PPARα and RXR-PPARγ heterodimers was investigated in human platelets using a Co-IP assay. Human washed platelets ($8x10^8$ cells/ml) were lysed in NP40 buffer before immunoprecipitation of RXR using a mouse monoclonal anti-RXR antibody overnight at 4°C in the presence of protein A/G magnetic beads. Isolated proteins were subjected to SDS–PAGE and western blotted onto a PVDF membrane. Immunoblot analysis was followed by the addition of rabbit polyclonal anti-LXR or anti-PPARα antibody or anti-PPARγ goat polyclonal antibody. Detection of primary antibody was done using a secondary antibody that does not recognise denatured IgG. Presence of RXR was also confirmed in the same samples. An equivalent amount of anti-RXR antibody was used as a negative control to exclude IgG contamination (-ve). Data are representatives of 3 separate experiments using platelets from different donors.

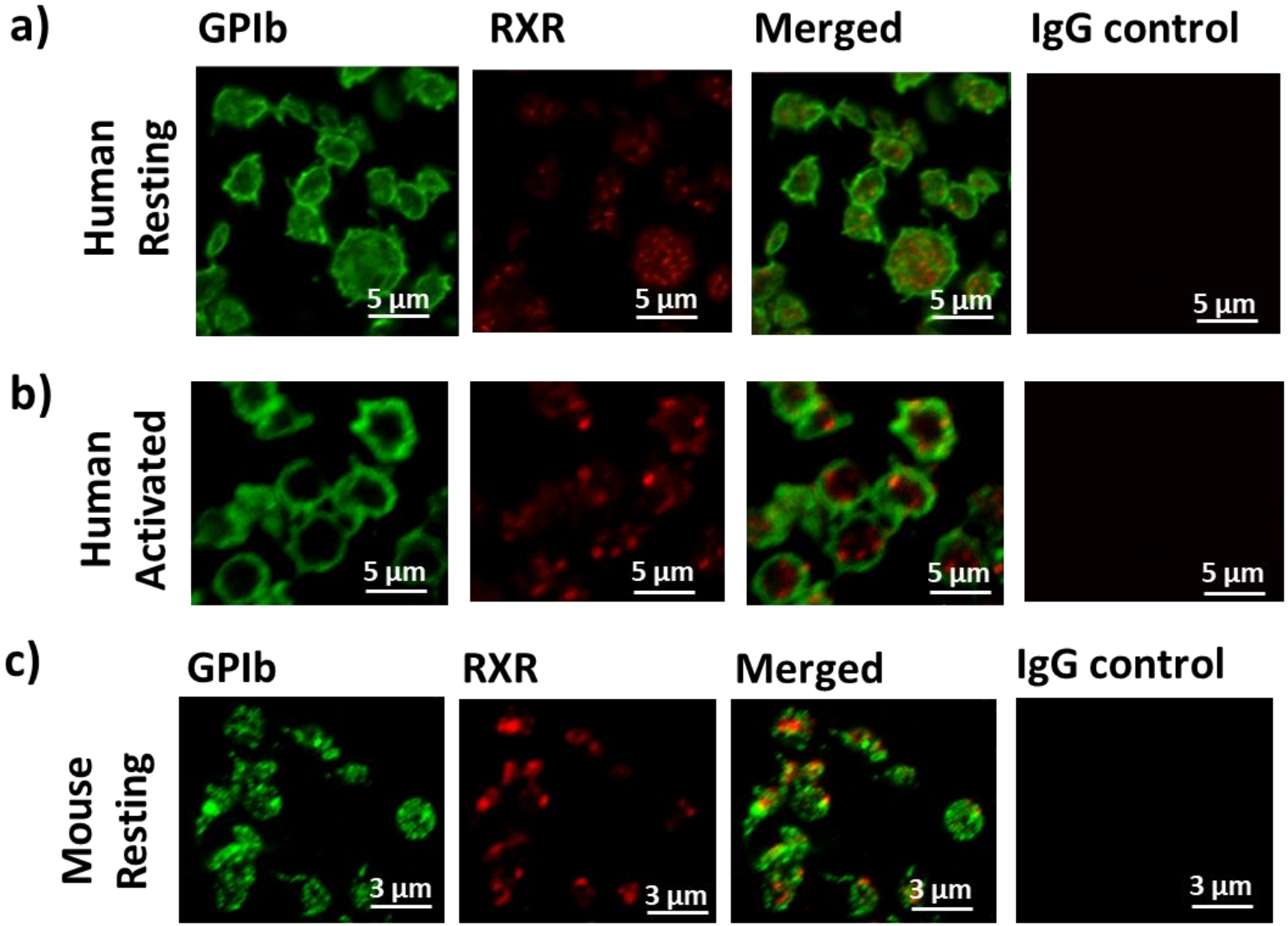
Since the distribution of FXR (Moraes et al., 2016) and PXR (chapter-3, section 3.3) was found altered in activated platelets in comparison to resting platelets, the subcellular localisation of RXR (which is their interacting partner) in resting and activated platelets was also explored using immunofluorescence microscopy. Unstimulated and stimulated (with 5 μM U46619 in the presence of integrilin) platelets (in PRP) were fixed with 4% paraformaldehyde and permeabilised using 0.1% Triton X-100. U46619 was used as an agonist because it stimulates a gentle activation of platelets with minimal shape change, which is helpful in studying the distribution of NRs. Samples were then incubated with a mouse monoclonal anti-RXR antibody (SantaCruz; sc-46659) to identify the distribution of RXR, while; platelets were stained using a goat polyclonal anti-GPIb antibody (SantaCruz; sc-6602), which marks the surface of platelets. The secondary antibodies conjugated with Alexa Fluor 647 and Alexa Fluor 488 (Life Technologies) were used for visualisation of RXR and GPIb respectively. Human platelets without any primary antibody treatment were used as a negative control. The samples were visualised using a Nikon A1-R confocal microscope (100X oil immersion lens).

The distribution of RXR (stained in red) in resting platelets appeared to be uniform in a punctate arrangement inside the cytosol of platelets (platelet surface is marked in green and represent surface GPIb receptors) (figure 5.2 a). Stimulation with U46619 appeared to redistribute RXR closer towards the plasma membrane (figure 5.2 b). Additionally, activated platelets exhibited fewer but larger spots of RXR in comparison to the uniformly distributed small spots of RXR in resting platelet cytosol. Although unclear, it gives an impression of RXR

becoming clustered close to the surface of platelets upon activation. The findings of RXR redistribution in activated platelets are quite similar to the observations made with PXR in chapter-3 (section 3.3) and reported in case of FXR (Moraes et al., 2016). Together, based on this evidence, translocation of NRs towards the plasma membrane can be proposed as a general feature shared by multiple NRs in platelets. Although, as mentioned earlier, super-resolution microscopy would be required to dissect this observation further and study the subcellular localisation of NRs more accurately.

A similar kind of punctate arrangement of RXR was also observed inside the cytosol of resting and permeabilised mouse platelets incubated with mouse monoclonal anti-RXR (SantaCruz; sc-46659) and goat polyclonal anti-GPIb (Santa Cruz; sc-6602) antibodies (Figure 5.2c).

Figure 5.2: The subcellular localisation of RXR in platelets. The distribution of RXR in human resting, activated (with 5 μM U46619 in the presence of integrilin) and resting mouse platelets was investigated using immunofluorescence microscopy. Platelets were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton-X-100. RXR (in red) and membrane GPIb receptors (in green) were stained using anti-RXR and anti-GPIb antibodies. Secondary antibodies conjugated to Alexa-647 and Alexa-488 were used to visualise RXR and GPIb, respectively. Platelets without primary antibody treatment were used as negative controls. Figures represent the distribution of RXR in **(a)** resting and **(b)** activated human platelets. **(c)** The localisation of RXR in resting mouse platelets. Data are representative of >3 separate experiments.


a)
GPIb
RXR
Merged
IgG control
Human Resting
5 µm
5 µm
5 µm
5 µm
b)
Human Activated
5 µm
5 µm
5 µm
5 µm
c)
GPIb
RXR
Merged
IgG control
Mouse Resting
3 µm
3 µm
3 µm
3 µm

5.3. RXR ligands inhibit platelet aggregation stimulated by a range of agonists

It has been previously reported that treatment of platelets with endogenous (9-*cis*-RA) or exogenous (methoprene acid) RXR ligands inhibit platelet aggregation. However, these inhibitory effects were predominantly noted for aggregation stimulated by GPCR agonists, ADP or U46619 (Moraes et al., 2007). The effects of RXR ligands on collagen stimulation were unclear, whereas, their influence on thrombin stimulation was not evaluated. Findings from our lab have demonstrated that NRs such as PPARγ, LXR, FXR and PXR, apart from down-regulating the effects of ADP or TxA_2, also substantially inhibit platelet activation stimulated by GPVI agonists (collagen or CRP-XL) and thrombin (Moraes et al., 2010b; Moraes et al., 2016; Spyridon et al., 2011). Based on this and recent observations, suggesting the ability of RXR to form heterodimers with several NRs in platelets (LXR, PXR, PPARα and PPARγ) enabled us to revisit and evaluate the effects of RXR ligands on collagen or thrombin stimulation.

Washed human platelets ($4x10^8$ cells/ml) were incubated with 9-*cis*-RA (10 or 20 μM) or vehicle-control (containing DMSO, 0.1% v/v) for 10 minutes prior to their stimulation with collagen (1 μg/ml). Aggregation responses were recorded using an optical aggregometer with constant stirring (1200 rpm) for 5 minutes at 37°C. Approximately 55% and 65% inhibition were observed with 10 or 20 μM 9-*cis*-RA, respectively, in comparison to vehicle-control (figure 5.3 ai, aii). Incubation with methoprene acid, which is structurally unrelated to 9-*cis*-RA also caused inhibition of collagen-stimulated platelet aggregation by 30% and 65% at 10 or 20 μM respectively (figure 5.3 bi, bii). Additionally, 10 and 20 μM of 9-*cis*-RA

attenuated aggregation mediated by CRP-XL (0.25 µg/ml) by nearly 40% and 60% respectively in comparison to vehicle-control (figure 5.3 ci, cii).

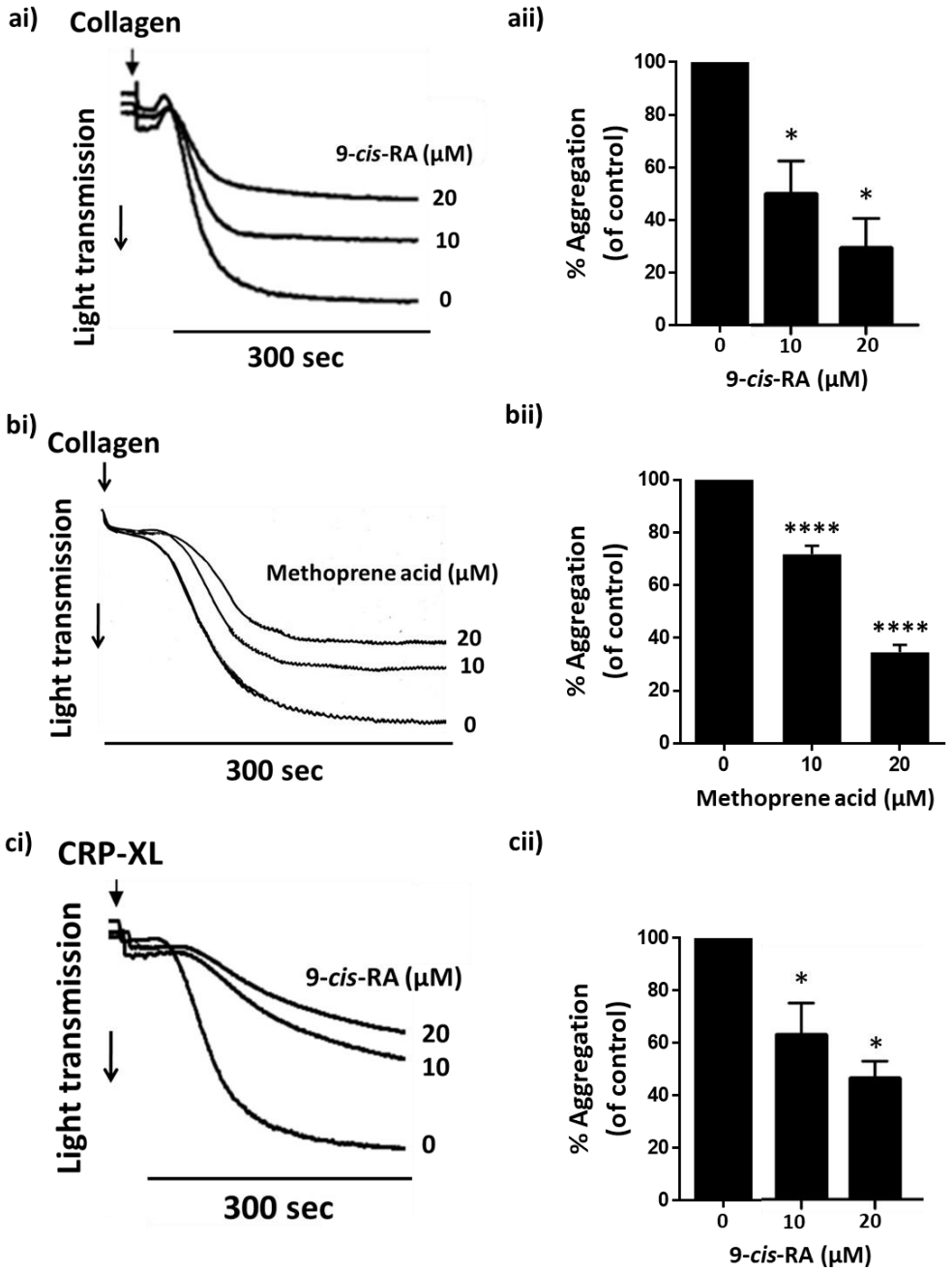


Figure 5.3: RXR ligands inhibit collagen or CRP-XL stimulated platelet aggregation. Washed human platelets (4×10^8 cells/mL) were incubated with 9-*cis*-RA, methoprene acid or vehicle (containing, DMSO 0.1% v/v) prior to their stimulation with **(a,b)** collagen (1 μg/ml) or **(c)** CRP-XL (0.25 μg/ml). Aggregation was measured as a change in light transmission and monitored for 300 seconds at 37°C under constant stirring (1200 rpm). Representative aggregation traces of platelets treated with **(ai, ci)** 9-*cis*-RA or **(bi)** methoprene acid for 10 minutes and stimulated with collagen or CRP-XL are shown. Quantified data displays the percentage of aggregation for **(aii, cii)** 9-*cis*-RA or **(bii)** methoprene acid treated samples (vehicle-treated samples represent 100% aggregation) at the end of 5 minutes. Data represent mean ± SEM (n≥3), *P ≤ 0.05 and ****P ≤ 0.0001 was calculated by one-way ANOVA.

