Regulation of platelet function: molecular pathways and targets

By

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CERTIFICATE OF APPROVAL

This is to certify that the PhD Dissertation of

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"Regulation of platelet function: molecular pathways and targets"

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AC	adenylyl cyclase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BAG	Bcl-2-associated athanogene
BCR-ABL	breakpoint cluster region-abelson
BSS	Bernard-Soulier syndrome
Btk	Bruton's tyrosine kinase
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CLEC-2	C-type lectin receptor-2
CLL	chronic lymphocytic leukemia
COX	cyclooxygenase
CRP	collagen-related peptide
CXCL	CXC motif ligand
DAG	1,2-diacylglycerol
DAMPs	damage-associated molecular patterns
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ER	endoplasmic reticulum
ERp57	endoplasmic reticulum resident protein 57
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FcR	Fc receptor

FII	factor II (prothrombin)
FIIa	factor IIa (thrombin)
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GPCR	G-protein coupled receptor
GPIb	glycoprotein Ib
GPIX	glycoprotein IX
GPV	glycoprotein V
GPVI	glycoprotein VI
Grp	glucose-regulated protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
Hsc	heat shock cognate protein
Hsp	heat shock protein
ICAM-1	intercellular adhesion molecule 1 (CD54)
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IL	interleukin
ILK	integrin-linked kinase
IP3	inositol 1,4,5-trisphosphosphate
ITAM	immunoreceptor tyrosine-based activation motif
LAT	linker for activation of T cells
LRR	leucine-rich repeat
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase

MCL	mantle cell lymphoma
MMP	matrix metalloproteinase
NE	neutrophil elastase
NEF	nucleotide exchange factor
NETs	neutrophil extracellular traps
PAMPs	pathogen-associated molecular patterns
PAR	protease-activated receptor
PBMCs	peripheral blood mononuclear cells
PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase
PDMS	polydimethylsiloxane
PECAM	platelet endothelial cell adhesion molecule (CD31)
PF4	platelet factor 4
PGI_2	prostacyclin
PI3K	phospoinositide 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMNs	polymorphonuclear leukocytes
PolyP	polyphosphate
PPACK	D-phenylalanyl-prolyl-arginyl chloromethyl ketone
PRD	proline-rich domain
PRP	platelet-rich plasma
PS	phosphatydylserine

PSGL-1	P-selectin glycoprotein ligand-1
RAGE	receptor for advanced glycation endproducts
RIAM	Rap1-GTP-interacting adapter molecule (RIAM)
SFKs	Src family kinases
SH2	Src homology 2
SH3	Src homology 3
SLP-76	SH2-containing leukocyte protein of 76 kDa
TF	tissue factor
TGF β	transforming growth factor beta
TLR	toll-like receptor
TRAP-6	thrombin receptor-activating peptide-6
TxA_2	thromboxane A_2
VCAM-1	vascular cell adhesion protein 1
vWF	von Willebrand factor
XLA	X-linked agammaglobulinemia

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To my mentor and PhD advisor, Dr. Owen J. T. McCarty, I cannot possibly express enough gratitude. From the beginning, Owen saw the scientific potential in me and nurtured that potential. He encouraged me and motivated me, using analogies about landing on the beach in Normandy, as needed. He always had a knack for pushing me when I needed motivation and stepping back when I needed space. Owen wrangled my writing into a more scientific style, particularly in regards to writing the most detailed results sections ever. Most importantly, I will always be grateful to Owen for showing me how to be an international traveler and opening my horizons through the Whitaker Fellowship. I look forward to our continued meetings around the world and to devising ever greater plans for the success of the McCarty lab.

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Abstract

Regulation of platelet function: molecular pathways and targets

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Platelets are the first defenders of vascular integrity, responding to vascular injury in a regulated sequence of adhesion, activation, and aggregation that enables formation of a hemostatic plug. Signaling pathways within the platelet are tightly regulated to orchestrate platelet response to injury while dampening platelet activity in quiescent conditions. Dysregulation of platelet function, however, may play a central role in thrombosis and inflammatory disease. The present research defines signaling pathways that activate platelets, with an emphasis on receptors and effectors that are potential targets for novel platelet therapeutics.