Similarly, incubation of washed platelets for 10 minutes with 9-*cis*-RA resulted in an inhibition of thrombin (0.05 U/ml) stimulated platelet aggregation. Approximately, 20% and 30% reduction was achieved by 10 and 20 μM 9-*cis*-RA in comparison to vehicle-control (containing, DMSO 0.1% v/v) (figure 5.4 ai, aii). Whereas, methoprene acid (20 μM) inhibited thrombin-mediated platelet aggregation by 25% (figure 5.4 bi, bii). These data, along with previously reported findings (Moraes et al., 2007), suggest that the inhibitory effects of RXR ligands are broader than previously anticipated. In addition to the inhibition of ADP and U46619 responses, RXR ligands can also modulate platelet activation evoked by collagen, CRP-XL or thrombin.

To further determine whether RXR ligands indeed affect collagen-evoked platelet activation or the inhibition is solely due to the attenuation of ADP and TxA_2-mediated effects that are released from platelets upon collagen-stimulation, aggregation was studied in the presence of saturated concentrations of indomethacin (blocks synthesis of TxA_2), cangrelor and MRS2179 (ADP receptor antagonists) to block secondary mediator effects. Given the inhibition of secondary mediator signalling, a higher concentration of collagen (10 μg/ml) was used to ensure 50% aggregation was still achieved in 5 minutes. As shown in figure 5.4 ci, treatment with indomethacin (I; 20 μM) or cangrelor (C; 1 μM) and MRS2179 (M; 100 μM) inhibited collagen-stimulated platelet aggregation by approximately 30% and 35% respectively. Collectively (C+M+I), they exhibited inhibition of 55%. Additional inhibition (≃15%) caused by 9-*cis*-RA (20 μM) to the primary reductions achieved by indomethacin, cangrelor and MRS2179

(C+M+I+9-*cis*-RA) post-stimulation by collagen suggested that that 9-*cis*-RA was able to inhibit collagen-evoked signalling directly (figure 5.4 ci, cii).

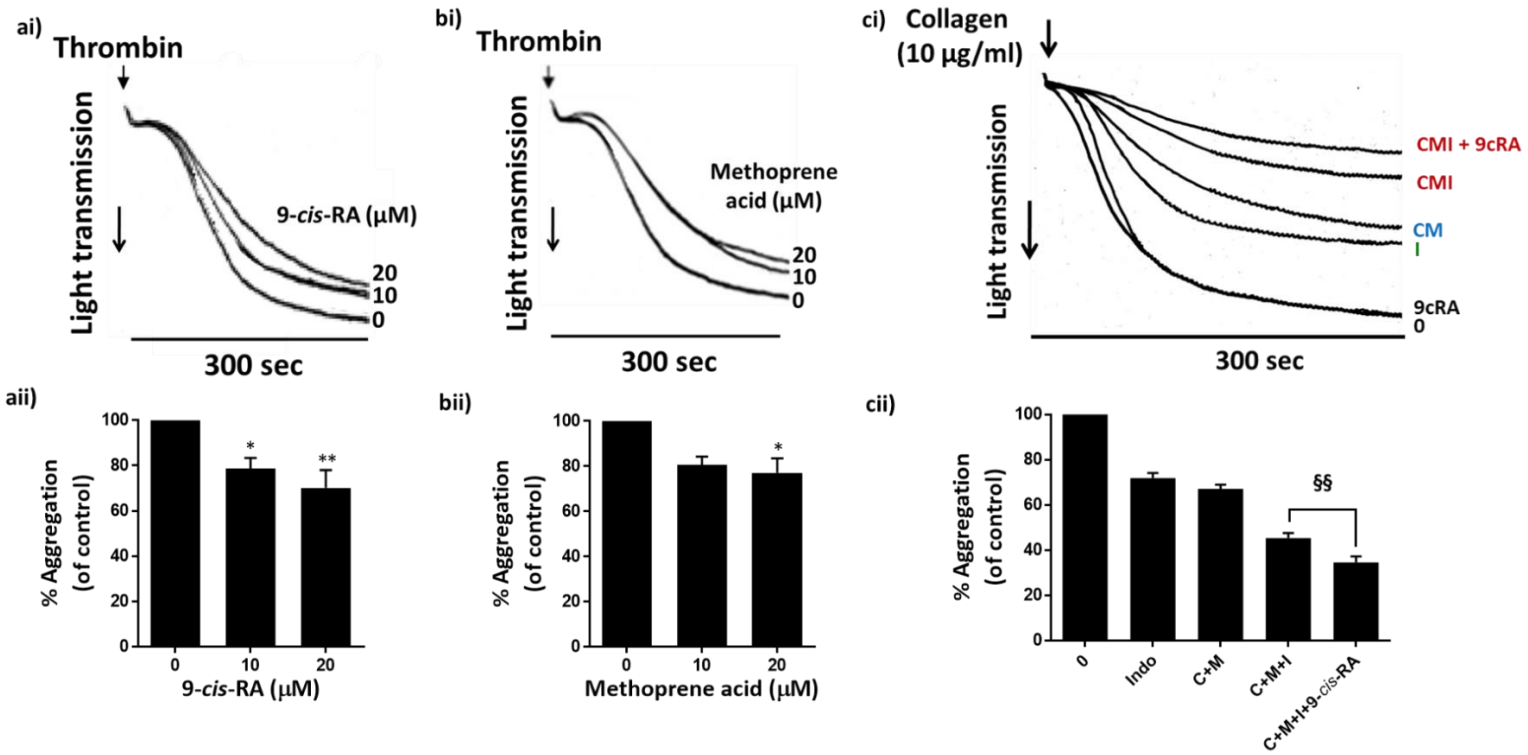


Figure 5.4: RXR ligands attenuate thrombin-mediated platelet aggregation and their effects on collagen-mediated platelet aggregation are not solely dependent on the inhibition of ADP and TxA2-mediated effects. Washed human platelets (4×10^8 cells/mL) were incubated with 9-*cis*-RA, methoprene acid or vehicle (containing, DMSO 0.1% v/v) before stimulation with thrombin (0.05 U/ml). Aggregation was measured as a change in light transmission and monitored for 300 seconds at 37°C under constant stirring (1200 rpm). Representative aggregation traces of platelets treated with **(ai)** 9-*cis*-RA or **(bi)** methoprene acid and stimulated with thrombin are shown. Quantified data displays the percentage of aggregation attained by **(aii)** 9-*cis*-RA or **(bii)** methoprene acid treated samples in 300 seconds upon stimulation with thrombin (vehicle-treated samples represent 100% aggregation). **(ci)** Representative aggregation trace display collagen-stimulated (10 μg/ml) platelet aggregation in presence or absence of 9-*cis*-RA (20 μM) in addition to indomethacin (20 μM), cangrelor (1 μM) and MRS2179 (100 μM). **(cii)** Quantified data displays the extent of collagen-stimulated platelet aggregation in the presence of 9-*cis*-RA, along with indomethacin or cangrelor and MRS2179 (C+M+I+9-*cis*-RA). 'O' signifies the sample stimulated with collagen in the absence of 9-*cis*-RA and secondary mediator signalling blockers. Data represent mean ± SEM (n≥3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by one-way ANOVA. §§P ≤ 0.01 was calculated by student t-test.
Abbreviations: I – Indomethacin, C – Cangrelor, M – MRS2179 and 9-*cis*-RA – 9-*cis*-retinoic acid

5.4. RXR ligands reduce integrin αIIbβ3 activation and α-granule secretion

Given the inhibition of platelet aggregation by RXR ligands, their effects on the activation of integrin αIIbβ3, which regulates its binding to fibrinogen (causing platelets to aggregate) were investigated using flow cytometry. The effects of RXR ligands on secretion from α-granules, essential for the amplification of platelet aggregation and thrombus formation were also evaluated.

Human PRP was treated with 9-*cis*-RA or methoprene acid (10 and 20 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes prior to the addition of FITC-conjugated anti-human fibrinogen antibody or anti-CD62P Cy5/PE conjugated antibody. PRP was stimulated with CRP-XL (0.25 μg/ml) or thrombin (0.05 U/ml) for 20 minutes at room temperature, with occasional gentle mixing. Samples were then fixed with 0.2% (v/v) formyl saline and fluorescence was measured using a flow cytometer. Data were collected for 10,000 events gated on the platelet population.

Consistent with inhibition of platelet aggregation, treatment with 9-*cis*-RA reduced CRP-XL-stimulated fibrinogen binding by approximately 40% and 50% at 10 and 20 μM respectively (figure 5.5 ai). Whereas, 20 μM methoprene acid caused inhibition of 40% in comparison to vehicle control (containing, DMSO 0.1% v/v) (figure 5.5 aii). Similarly, both 9-*cis*-RA (figure 5.5 bi) and methoprene acid (figure 5.5 bii) attenuated thrombin-mediated fibrinogen binding by approximately 30% at 20 μM in comparison to vehicle control. The level of inhibition obtained with CRP-XL or thrombin were of similar magnitude as observed with the inhibition of collagen/CRP-XL or thrombin-mediated platelet aggregation by RXR ligands.

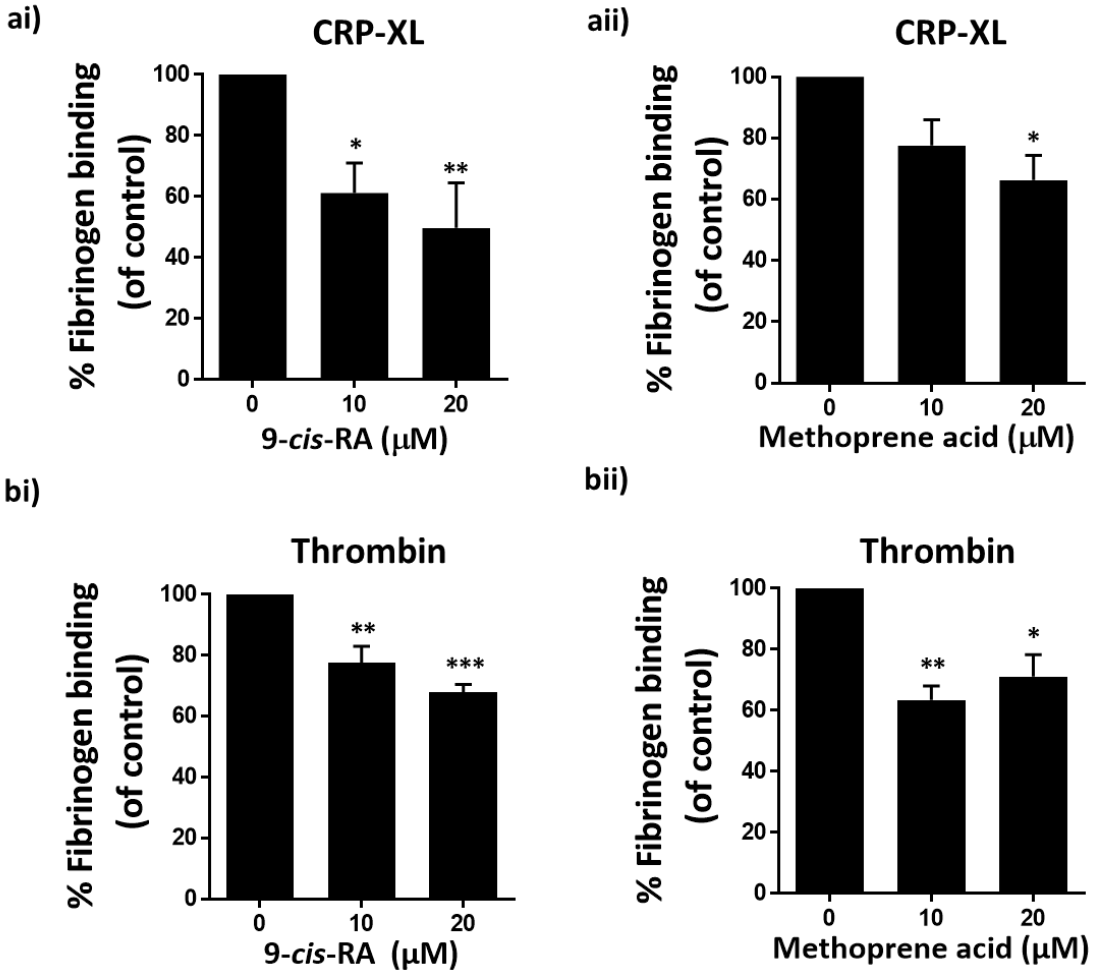


Figure 5.5: RXR ligands down-regulate fibrinogen binding to integrin αIIbβ3. Human PRP was incubated with 9-*cis*-RA or methoprene acid (10 and 20 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 minutes. This was followed by the addition of FITC-labelled rabbit anti-fibrinogen antibody. Post-stimulation by CRP-XL or thrombin, samples were fixed with 0.2% formyl saline (v/v) and analysed by flow cytometry. The effects of RXR ligands on samples stimulated with **(ai, aii)** CRP-XL (0.25 μg/mL) or **(bi, bii)** thrombin (0.05 U/ml) are shown. Data represent percentage fibrinogen binding compared with vehicle-treated control, which is defined as 100% fibrinogen binding. Data represent mean ± SEM (n=4), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