In our first study, we examine the role of the tyrosine kinase Btk in platelet function, demonstrating that Btk antagonists similar to the leukemia drug ibrutinib have broad inhibitory effects on platelet

function. Btk inhibitors impaired phosphorylation of Btk but also the tyrosine kinase Lyn and targets downstream of glycoprotein (GP)VI receptor activation, demonstrating off-target effects of the inhibitors. Functional studies demonstrated Btk inhibition dampens platelet activation, spreading, and aggregation. A short-term oral dosing study showed that Btk inhibitors abrogated platelet aggregation with no effect on plasma clotting times or bleeding times. Overall, this study demonstrates Btk inhibitors analogous to imatinib impair platelet function. This is a timely study in light of reports of bleeding side effects seen in patients taking imatinib, and these results provide rationale for the development of second-generation Btk inhibitors, which is now underway.

Next, we demonstrate a novel role for the molecular chaperone heat shock protein 70 (Hsp70) in coordinating assembly of signaling complexes required for platelet function. While other molecular chaperones are known to coordinate platelet integrin activation required for aggregation, it was not previously known whether Hsp70 also played a role in orchestrating platelet activation. This study showed that targeted inhibition of Hsp70 impaired platelet signaling downstream of GPVI receptor activation. Biochemical investigations determined that Hsp70 associates with specific proteins in the LAT signalosome and that inhibition of Hsp70 impaired these associations, preventing platelet activation. These results demonstrate for the first time that Hsp70 plays an important function in coordinating assembly of a signaling complex required for platelet activation, which is of interest for the development of Hsp70 antagonists for cancer and other diseases.

Finally, we describe a role for the platelet protease-activated receptor 4 (PAR4) in promoting platelet granule release and platelet-leukocyte interactions initiated by the blood proteases thrombin, cathepsin G, or plasmin. The platelet PAR4 receptor is an emerging target in thrombosis, with PAR4 antagonists being tested in clinical studies, but less is known about the role of platelet PAR4 in inflammation. Therefore, we performed functional studies to interrogate the role of PAR4 activity in

platelet dense granule release and platelet-leukocyte interactions. PAR4 inhibitors impaired platelet dense granule release by the blood proteases thrombin, cathepsin G, and plasmin and also impaired platelet-leukocyte interactions in a purified system or in whole blood. These results suggest that PAR4 may be a useful target for diseases involving inflammation in which platelet-leukocyte interactions are present.

Overall, these studies define new roles for signaling molecules and receptors in platelet function and provide insights into the development of antiplatelet therapies for thrombosis and inflammatory disease. Future studies will investigate how pharmacological inhibition of platelet function impacts receptor-mediated interactions between platelets and leukocytes and platelets and endothelial cells, to better define and predict the hematological outcomes of clinical treatments.

Chapter 1. Introduction to platelet signaling and activation

1.1 Overview

The circulatory system experiences micro-tears, cuts, and traumatic injuries on a recurring basis. These injuries require a fast, internal response to restore blood flow and prevent further damage. Platelets are the smallest cellular elements of circulating blood and play a fundamental role in the process of stopping blood loss, known as hemostasis [1, 2]. Platelets are anuclear, discoid fragments of bone marrow megakaryocytes that circulate at 150,000-450,000 per microliter of blood, with a lifespan of 7-10 days. Platelets patrol the vessel wall or endothelium, which under normal conditions secretes compounds such as prostacyclin (PGI_2) and nitric oxide to inhibit platelet activation. Upon vessel injury, platelets rapidly respond by tethering to exposed extracellular matrix proteins, releasing chemical messengers, changing shape, and recruiting additional platelets and blood cells to the site of injury.[3] While platelets are responding, the blood coagulation cascade begins in parallel, involving sequential activation of blood coagulation proteins that circulate in inactive or zymogen form. The cascade culminates in the generation of thrombin, a protease that further activates platelets and ultimately facilitates the conversion of fibrinogen into fibrin strands to bind the platelet aggregate into a tightly-packed hemostatic plug. In the following sections, we will explore fundamental aspects of platelet function in hemostasis and inflammation, examine the role of platelets in disease, and finish with an overview of this thesis.