To estimate the effect of RXR ligands on α-granules secretion, CRP-XL or thrombin-stimulated P-selectin exposure on the platelet surface, in the presence or absence of RXR ligands was studied using flow cytometry. CRP-XL stimulated P-selectin exposure was observed to be significantly decreased by approximately 40% in the sample treated with 20 μM of 9-*cis*-RA, in comparison to vehicle-treated control (containing, DMSO 0.1% v/v) (figure 5.6 ai). Incubation with methoprene acid also caused inhibition of 20% and 30% at 10 and 20 μM respectively (figure 5.6 aii). Likewise, P-selectin exposure stimulated by thrombin was also negatively-regulated by 30% and 35% by 10 and 20 μM of 9-*cis*-RA (figure 5.6 bi) and approximately similar degree of inhibition was exhibited by samples treated with methoprene acid (figure 5.6 bii).

Together these findings propose a potential role of RXR ligands in altering both integrin αIIbβ3 activation along with a reduction in the extent of secretion from α-granules. Down-regulation of both of these vital aspects of platelet activation would be anticipated to impact the development and stability of thrombus formation *in vitro* along with its their influence on thrombosis and haemostasis.

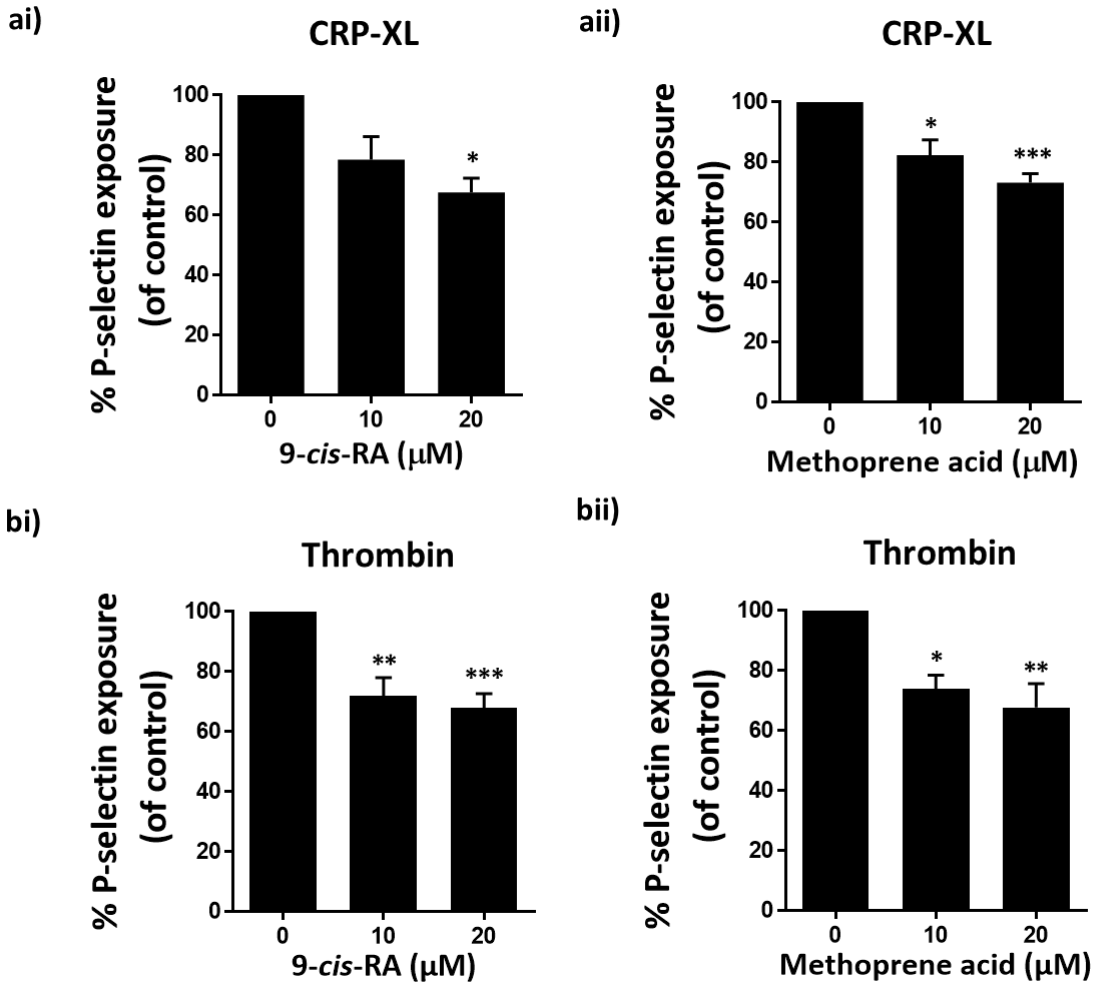


Figure 5.6: RXR ligands attenuate α-granule secretion. Human PRP was incubated with 9-*cis*-RA or methoprene acid (10 and 20 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 minutes. This was followed by the addition of anti-CD62 Cy5/PE conjugated antibody. Post-stimulation with CRP-XL or thrombin, samples were fixed with 0.2% formyl saline (v/v) and analysed by flow cytometry. The effects of RXR ligands on samples stimulated with **(ai, aii)** CRP-XL (0.25 μg/mL) or **(bi, bii)** thrombin (0.05 U/ml) are shown. Data represent percentage P-selectin exposure in comparison to vehicle-treated control, which is defined as 100% P-selectin exposure. Data represent mean ± SEM (n=4), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

5.5. RXR ligands inhibit elevation of intracellular calcium levels

Both CRP-XL and thrombin stimulation can induce an elevation of intracellular calcium concentrations through distinct pathways. As explained in chapter-3 (section 3.11), calcium mobilisation is one of the most fundamental events that determine platelet activation by regulating several vital steps of this dynamic process such as the reorganisation of the actin cytoskeleton necessary for shape change, degranulation and affinity upregulation of integrin αIIbβ3. Therefore, studying calcium mobilisation would be crucial for understanding whether RXR ligands possess the ability to modulate calcium signalling and thus facilitate negative regulation of platelet aggregation, degranulation and integrin αIIbβ3.

The extent of calcium mobilisation was determined using a ratiometric membrane permeable fluorescent dye, Fura-2AM, which binds to free intracellular calcium. PRP was incubated with Fura2-AM and washed platelets ($4x10^8$ cells/ml) were prepared, which were then incubated with RXR ligands (10 or 20 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes in a 96 well plate at 37^oC prior to activation with either CRP-XL (0.25 μg/ml) or thrombin (0.05 U/ml). Fluorescence measurements were made using a plate reader for 5 minutes after the addition of the agonist, and calcium mobilisation was estimated using the equation described in Chapter 2 (section 2.2.8).

As shown in figure 5.7, stimulation of vehicle-treated (containing, DMSO 0.1% v/v) samples with CRP-XL caused a steady rise in calcium levels, achieving peak concentration in approximately 3-4 minutes. This CRP-XL-mediated rise in

intracellular calcium levels was noted to decrease significantly in samples treated with RXR ligands in a concentration-dependent manner. Approximately, 40% inhibition in peak calcium concentration was caused by 20 μM 9-cis-RA (figure 5.7 ai, aii), whereas, methoprene acid resulted in a reduction of 23% and 40% at 10 and 20 μM respectively in comparison to vehicle-control (figure 5.7 bi, bii).

Treatment with RXR ligands also caused a significant decrement in thrombin-stimulated elevation of intracellular calcium concentration. As shown in figure 5.8ai, stimulation with thrombin caused a marked increase in calcium mobilisation in the vehicle-treated sample, which was observed to reduce by approximately 40% and 60% (peak calcium level) in samples treated with 10 and 20 μM 9-cis-RA respectively (figure 5.8 aii). Similarly, 50% reduction in calcium mobilisation was exhibited by 20 μM of methoprene acid (figure 5.8 bi, bii).

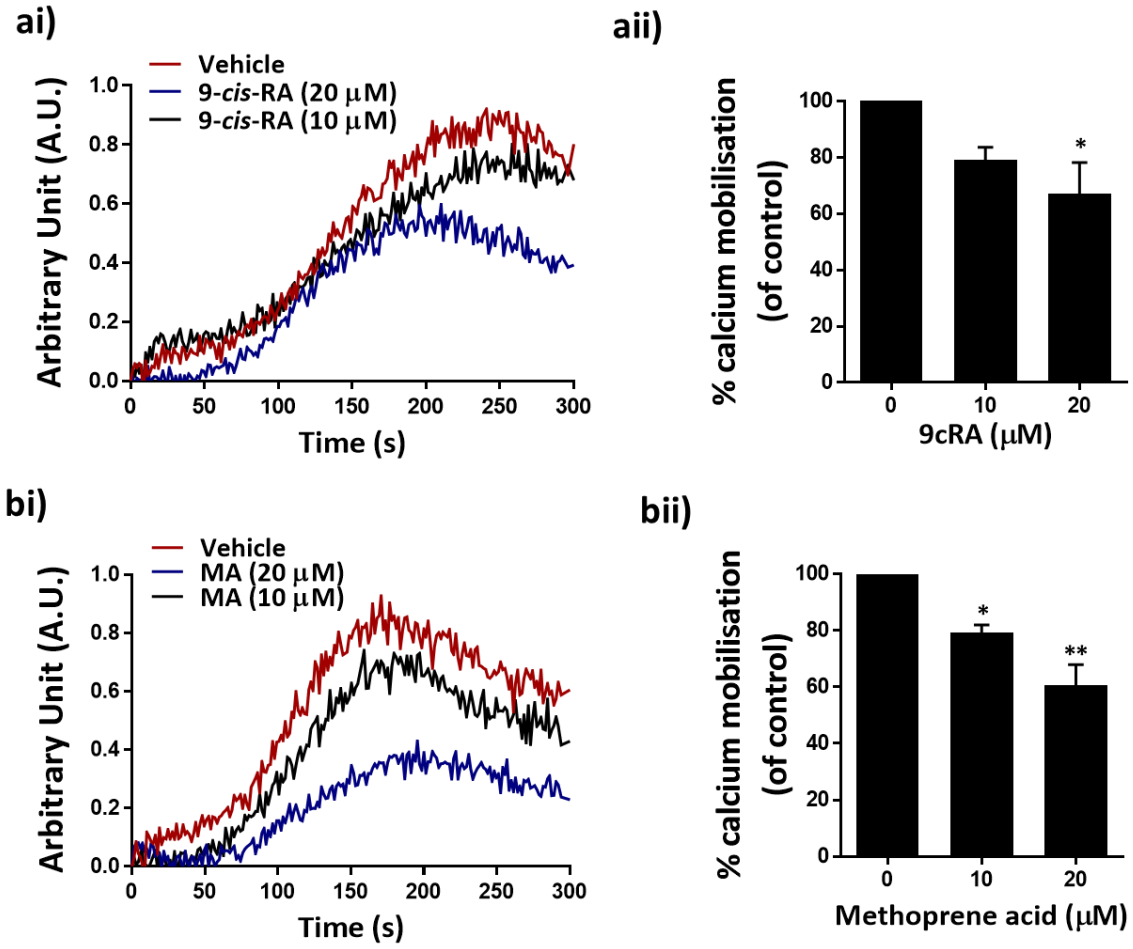


Figure 5.7: RXR ligands inhibit CRP-XL-stimulated calcium mobilisation. Fura-2AM loaded platelets (4x10^8 cells/ml) were incubated with **(ai, aii)** 9-*cis*-RA or **(bi, bii)** methoprene acid (MA; 10 and 20 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 min at 37^{o}C prior to the addition of CRP-XL (0.25 μg/ml). Fluorescence measurements were made with excitation at 340 nm and 380 nm and emission at 510 nm using a NOVOstar plate reader. Ca^{2+} was estimated from the ratio of the 340 nm and 380 nm excitation signals. **(ai, bi)** Traces of calcium mobilisation over a period of 5 minutes following CRP-XL-stimulation are shown. **(aii, bii)** Cumulative data (peak calcium levels) of calcium mobilisation in the presence or absence of RXR ligands after stimulation with CRP-XL. Peak calcium levels achieved in the presence of vehicle-control defines 100%. Data represent mean ± SEM (n≥3), *P ≤ 0.05, and **P ≤ 0.01 was calculated by one-way ANOVA.
Abbreviations: 9-*cis*-RA – 9-cis-retinoic acid and MA – Methoprene acid

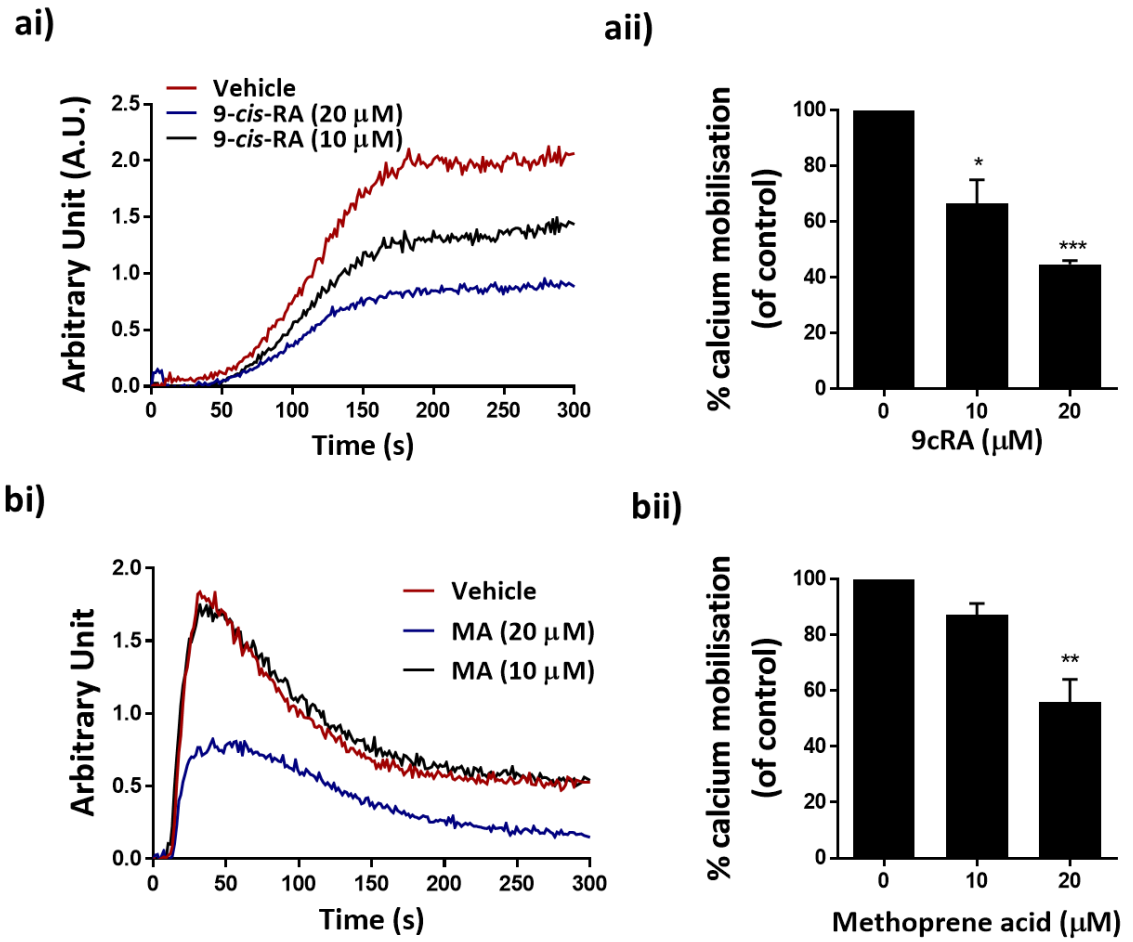


Figure 5.8: Thrombin-stimulated elevation of intracellular calcium level is negatively-regulated by RXR ligands. Fura-2AM loaded platelets ($4x10^8$ cells/ml) were incubated with **(ai, aii)** 9-*cis*-RA or **(bi, bii)** methoprene acid (MA; 10 and 20 μM) or vehicle (containing, DMSO 0.1% v/v) for 10 min at 37°C prior to the addition of thrombin (0.05 U/ml). Fluorescence measurements were made with excitation at 340 nm and 380 nm and emission at 510 nm using a NOVOstar plate reader. Ca^{2+} was estimated from the ratio of the 340 nm and 380 nm excitation signals. **(ai, bi)** Traces of calcium mobilisation over a period of 5 minutes following thrombin-stimulation are shown. **(aii, bii)** Cumulative data (peak calcium levels) of calcium mobilisation in the presence or absence of RXR ligands after stimulation with thrombin. Peak calcium levels achieved in the presence of vehicle-control defines 100%. Data represent mean ± SEM (n≥3), *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 was calculated by one-way ANOVA.

Abbreviations: 9-*cis*-RA – 9-cis-retinoic acid and MA – Methoprene acid

5.6. 9-*cis*-RA inhibit thrombus formation but not adhesion to collagen under flow

Since numerous aspects of platelet activation were observed to be down-regulated following treatment with 9-*cis*-RA, its influence on thrombus formation under flow on collagen was investigated. Testing the effects of 9-*cis*-RA using this assay enabled examination of the effects of RXR ligands in the presence of plasma proteins, blood cells and arterial shear rate. Moreover, this provided insight on the role of 9-*cis*-RA in regulating thrombus formation, prior to assessing its effects *in vivo*.

To perform the assay, citrated human blood (collected in vacutainers) was incubated with the lipophilic dye DiOC6 (5 μM) for an hour at 30^{o}C and Vena8 Biochips were coated with type-I collagen (100 μg/ml). Excess collagen in the channel of the microfluidic chip was washed with modified Tyrodes-HEPES buffer. Whole blood was incubated with 9-*cis*-RA (20 μM) or vehicle-control (containing, DMSO 0.1% v/v) for 10 minutes at 30^{o}C before perfusion through the collagen-coated microfluidic channels under arterial flow condition (shear stress: 20 dyne/cm^{2} or shear rate: 500 s^{-1}). Fluorescence was excited at 488 nm with an argon laser and emission was detected at 500-520 nm. Thrombus formation was observed using a Nikon A1-R confocal microscope with a 20X objective and images were captured (focused on a single section) every 1 second for 600 seconds. Median thrombus fluorescence intensity was calculated using NIS elements software (Nikon) and normalised to the level of fluorescence at the end of the assay in the vehicle-treated sample.

As shown in figure 5.9ai, large and stable thrombi were formed in the vehicle-treated whole blood sample following perfusion through the collagen-coated microfluidic channel. Treatment with 9-*cis*-RA prevented platelets from forming large and stable thrombi and resulted in an approximate reduction of 35% in comparison to vehicle-treated control (figure 5.9aii). This observation was consistent with the previously observed inhibition of platelet aggregation by 9-*cis*-RA following stimulation with collagen.

To investigate whether this reduction in thrombus formation by 9-*cis*-RA is due to the inability of platelets to adhere to collagen, the flow assay was performed by treating platelets with integrillin (4 μM; an integrin αIIbβ3 antagonist), which prevent platelet-platelet interactions. No significant difference in adhesion of platelets to collagen was observed in blood samples treated with 9-*cis*-RA (20 μM) or vehicle-control (both in the presence of integrillin) (figure 5.9b). This suggests that 9-*cis*-RA indeed affects thrombus stability and its growth but it does not modulate adhesion of platelets to collagen. These experiments were performed in collaboration with Dr A. Unsworth.

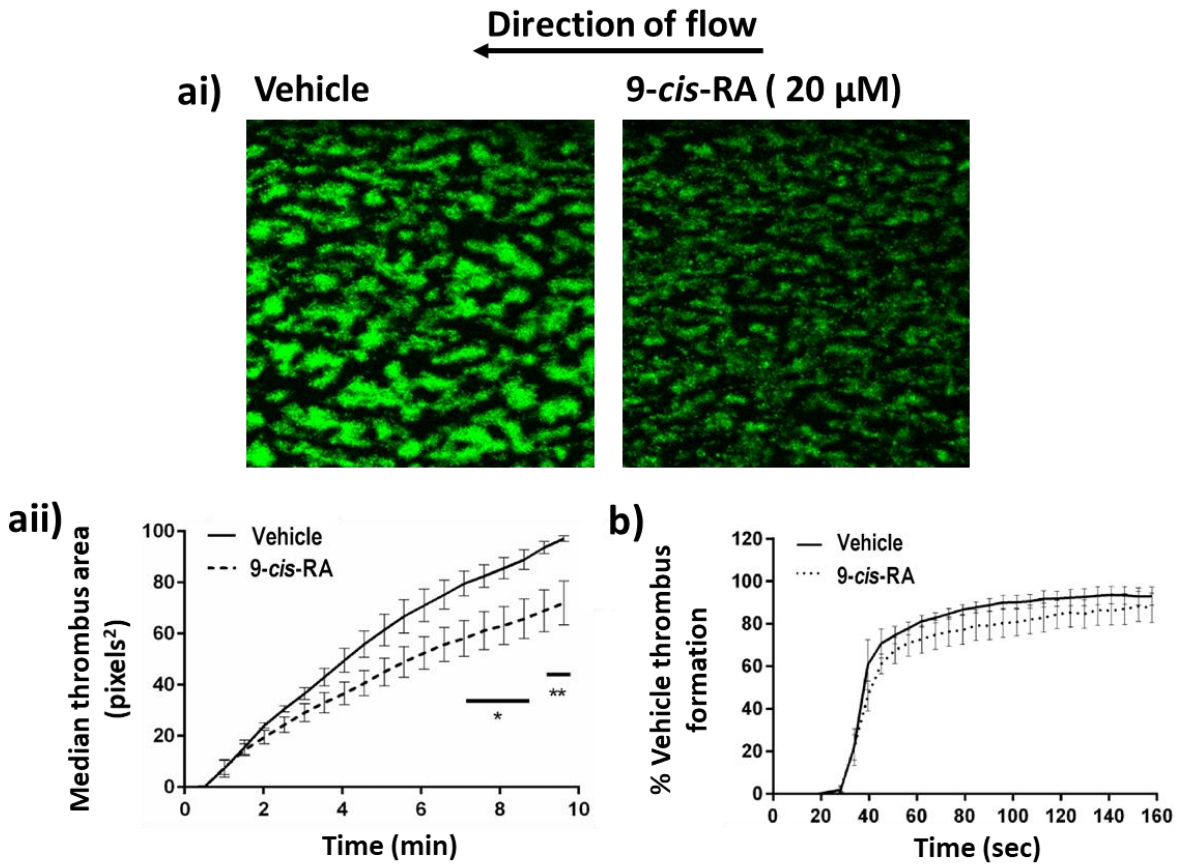


Figure 5.9: 9-*cis*-RA negatively-regulates thrombus formation but not adhesion to collagen *in vitro*. Citrated human blood, incubated with DiOC6 (5 µM) for an hour at 30°C was perfused through collagen-coated (100 µg/ml) Vena8 microfluidic Chips under arterial flow conditions (20 dyne/cm²), after treatment with vehicle (containing, DMSO 0.1% v/v) or 9-*cis*-RA (20 µM) for 10 minutes. **(ai)** Representative image of thrombus formation (endpoint) in samples treated with vehicle or 9-*cis*-RA is shown. Fluorescence was excited at 488 nm with an argon laser and emission was detected at 500-520 nm. The thrombus formation was observed using a Nikon A1-R confocal microscope (20X objective), and images were captured (focused on a single section) every 1 second for 600 seconds. **(aii)** Quantified data represent median fluorescence intensity for vehicle and 9-*cis*-RA treated samples; and **(b)** vehicle and 9-*cis*-RA treated samples in the presence of integrillin (4 µM), calculated using NIS elements software (Nikon) and normalised to the level of fluorescence of the vehicle-treated sample. Data represent mean ± SEM (n≥3), *P ≤ 0.05 and **P ≤ 0.01 was calculated by two-way ANOVA.

5.7. RXR ligands inhibit thrombosis and haemostasis

Having observed a significant reduction of thrombus formation *in vitro* by 9-*cis*-RA, its acute effects were investigated *in vivo* to determine its influence on thrombosis and haemostasis.

The thrombosis assay was performed using a laser-induced injury model of mouse (C57BL/6) and visualised using intravital microscopy, as described by Falati *et al.* (2002). The details of the procedures followed are described in chapter-2 (section 2.2.15). As shown in figure 5.10ai, the initial kinetics of thrombus formation were similar in both vehicle (treated with DMSO, 0.1% v/v) and 9-*cis*-RA (final concentration 20 μM) treated mice. However, the size of thrombi in mice treated with 9-*cis*-RA were observed to be smaller (figure 5.10aii). As shown previously, RXR ligands did not affect adhesion of platelets to collagen; this observation can account for the similarity noted in the initial kinetics of thrombus formation between 9-*cis*-RA and vehicle-treated mice. 9-*cis*-RA reduced the overall size of the thrombus by approximately 35%, which is similar to the reduction observed in thrombus formation *in vitro* (figure 5.10aiii). These experiments were performed in collaboration with Dr P. Sasikumar.

The impact of RXR ligands-dependent platelet inhibition on haemostasis was observed using a tail-bleeding assay on C57BL/6 mice. 9-*cis*-RA or vehicle-control was injected into the femoral vein of mice based on body weight and blood volume. The volume of 9-*cis*-RA (10 mM stock) or DMSO (10% v/v stock) injected was expected to give a concentration of 20 μM and 0.1% v/v respectively in the blood assuming 2 ml of blood is present in 25 gms of mouse. The tip of the tail was

removed using a sharp razor blade and placed in sterile saline (37°C) after 10 minutes post injection of vehicle-control or 9-*cis*-RA. The time to cessation of bleeding (secs) was measured thereafter. The mean time to cessation of bleeding after removal of the tail-tip was significantly prolonged in mice treated with 9-*cis*-RA in comparison to vehicle control. Vehicle-treated mice were observed to bleed for approximately 120 secs, while treatment with 9-*cis*-RA increased the cessation time of bleeding to approximately 500 seconds, which represents an impaired haemostatic response (figure 5.10b). These experiments were performed in collaboration with Ms T. Sage.

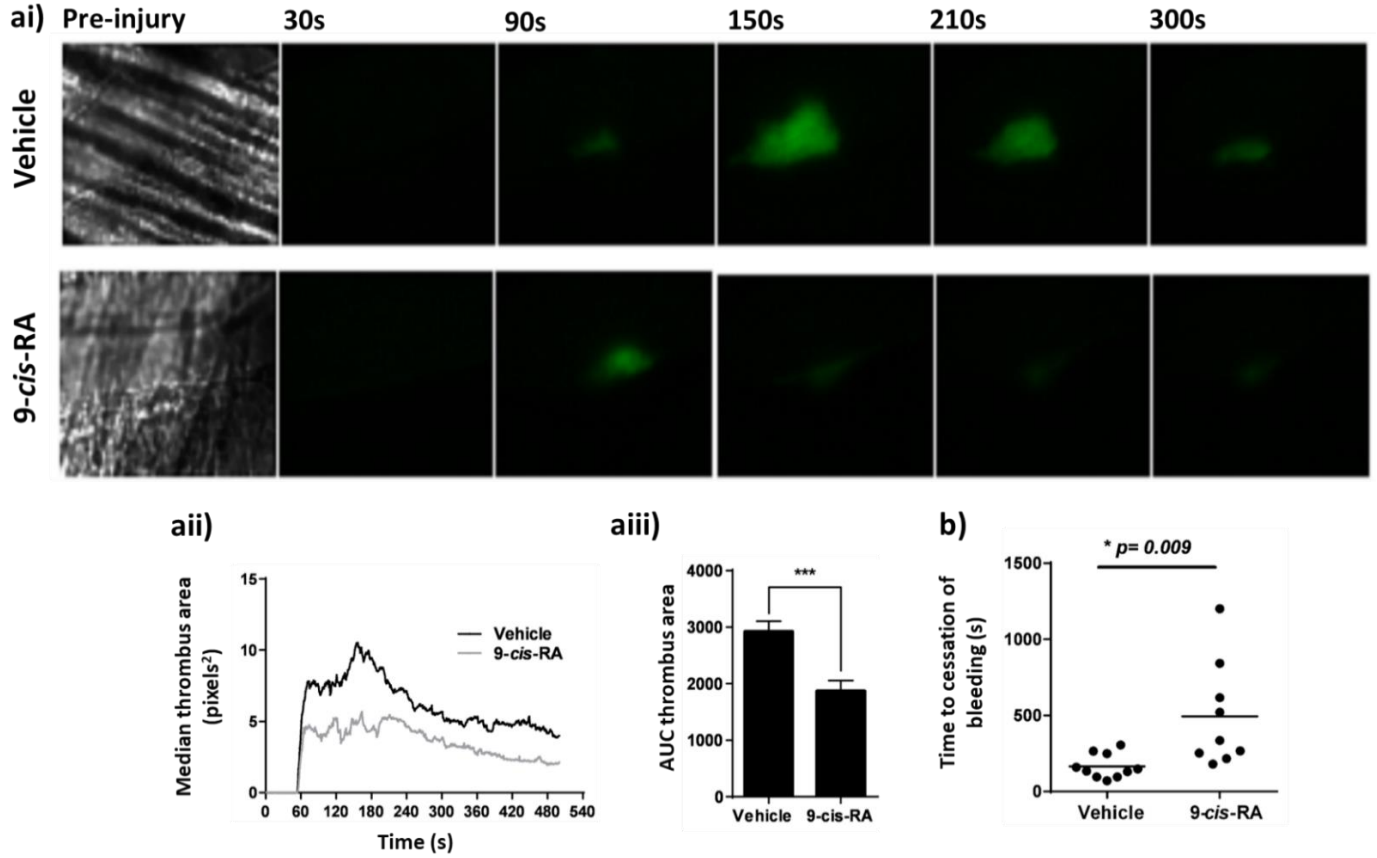


Figure 5.10: 9-*cis*-RA inhibit thrombus formation and increase bleeding time in mice. *In vivo* thrombosis assay was performed on C57BL/6 mice through intravital microscopy using the laser-induced injury model. Vehicle-control (DMSO 10% v/v for 0.1% v/v final concentration) or 9-*cis*-RA (20mM for 20 μM final concentration) was administered intravenously 10 minutes prior to the initial injury. Platelets were fluorescently labelled with DyLight 649–conjugated anti-GPIbα antibody. The injury was induced by laser to assess platelet accumulation and thrombus formation. **(ai)** Representative images of thrombi obtained at different time intervals are shown. Data represent **(aii)** median fluorescence intensity measured for 8 to 10 thrombi from 4 mice each of control and 9-*cis*-RA treated groups. **(aiii)** Thrombus-size was determined by calculating the area under the median fluorescence intensity curve of each thrombi (AUC). **(b)** Tail bleeding was determined as time to cessation of bleeding in mice pre-treated with vehicle or 9-*cis*-RA (estimated concentration; 20 μM) for 10 minutes (n=10 for vehicle and 9 for 9-*cis*-RA–treated samples). Results are mean ± SEM for n≥3. Results are mean ± SEM. ***P ≤ 0.001 was calculated by the nonparametric Mann–Whitney test.

5.8. Chapter discussion

RXR is one of the most widely expressed NR in human cells and tissues, where it forms heterodimer with several NRs such as LXR, FXR, RXR and PPARs, and elicit genomic responses to regulate a range of biological processes such as glucose, lipids, cholesterol and bile acid metabolism (Evans and Mangelsdorf, 2014). Consequently, its dysregulation is associated with metabolic and cardiovascular disorders that include type-2 diabetes mellitus, obesity, hyperlipidaemia and atherosclerosis (Meissburger and Wolfrum, 2008). Recently, the presence of RXR was reported in human and mouse platelets and its ligands were observed to cause anti-platelet effects (in response to ADP or U46619) in a non-genomic manner (Moraes et al., 2007). Additionally, RXR ligands have also been proposed to reduce the development of atherosclerosis in apolipoprotein E knockout mice (Claudel et al., 2001), a pathological condition where platelets are known to be major contributors. Based on this, we further examined the role of RXR ligands in human platelets with emphasis on their ability to regulate collagen-stimulated platelet activation. 9-*cis*-RA is commercially used (marketed as Alitretinoin) for the treatment of Kaposi sarcoma and eczema and decreased blood clotting is currently listed as one of its side effects (Ghasri and Scheinfeld, 2010; Walmsley et al., 1999). Therefore, we also investigated the effects of 9-*cis*-RA on thrombus formation (*in vitro* and *in vivo*) and haemostasis. Major findings of this chapter include:

I. The physiological role of RXR ligands in platelets

Inhibitory effects of RXR ligands were observed on several aspects of platelet activation evoked by collagen, CRP-XL or thrombin. For instance, treatment of platelets with 9-*cis*-RA or methoprene acid significantly inhibited CRP-XL, collagen or thrombin-mediated platelet aggregation. Although, the extent of inhibition was of a lower magnitude, in comparison with ADP or U46619 instigated aggregation. This might be an outcome of apparently stronger platelet-activation potency of CRP-XL, collagen and thrombin, in comparison to ADP and U46619. The amplitude of collagen, CRP-XL or thrombin-stimulated integrin αIIbβ3 activation, α-granule secretion and mobilisation of intracellular calcium were also found to be reduced significantly after treatment with RXR ligands.

Under physiological conditions, the plasma concentration of endogenous ligand of RXR (9-*cis*-RA) is close to picomolar range because of its rapid metabolism in intestines and liver (Vogel et al., 1999; Wolf, 2006). Moreover, it is not stored in the liver or any other organ, therefore, it does not accumulate over time (Bidlack, 1994). However, treatment with alitretinoin (commercially available 9-*cis*-RA), prescribed at a maximum dose of 40 mg/day may lead to higher 9-*cis*-RA plasma concentrations (English, 2009; Walmsley et al., 1999). Thus, it is plausible that 9-*cis*-RA at clinical and therapeutic doses may facilitate non-genomic effects on platelets. Moreover, the possibility of the occurrence of non-genomic effects at the site of 9-*cis*-RA biosynthesis (where concentration would be higher than rest of the recipient tissues) cannot be ruled out.

Inhibition of platelet activity by 9-*cis*-RA also correlated with inhibition of thrombus formation *in vitro* and *in vivo*, suggesting that the previously described

cardioprotective effects of RXR agonists in reducing atherosclerosis could also potentially be attributed to their negative regulation of platelet function (Claudel et al., 2001). Moreover, increased bleeding time in mice treated with 9-*cis*-RA is consistent with the observation of reduced blood clotting in patients administered with alitretinoin (Ghasri and Scheinfeld, 2010; Walmsley et al., 1999).

II. RXR ligands upregulate PKA activity to inhibit platelet activation (Unsworth, Flora et al., 2017)

Upregulation in the levels of cAMP or activation of PKA in platelets has been associated with ligands of PPARα, PPARβ or PPARγ that are common binding partners for RXR (Ali et al., 2009a; Ali et al., 2006; Unsworth et al., 2017d). Likewise, RXR ligands were also found to cause upregulation of PKA activity, as treatment of platelets with RXR ligands resulted in an increase in VASP phosphorylation at S157 (the PKA phosphorylation site) in both resting and agonist-stimulated platelets. This increase was reversed after treatment with the PKA inhibitors H89 and Rp-8-CPT-cAMPs or adenylyl cyclase inhibitor SQ22358 but not after treatment with an IP receptor antagonist (Ro1138452). This suggests that RXR agonists activate PKA through a mechanism that is dependent on cAMP, although no major alterations in cAMP levels were observed after treatment with the different RXR ligands. It is possible that treatment with RXR agonists does cause small increases in platelet cAMP levels that are not detected given current limitations in sensitivity of the assays used. It has been shown that even minor increases in cAMP levels can cause significant activation of cellular PKA and large increases in VASP S157 phosphorylation (Eigenthaler et al., 1992). As such, a role

for RXR ligands in the upregulation of adenylyl cyclase activity cannot be ruled out.

The effects of RXR ligands reported on PKA activation demonstrate a novel mechanism that may act as a potential target for antiplatelet therapy (Unsworth, Flora et al., 2017). Therefore, it can be suggested that RXR ligands could offer extra protective effects *in vivo* if developed as drug targets, although these effects would need to be carefully balanced to ensure there is no increased risk of bleeding.

Chapter-6

General Discussion

6.1. Platelets as model systems for studying non-genomic actions of nuclear receptors

Auto-regulation of platelet activation arbitrated by negative feedback mechanisms in platelets is vital to prevent uncontrolled and rampant thrombus formation, following vascular injury. Dysregulation of these mechanisms can lead to occlusion of the artery, leading to life-threatening conditions such as heart attack or stroke (Bye et al., 2016). Besides the well characterised inhibitory mechanisms instigated by PGI_2 and NO in platelets, several other receptors that contribute to negative regulation have been reported in recent years. These include immunoreceptor tyrosine-based inhibition motif (ITIM) containing receptors (such as PECAM-1, CEACAM-1, CEACAM-2, G6b-B), Wnt–β-catenin, semaphorin 3A and intracellular nuclear receptors (Unsworth et al., 2017a). Of these, our lab has extensively investigated and reported the role of PECAM-1 (Moraes et al., 2010a; Moraes et al., 2013) and several NRs in platelets such as RXR, LXR, FXR, PPARγ and through this study PXR (Moraes et al., 2016; Unsworth et al., 2017c; Moraes et al., 2010b; Moraes et al., 2007; Spyridon et al., 2011).

As explained in chapter-1, NRs have been well characterised for their genomic roles (regulation of transcription and gene expression), while little is known about their non-genomic functions that are mediated independently of transcriptional regulation. Non-genomic effects have mostly been investigated in cell types that lack a functional nucleus such as erythrocytes and platelets (Losel and Wehling, 2003), though, these effects have also been reported in nucleated cells (Simoncini et al., 2004). The non-genomic functions of NRs vary and whilst it is thought these functions are initiated by physical interactions of NRs with

cofactors and binding partners that initiate rapid signalling events (Hammes and Levin, 2007; Losel et al., 2003; Nadal et al., 2001), the exact mechanisms are not well understood. One possible explanation is that the different cellular localisation of NRs influences the availability of cofactors and substrates, which leads to varying degree of binding-partner interactions. For instance, localisation of NRs towards the cytosol, plasma membrane or other intracellular organelles such as mitochondria increases the likelihood of initiation of non-genomic effects, whilst, genomic functions are more restricted with NRs localised in the nucleus (Boonyaratanakornkit and Edwards, 2007; Ordonez-Moran and Munoz, 2009; McKenna and O'Malley, 2002; Nathan et al., 2017). The formation of different multi-protein signalling complexes along with different localisation and distribution of proteins across multiple cell types could offer a high degree of cell and tissue-selective action but these are currently poorly defined (McKenna and O'Malley, 2002; Nathan et al., 2017).

Although devoid of a nucleus, platelets still contain different forms of RNA (mRNA, rRNA, tRNA and miRNA) and components of the transcription and translation machinery that are derived from megakaryocytes during thrombopoiesis (Schubert et al., 2014). There is a growing consensus that these RNAs are not subjected to a random transfer by megakaryocytes but are specifically sorted and are competent for translation within platelets (Rowley et al., 2012; Cecchetti et al., 2011). Moreover, there is evidence to suggest that platelet-derived microparticles may deliver platelet mRNAs into other nucleated cells, such as monocytes and endothelial cells, where they then undergo translation (Risitano et al., 2012). Intracellular NRs recently identified in platelets

is another addition to various components of transcription machinery existing inside platelets. Due to their anucleate nature and mechanistically well-characterised and rapid responses, such as aggregation and adhesion, platelets provide an excellent model system to study the acute non-genomic effects of the NRs (Bishop-Bailey, 2010; Jones et al., 2012).

This study identifies the presence of an additional nuclear receptor, PXR, in platelets and the ability of its ligands to regulate platelet activation by modulating GPVI signalling. Furthermore, this investigation also characterises previously unknown abilities of RXR ligands to regulate platelet function stimulated by collagen, CRP-XL or thrombin along with their mode of action (Unsworth, Flora et al., 2017a).

6.2. Role of PXR as a modulator of platelet activation

The effects of PXR on vascular biology through its cardio-protective effects have been uncovered in the last few years. Conventionally acting as a metabolic sensor, PXR is highly expressed in liver and intestines. Recently its expression was also reported in the human vasculature, where it was suggested to regulate vascular function, inflammation, and cholesterol and lipid homeostasis (Swales et al., 2012). These findings are relevant considering the involvement of cholesterol, lipids and inflammation towards the development of atherosclerosis in the arterial wall. Additionally, PXR has also been associated with anti-atherosclerotic effects (mainly by promoting cholesterol efflux and HDL synthesis) in several studies performed on murine models of atherosclerosis (de Haan et al., 2009; Li et al., 2007; Zhou et al., 2009a; Masson et al., 2005). Considering the role of platelets in

the progression of atherosclerosis and the presence of several NRs in platelets, the presence of PXR in platelets was evaluated. The expression of PXR was observed in both human and mouse platelets. Immunofluorescence studies suggested the distribution of PXR in a punctate arrangement inside the resting platelet cytosol, which appeared to redistribute towards the plasma membrane upon platelet activation. The potential significance of such rearrangement of PXR is still speculative and may be associated with the regulation of platelet signalling through the potential interaction (still unknown) of PXR with signalling molecules. For instance, several signalling molecules such as Syk, Gαq, Btk and PLCγ2, upon platelet activation migrate close to their respective receptors on the plasma membrane to initiate signalling (Pula et al., 2005; Sarkar, 1998) and are also known to interact with RXR (with $G\alpha_q$), LXR and PPARγ (with Syk, LAT, PLCγ2) (Moraes et al., 2010b; Moraes et al., 2007; Spyridon et al., 2011). Nonetheless, translocation of NRs in activated platelets appears to be a common feature shared by different NRs in platelets including PXR, RXR and FXR.

Ligation of PXR with SR12813 or rifampicin resulted in down-regulation of a range of platelet functions such as aggregation, affinity upregulation of integrin αIIbβ3, TxB_2 production and degranulation stimulated by both GPVI agonists (CRP-XL or collagen) and GPCR agonist (thrombin). We also observed that treatment of human blood with PXR ligands caused a substantial reduction in the development of thrombus *in vitro*. The lack of platelet adhesion to collagen in samples treated with PXR ligands in both arterial flow and static conditions was attributed to be partly responsible for this reduction. Given these observations, the reported anti-atherogenic effects of PXR ligands might be platelet-orchestrated

that may be mediated at least in following three ways: (i) Inflammatory events during the initial stages of atherosclerosis lead to the activation of endothelial cells (Liao, 2013). Platelets via P-selectin, integrin αIIbβ3 and GPIb receptors are prone to interact with these activated cells and become stimulated to secrete proinflammatory chemokines and cytokines that accelerate the inflammatory process and plaque development (Lievens and von Hundelshausen, 2011). PXR ligands mediated inhibition of P-selectin exposure on the platelet surface and reduced affinity-upregulation of integrin αIIbβ3 may prevent platelet-endothelial cells and platelet-monocyte interaction (via reduced platelet P-selectin mediated monocytic PSGL-1 ligation) (Seizer et al., 2008) and thus reduce the progression of atherosclerosis. (ii) Moreover, reduced secretion from activated platelets following treatment with PXR ligands can potentially avert the release of proinflammatory chemokines and cytokines that can further reduce inflammation at the atherosclerotic site, thereby slowing the development of atherosclerosis (Nording et al., 2015). (iii) Lastly, the fibrous cap of atherosclerotic plaque exposes collagen fibres, allowing platelets to adhere, get activated and promote thrombus formation (Nadkarni et al., 2009). The lack of platelet adhesion to collagen following treatment with PXR ligands may attenuate this interaction, which may prevent the formation of a blood clot at the site of atherosclerotic plaque.

Due to differences in the sequence of the LBD of mouse and human PXR, ligands that bind to PXR are prone to function in a species-specific manner, as reported in several nucleated cells (Iyer et al., 2006). Similar observations were made in platelets, where human PXR ligands (SR12813 or rifampicin) inhibited thrombus formation in human blood while no effect was observed in mouse blood.

Similarly, mouse PXR ligand (PCN) attenuated thrombus formation in mouse blood, while no effect was observed in human blood. These findings also indirectly confirm that the effects of PXR ligands are likely to be mediated through PXR in human and mouse platelets. Due to the species-specific profile of PXR ligands, the effects of human PXR ligands were evaluated *in vivo* using 'humanised' PXR mice (hPXR) (Taconic Biosciences), in which the endogenous mouse PXR gene has been replaced with human PXR gene (Scheer et al., 2008; Scheer et al., 2010). Similar to inhibition of *in vitro* thrombus formation, SR12813 significantly down-regulated laser-induced arteriolar thrombosis. A smaller thrombus, with initial kinetics similar to the vehicle-treated sample was still able to form upon treatment with SR12813. The initial rapid kinetics of thrombus formation in SR12813 treated mice might be due to (i) an initial burst of numerous platelet adhesive stimulants (such as vWF, P-selectin, angiopoietin-2, tissue plasminogen activator, and endothelin-1) from the injured endothelial cells and (ii) rapid stimulation of platelets through their GPCRs by locally synthesised thrombin (via coagulation pathway) at the injured site and secretion of TxA_2, ADP and thrombin from activated platelets. Additionally, a substantial increase in tail bleeding time was observed in the hPXR mice treated with SR12813, indicative of impaired haemostasis.

PXR ligands were noted to substantially inhibit activation of the GPVI-mediated signalling pathway, while there was no modulation of inhibitory signalling arbitrated by cyclic nucleotides or via PKA and PKG. An attenuation of both early and late phases of GPVI signalling pathway was observed. As described in chapter 1 (section 1.5.1), GPVI receptor clustering after its binding to

collagen/CRP-XL initiate signalling pathway that begins with the autophosphorylation of the SFKs, which subsequently phosphorylate the ITAM-containing FcRγ-chain that provide a binding site to Syk to become auto-phosphorylated. This is followed by Syk-mediated phosphorylation of LAT, resulting in the formation of the LAT-signalosome and consequent phosphorylation and activation of PLCγ2. Calcium mobilisation and PKC activation follow next, which stimulate degranulation and activation of integrin αIIbβ3, resulting in platelet aggregation (Li et al., 2010; Watson et al., 2005). Inhibition of GPVI signalling was observed from the beginning of the pathway with reduced phosphorylation levels of SFKs, which was followed with down-regulation of Syk, LAT and PLCγ2 phosphorylation. These findings add PXR to the list of NRs such as LXR and PPARγ that can regulate GPVI mediated signalling in platelets (Moraes et al., 2010b; Spyridon et al., 2011). The ability of PXR ligands to inhibit SFKs is a novel observation, which has not been previously reported for other NRs expressed in platelets. Besides the regulation of GPVI signalling, SFKs also function to initiate signalling via rhodocytin or podoplanin receptor CLEC-2 that share considerable structural similarity with GPVI (Watson et al., 2010). PXR ligands were also able to inhibit phosphorylation of Src, downstream of CLEC-2, which indicates SFKs as the general target of PXR ligands. Furthermore, reduction in the phosphorylation of Src proximal to integrin αIIbβ3 receptors was also noted, which provide additional evidence that PXR ligands indeed mediate their action by regulating SFKs. Since SFKs play a crucial role in the regulation of outside-in signalling via integrin αIIbβ3; its inhibition by PXR ligands was also reflected by diminished levels of platelet spreading on fibrinogen and reduced fibrin clot retraction. Besides this, PXR ligands also inhibited calcium mobilisation, PKC and

MLC phosphorylation, which demonstrate their ability to modulate late events of GPVI signalling cascade (Figure 6.1). While the underlying mechanism that contributes towards the inhibition of GPVI signalling components is still unknown. One possibility is through the direct interaction of PXR with one or more of GPVI-stimulated signalling molecules, as reported in case of LXR and PPARγ, which interact with Syk/PLCγ2 and Syk/LAT respectively (Moraes et al., 2010b; Spyridon et al., 2011). Treatment with LXR ligand GW3965 substantially increased the LXR-Syk and LXR-PLCγ2 interactions, which was associated with the attenuation of platelet function (Spyridon et al., 2011). Similarly, PPARγ in its unliganded state interacts with Syk and LAT and plausibly facilitate the phosphorylation and activation of proteins downstream within the GPVI pathway. However, upon ligation of PPARγ, these interactions were identified to be reduced and may account for the inhibition of GPVI signalling and subsequent platelet activation (Moraes et al., 2010b). However, immunoprecipitation of PXR did not reveal interactions with Src, Syk, LAT or PLCγ2. There are challenges associated with co-immunoprecipitation assays, which mainly depend on the quality, specificity and efficiency of antibodies targeting a specific protein to study protein-protein interactions (Bordeaux et al., 2010). Therefore the interaction of PXR with these and other components of GPVI signalling cannot be ruled out yet and would require further examination by other techniques such as mass spectrometry. Moreover, PXR ligands were observed to inhibit calcium mobilisation stimulated by low concentrations of thrombin, suggesting regulation of underlying signalling. Therefore, the influence of PXR ligands on GPCR signalling at such concentrations cannot be excluded.

Collectively, these findings suggest that PXR ligands, besides their potential anti-atherosclerotic properties, may produce additional cardioprotective effects through anti-thrombotic effects. However, development of PXR ligands into potential therapeutic agents requires two important considerations: Firstly, the anti-thrombotic effects of these ligands require careful balancing with the associated risk of bleeding. Secondly, drug-drug interactions (DDIs) associated with the activation of PXR should be considered. PXR facilitates the induction of key enzymes such as CYP2B6, CYP3A4, and UGT1A1 that are chiefly involved in the metabolism of approximately 80% of clinically used drugs (Zhou et al., 2009b). Metabolism typically inactivate drugs, though, some drugs when metabolised demonstrate higher pharmacological activity than the parent compound (prodrug). It has been reported that several drugs that are PXR activators when co-administered at therapeutic doses with other drugs (such as anti-HIV protease inhibitors, oral contraceptive, thiazolidinediones and benzodiazepines) are predicted to cause DDIs (Sinz, 2013; Wang et al., 2014a; Chai et al., 2013). The most common DDIs arise when one drug either enhances or reduces the effective concentration of another. This can have adverse effects because PXR activation by their ligands can reduce the metabolism of the other compound, causing it to accumulate at toxic levels. Whereas, increased metabolism can decrease the therapeutic concentration of the other drug or increase the accumulation of toxic metabolites (Moore and Kliewer, 2000). For instance, co-administration of rifampicin with rosiglitazone (PPARγ activator) was reported to decrease the therapeutic concentration of rosiglitazone significantly, making it no longer efficacious (Park et al., 2004). Recently, PXR induction in a cultured rat aorta system was found to up-regulate the transcription of CYPs involved in the

metabolism of clopidogrel (irreversible inhibitor of platelet ADP receptor, P2Y12), leading to the metabolic conversion of its prodrug form to its bioactive metabolite that inhibited platelet aggregation (Swales et al., 2012). Therefore, enhanced metabolism of clopidogrel through PXR activation can lead to bleeding disorders (Sibbing et al., 2010). Therefore, DDIs mediated by PXR activation are an important consideration that needs to be taken into account, should PXR ligands be developed into potential anti-thrombotic agents.

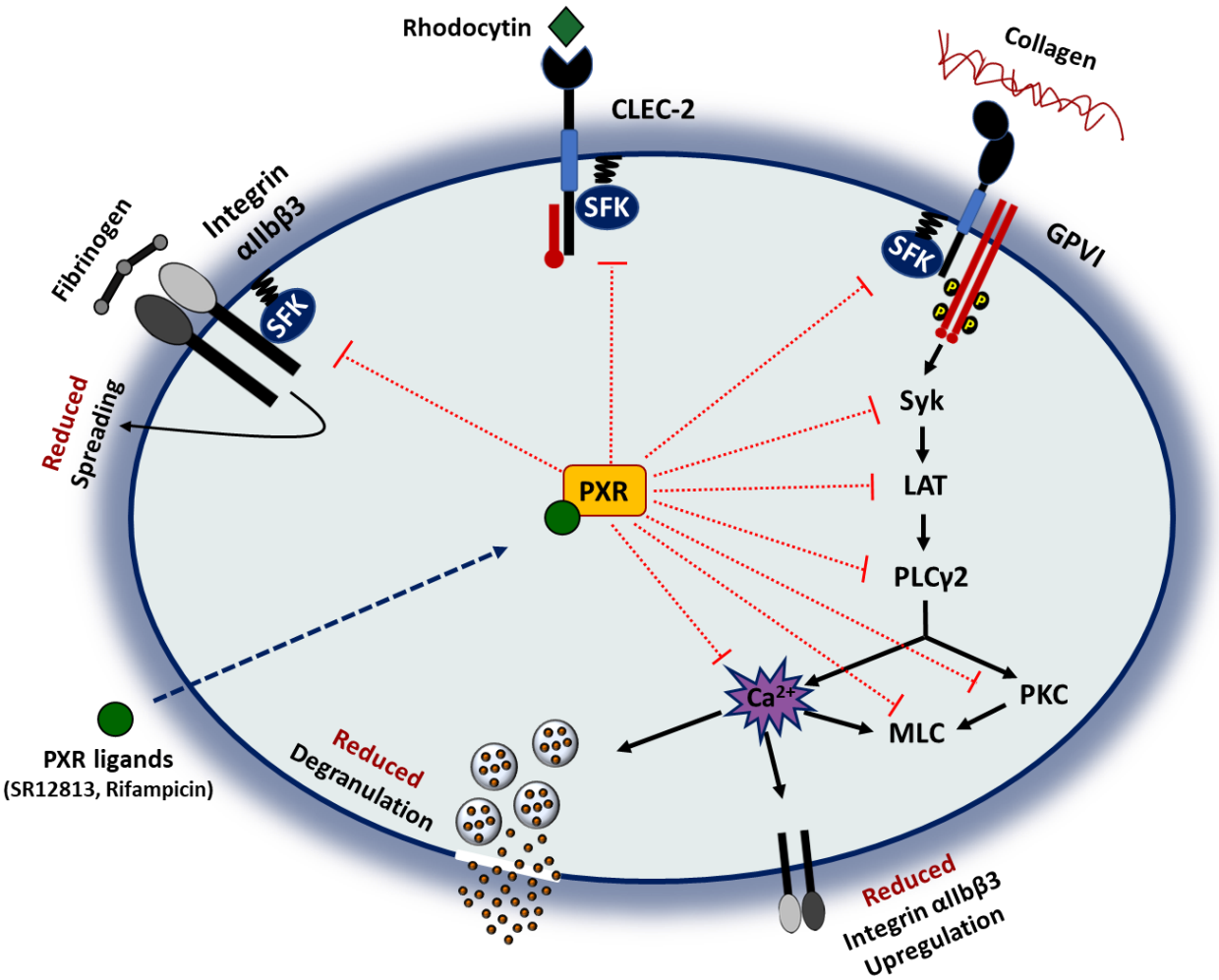


Figure 6.1: Schematic representation of the effects of PXR ligands on platelet signalling. Treatment of platelets with PXR ligands inhibit both early and late stages of GPVI signalling pathway. Exposure of platelets to PXR ligands reduced phosphorylation of Syk (at Y525/526), LAT (at Y200) and PLCγ$_2$ (at Y1217). PXR ligands also decreased CRP-XL-mediated rise in intracellular calcium levels, which was followed by a decrease in the phosphorylation of PKC and MLC (S19). Treatment with PXR ligands also inhibited phosphorylation of SFKs downstream of GPVI (Src at Y418 and Lyn at Y396), CLEC-2 (Src at Y418) and integrin αIIbβ3 (Src at Y418) receptors, which indicates SFKs to be general targets of PXR ligands. The inhibition of the activity of these signalling proteins was associated with reduced affinity upregulation of integrin αIIbβ3, aggregation, degranulation and platelet spreading. The underlying mechanisms through which PXR mediate inhibition of these signalling components are not yet clear.

(Abbreviations- SFK: Src family kinases, Syk: Spleen tyrosine kinases, LAT: Linker for activated T cells, PLCγ2: Phospholipase Cγ2, PKC: protein kinase C, MLC: Myosin light chain, Ca^{2+}: Calcium, CLEC-2: C-type lectin-like receptor 2, GPVI: Glycoprotein VI)

6.3. Non-genomic regulation of platelet activation by RXR ligands

RXR in its genomic role is known to form a heterodimer with numerous non-steroid NRs such as LXR, FXR, PPARs and PXR. The interaction of these non-steroid NRs with RXR is vital to facilitate their binding to DNA and subsequent transcription of their target genes (Evans and Mangelsdorf, 2014). Additionally, heterodimers can also regulate a wide range of signalling pathways associated with RXR and its binding partner by their ability to initiate signalling in response to stimulation by ligands of both, RXR or the other binding partner forming the dimer (Li et al., 2004). As a consequence of these interactions, RXR is associated with the regulation of a range of physiological processes including the regulation of glucose, triglyceride, cholesterol, and bile acid homeostasis. RXR ligands have been proposed to exhibit athero-protective effects in mouse models of atherosclerosis (Claudel et al., 2001).

Moraes et al. (2007) reported the expression of RXR in platelets and treatment with 9-*cis*-RA resulted in an interaction between RXR and Gq, causing a reduction in U46619 or ADP-mediated platelet aggregation. In the present study, a novel mechanism was proposed, where RXR ligands elicit anti-platelet effects through the activation of PKA via cAMP and NFκβ upregulation (Figure 6.2) (Unsworth, Flora et al., 2017). Treatment of platelets with RXR agonists, 9-*cis*-RA or methoprene acid, inhibited a range platelet activation processes such as integrin αIIbβ3 activation, platelet aggregation, degranulation, calcium mobilisation that were stimulated by both GPVI receptor agonists (collagen and CRP-XL) or GPCR receptor agonist (thrombin). This was found to be associated with a substantial reduction in thrombus development *in vitro* and *in vivo*

(Unsworth, Flora et al., 2017). Therefore, considering the role of platelets towards the initiation of atherosclerosis, the previously described anti-atherosclerotic effects of RXR ligands might be partly due to their antiplatelet effects. Additionally, 9-*cis*-RA treatment also prolonged the tail-bleeding time in mice, which is consistent with the observations of reduced blood clotting in patients taking Alitretinoin (9-*cis*-RA) for the treatment of Kaposi sarcoma and eczema (Ghasri and Scheinfeld, 2010; Walmsley et al., 1999).

In the present study, RXR was also found to form heterodimers in platelets with LXR, PPARα, PPARγ and PXR. While unexplored, it can be speculated that these interactions may lead to potential cross-talk between the non-genomic mechanisms of action of these NRs in platelets, leading to the transformation of a simple linear signalling pathway to a complex network. Treatment with multiple NR ligands may indulge in the stimulation of different signalling pathways in platelets (Boonyaratanakornkit and Edwards, 2007), facilitating platelet inhibition in an additive, cooperative or synergistic manner (Rőszer et al., 2013). For instance, patients co-administered with rifampicin (PXR activator) and alitretinoin (RXR activator) may cause a stronger inhibition of platelet activation by simultaneous downregulation of GPVI signalling and upregulation in PKA activity respectively. Similarly, treatment with RXR and FXR ligands may exhibit stronger effects via co-activation of PKA and PKG respectively. Therefore, combinatorial stimulation of NRs might modulate each other's inhibitory mechanisms and generate responses that are more pronounced than individual responses.

Although these results look promising with respect to the antiplatelet actions of RXR ligands, potential bleeding risk and the specificity of RXR ligands

towards RXR in platelets are important considerations that need further evaluation before developing RXR ligands into potential anti-thrombotic agents. Experiments employing mice that lack a functional RXR gene is the ideal way to confirm this. RXR-deficient mice, however, are not viable, which makes their usage complicated. For instance, systemic loss of the RXRα (expressed in platelets) gene is embryonically lethal due to defective cardiac ventricles and ocular abnormalities (Pinaire and Reifel-Miller, 2007). Approximately 50% of RXRβ (expressed in platelets) null mutant mice die before or at birth (Meissburger and Wolfrum, 2008).

Treatment with RXR ligands in the presence of their antagonists such as HX531 and PA452 (Kanayasu-Toyoda et al., 2005; Takahashi et al., 2002) could be an alternative strategy to evaluate the specificity of RXR ligands in platelets. Most of the NR antagonists (including RXR), however, are defined based on their ability to regulate genomic functions of the NRs by preventing their binding to the DNA, which antagonises transcription processes. However, platelets lack genomic DNA and exhibit minimal protein translation, therefore, in the absence of nuclei, the actions of NRs in platelets are chiefly non-genomic in nature. Consequently, non-genomic effects may not respond to antagonists in the same way as genomic effects. Moreover, the mechanisms of action of antagonists in a non-genomic context are unclear. This limits the use of NR antagonists in studies targeting non-genomic effects of NR ligands. For instance, in the present study, it was reported that HX531, which is an RXR antagonist (genomically), functions as an RXR agonist in platelets. Treatment with HX531 inhibited platelet aggregation stimulated by collagen, thrombin or U46619 in a manner similar to 9-*cis*-RA and methoprene

acid (Unsworth, Flora et al., 2017). HX531 shares the same ligand binding site on RXR as 9-*cis*-RA and methoprene acid, and under genomic regulation, HX531 induces a conformational change in the receptor and blocks its DNA-binding ability (Unsworth et al., 2017c). However, HX531-induced conformational change of RXR, preventing its DNA binding should not affect its non-genomic effects, in the absence of DNA. Therefore, the antiplatelet effects of HX531 may be due to other conformational changes that occur after ligand interaction (similar to RXR ligands).

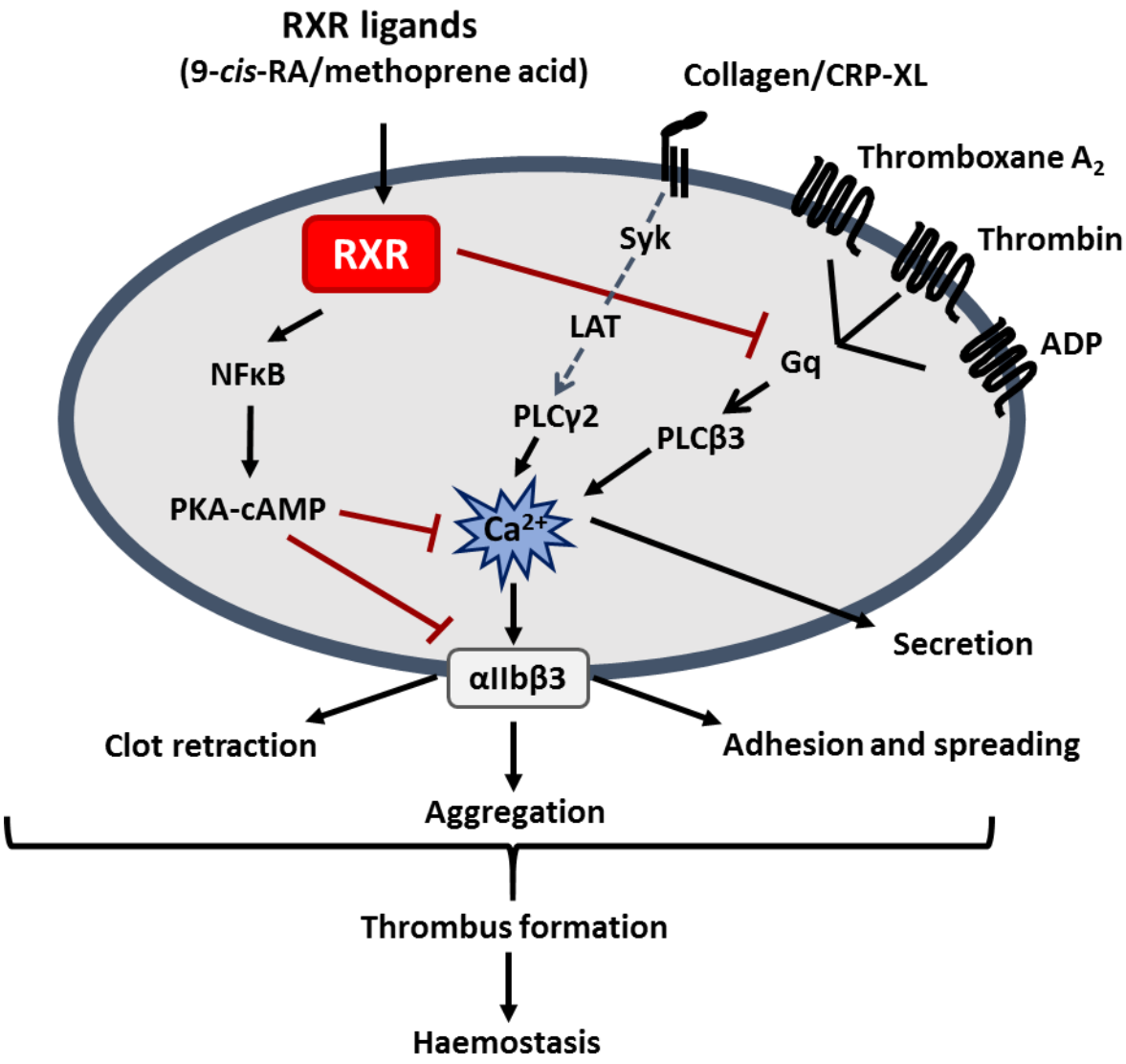


Figure 6.2: Schematic representation of the effects of RXR ligands on platelet signalling. RXR ligands, 9-cis-RA or methoprene acid inhibit platelet activation stimulated by a range of platelet agonists that include GPCR agonists (ADP, U46619 or thrombin) and GPVI agonists (collagen or CRP-XL). Interaction of RXR with Gq and subsequent negative regulation of Rac activation is one of the probable explanations for the reduction in GPCR mediated platelet activation. These ligands have also been shown to upregulate PKA activity in a cAMP and NFκβ dependent manner providing a more generalised mechanism of inhibition. Despite the inhibition of collagen/CRP-XL mediated platelet activation by RXR ligands, there was no effect on GPVI mediated signalling (Figure courtesy of Dr A. Unsworth)

6.4. Could NRs offer anti-platelet therapeutic targets?

Platelets are known to contribute directly towards the development of cardiovascular diseases (CVDs) such as thrombosis and atherosclerosis (Lin et al., 2009), whereas, they exhibit hyperactivity in cases of hyperlipidaemia (Wang and Tall, 2016), diabetes mellitus (Schneider, 2009) or hypertension (El Haouari and Rosado, 2009). This makes platelets therapeutic targets for the treatment of CVDs, particularly atherothrombosis (Badimon et al., 2012). Significant advances have been made towards the development of effective anti-thrombotic therapeutics, however, they are still known to pose bleeding risks and their efficacy becomes substantially reduced in patients suffering from pathological conditions such as hypertension and diabetes (Nathan et al., 2017). Therefore, development of effective therapeutics that ensure a balance between the prevention and treatment of thrombosis and related complications is needed.

Non-genomic effects of NR ligands (both natural and synthetic) have been reported to modulate platelet function through different mechanisms, several of which appear to be shared by different NR family members. NRs are known to act as therapeutic targets of approximately 13% of FDA approved drugs for the treatment of several pathological conditions including the CVDs (Table 6.1) (Overington et al., 2006). Therefore, drugs targeting NRs are likely to have effects on platelet activity as well and should not be ignored. However, development of NRs as anti-platelet therapeutic targets requires a few important considerations. Firstly, studies so far have only considered the acute effects of NR ligands on platelet functions. Therefore, it would be important to evaluate the consequences of chronic exposure of NR ligands on platelet activity, prior to the development of

these ligands as potential anti-platelet agents. Secondly, the possibility of genomic effects occurring in platelets cannot be denied completely, given the existence of mRNA in platelets and their limited ability to perform translation (Zimmerman and Weyrich, 2008; Rowley et al., 2012). The ability of NRs to interact with mRNA and regulate the genomic activity of a cell has been reported (Ottaviani et al., 2014; Xu and Koenig, 2004). Therefore, it is important to distinguish genomic and non-genomic effects in platelets. There are few features that can make this distinction: (i) The difference in time frames to mediate genomic effects (hours) and non-genomic effects (minutes) is the first parameter that can differentiate between these two regulatory mechanisms (Harvey et al., 2001). (ii) Non-genomic effects, unlike genomic regulation, are non-responsive to the inhibitors of transcription or translation (Losel and Wehling, 2003). Therefore, future studies evaluating the role of NRs in platelets should consider including these inhibitors that may help to differentiate between genomic and truly non-genomic actions of these receptors. Schwertz *et al.* (2017) recently described such a mechanism demonstrating RARα-dependent translational control in human platelets, which resulted in the synthesis of several transcripts (Schwertz et al., 2017). Whether other NRs (such as RXR, PXR, FXR, LXR or PPARs), identified in platelets, can also replicate such a mechanism is still unknown. Finally, it is important to note that the existence of a substantial level of structural similarity between different NRs makes them potentially promiscuous (Ng et al., 2014; Kwon et al., 2014; Noy, 2007; Sepe et al., 2016; Krasowski et al., 2011). Numerous studies examining the genomic functions have reported this feature of NRs, for example; 15d-PGJ2 is an endogenous PPARγ ligand, which can also act as an FXR antagonist (Xu et al., 2013), whereas, phytanic acid can activate both PPARα and RXR (Hellgren, 2010).

Hyperforin is a PXR agonist but functions as an FXR antagonist (Jonker et al., 2012). Similarly, LG100754 is a highly specific RXR:PPARγ agonist while it acts as an antagonist of RXR homodimers (Cesario et al., 2001). This makes the selective targeting of the NRs challenging and as such identification of ligands that function in a receptor- and gene-specific manner is important.

Table 6.1. Commercially available nuclear receptor drugs (Flora et al., 2018)

Nuclear Receptor	Disease	Drug generic name (Marketed Drug)
GR	Metabolic and immunological disorders	Dexamethasone (Dexasone), Prednisolone (Orapred) (Sundahl et al., 2015; Kadmiel and Cidlowski, 2013)
ER	Breast cancer, obesity	Tamoxifen (Nolvadex), Raloxifene (Evista) (Muchmore, 2000; Y Maximov et al., 2013)
PPARα	Dyslipidemia, atherosclerosis	Fenofibrate (Tricor) (Filippatos and Milionis, 2008)
PPARγ	Diabetes, obesity	Pioglitazone (Actos), Rosiglitazone (Avandia) (Kersten et al., 2000; Ahmadian et al., 2013)
RAR	Leukaemia, acne	13-cis-retinoic acid (Isotretinoin) (Layton, 2009)
RXR	Leukaemia, Kaposi sarcoma, eczema	9-cis-retinoic acid (Alitretinoin), Bexarotene (Targretin) (Ghasri and Scheinfeld, 2010; Njar, 2008; Walmsley et al., 1999)
VDR	Osteoporosis, calcium homeostasis	Calcitriol (Calcijex), Paricalcitol (Zemplar) (Wu-Wong, 2009; Makishima and Yamada, 2005)

6.5. Future Work

The work presented here provides substantial insights into the role of PXR and RXR in platelets and begins to address underlying mechanisms of action. However, several questions need to be investigated in platelets prior to the development of RXR and PXR ligands as potential anti-thrombotic agents:

1. Specificity of PXR and RXR ligands

Given the promiscuous nature of NR ligands, evaluating their specificity is of vital importance. This is because of the cross-reactivity of ligands between different members of the NR family may lead to undesired side-effects. Development of NR knockout (KO) mice is challenging due to their involvement in the regulation of a range of genes, some of which controls embryogenesis, cellular differentiation and metabolism, the deletion of which can be embryonically lethal (Gray et al., 2005; Sucov et al., 1994). Based on these reasons, mice lacking RXR gene are not viable (Meissburger and Wolfrum, 2008; Pinaire and Reifel-Miller, 2007) and thus the development of platelet-specific conditional compound KOs devoid of both RXRα and RXRβ isoforms (expressed in platelets) would be ideal to test the specificity of RXR ligands towards RXR. In the absence of nucleus in platelets, a genomic deletion of RXR in platelets must be accompanied by its deletion in the parent cell, megakaryocyte. This might add another challenge as the expression of NRs might have crucial roles to perform in megakaryocytes. For instance, PPARγ in megakaryocytes is associated with platelet production (Sahler et al., 2012). Our lab recently identified an impaired platelet-production in megakaryocyte/platelet-specific conditional PPARγ KO mice (unpublished

finding). Given the ability of RXR to interact with PPARγ, deletion of RXRα/β isoforms may also alter platelet production and need to be investigated.

The species-specific effects of PXR ligands indirectly explain their specificity in platelets. However, the specificity of PXR ligands, in addition to this can also be tested using PXR KO mice that are commercially available (Taconic Biosciences). Evaluating this was beyond the scope of this study due to financial licencing and time constraints.

2. Chronic effects of PXR and RXR ligands

The studies reported so far have addressed the acute effects of NR ligands. However, physiologically NRs are exposed to drug/ligands targeting NRs for longer durations. Consequently, it is important to understand the influence of chronic exposure of NR ligands on platelets. For example, under chronic treatment, metabolism may alter the effective therapeutic concentrations of PXR and RXR ligands, which may affect their efficacy on platelets. Under chronic exposure, the effective therapeutic concentration of NR ligands might be different than evaluated under acute exposure. For instance, in this study 10 or 20 μM of PXR ligands were found to be largely ineffective in regulating platelet activation. However, prolonged exposure to these concentrations *in vivo* may exhibit better efficacy in comparison to their acute exposure. Apart from this, studying chronic exposure would also enable us to determine potential side-effects of NR ligands on other cell types apart from platelets. Additionally, a chronic study *in vivo* would also take into account the effects of NR ligands (at pharmacologically relevant

concentrations) on megakaryocytes and how they regulate platelet production in the presence of NR ligands.

3. Distribution of PXR and RXR in platelets

A redistribution and release of RXR (Ray et al., 2008; Unsworth et al., 2017c) and PXR from platelets have been proposed following their activation. It is speculated that such a rearrangement of NRs might be due to their role in the regulation of platelet signalling (discussed in section 6.2) and their release might lead to their delivery in other cell types, plausibly in the form of microparticles (Ray et al., 2008). In order to understand the relevance of this observation, it would be helpful to devise the exact location of NRs in both resting and activated platelets using electron microscopy with immunogold labelling or super-resolution microscopy such as Stochastic Optical Reconstruction Microscopy (STORM).

Immunogold labelling is a useful technique that can probe into the ultrastructure of a cell and can prove to be effective in the identification of the precise location of RXR and PXR in platelets (Murtey, 2016). Conventional fluorescence microscopy due to a relatively low spatial resolution limits the visualisation of small cells such as platelets (2-4 μm) (Huang et al., 2009). STORM super-resolution microscopy breaks this resolution limit and allows the visualisation of cellular structures, individual proteins and entire organelles at a nanometer scale (Rust et al., 2006), which would again be beneficial to study the distribution of NRs in platelets.

4. Consequences of potential cross-talk between NRs

Given the prospects of the formation of RXR heterodimers in platelets, there is a possibility of potential cross-talk between the mechanisms of actions of NRs in platelets. Combinatorial studies of NR ligands in platelets targeting different NRs might be helpful in dissecting such effects and determining how exposure to different NR ligands can influence multiple signalling pathways in platelets and consequently regulate their activation. Testing several combinations of low medium and high concentrations of different NR ligands through high-throughput 96-well platelet-based aggregations or flow cytometry analysis (fibrinogen binding or P-selectin exposure) may enable the identification of their plausible additive, cooperative and synergistic effects in modulating platelet activation. Such an investigation would also determine the likely effects of NR drugs/ligands in patients taking multiple drugs targeting different NRs.

5. Additional mechanistic insights

PXR ligands have been shown to inhibit the phosphorylation of several components of the GPVI-mediated signalling in platelets. However, the mechanisms that mediate this inhibition are not entirely clear, although they are likely to act at the level of SFKs. Given the previous studies that indicate a direct interaction of NRs with GPVI signalling molecules to facilitate platelet inhibition (Moraes et al., 2010b; Spyridon et al., 2011), a similar investigation on PXR using mass spectrometry could be utilised to gain additional insights into the mechanisms of action of PXR ligands.

The conventional co-immunoprecipitation assays are effective to investigate protein-protein interaction, but they may fail to detect low-affinity and instantaneous protein-protein interactions, which is highly plausible in the case of platelet-signalling, which is characterised by rapid and dramatic responses upon agonist-stimulation. Moreover, this assay requires the use of antibodies with high specificity and avidity along with the prior prediction of the target interactive protein (Miernyk and Thelen, 2008; Free et al., 2009). Considering this, analysis of protein-protein interactions using mass spectrometry may be possible, because by targeting a known member of the complex, the entire protein complex can be isolated and then subjected to the identification of protein composition with high sensitivity, in comparison to co-immunoprecipitation (Figeys et al., 2001; Han et al., 2008).

6.6. Major outcomes of the study

This study identifies the presence of a novel nuclear receptor, PXR, in platelets and the ability of its ligands to negatively regulate platelet functions and thrombus formation in a non-genomic manner. These ligands were also found to exhibit their effects in a species-specific manner in human and mouse platelets, a feature observed in other cell types. Regulation in the phosphorylation levels of SFKs, downstream of GPVI, CLEC-2 and integrin αIIbβ3 receptor was identified as a general mechanism by which PXR ligands inhibit platelet activation, with GPVI signalling being the major pathway affected. The potential mode of action contributing towards the inhibition of signalling is unclear. However, it is plausible

that PXR interacts directly with GPVI signalling molecules such as SFKs and thus facilitate their inhibition.

This study also identifies that RXR ligands besides their previously reported ability to inhibit platelet activation stimulated by U46619 and ADP, also attenuate collagen/CRP-XL and thrombin-mediated platelet activation. Thrombus formation *in vivo* was inhibited by RXR ligands and RXR was also found to form heterodimers with PXR, LXR, PPARα and PPARγ. RXR ligand-mediated inhibition of platelet activation was found to be an outcome of the upregulation of PKA activity in a cAMP- and NFκB-dependent manner (Unsworth, Flora et al., 2017).

In conclusion, the work presented here identifies the ability of PXR and RXR ligands to regulate platelet function and provides novel mechanistic insights that facilitate these effects. Previously proposed anti-atherosclerotic effects of PXR and RXR ligands along with their newly discovered anti-thrombotic effects suggest that PXR and RXR may represent potential candidates for the development of cardioprotective drugs.

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