1.2 Platelet adhesion to ECM proteins

In the occurrence of a vascular injury, a tear through the vessel wall exposes the extracellular matrix (ECM), the connective tissue surrounding the inner blood vessel layer or endothelium. Exposure of

ECM proteins to the flowing blood quickly recruits platelets to the site of injury, where platelets bind ECM proteins via specialized receptors (Figure 1.1).[4]

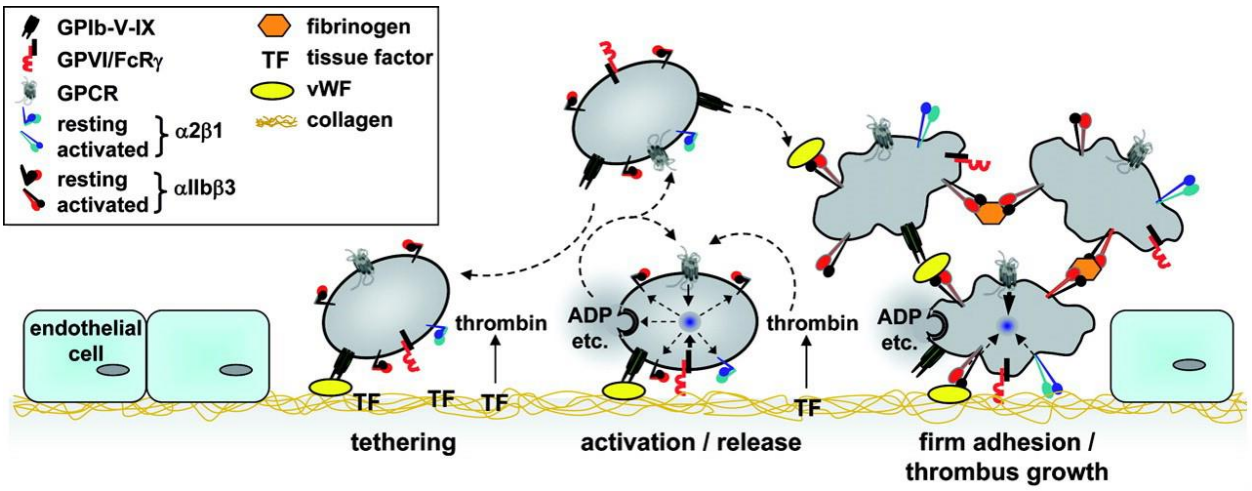

Figure 1.1. Platelet adhesion, activation, and aggregation. Opon injury to the endothelium, platelets bind exposed von Willebrand factor (vWF) via GPIb-V-IX receptors and collagen via GPVI receptors and $\alpha_2\beta_1$ integrins. Platelets become activated and release soluble agonists such as ADP, which feeds back to activate platelet G-protein coupled receptors (GPCRs). Integrin $\alpha_{IIb}\beta_3$ becomes activated and binds fibrinogen, facilitating platelet-platelet aggregation. Figure adapted from Nieswandt *et al*., *Arterioscler Thromb Vasc Biol*, 2008 Mar;28(3):403-12. Reprinted with permission.

1.2.1 *Von Willebrand factor*

One of the first and most fundamental platelet-ECM interactions is with von Willebrand factor (vWF).[5] Von Willebrand factor is a large, multimeric glycoprotein present in the ECM, in endothelial cell granules called Weibel-Palade bodies, in platelet α-granules, and in the circulation. Each mature vWF monomer is 2050 residues in length and contain numerous domains, as shown in Figure 1.2.[6] Binding sites are found on domain D' for FVIII, A1 for platelet glycoprotein Ibα and collagen type IV, A2 (cleavage site) for the degradative enzyme ADAMTS-13, A3 for collagen types I and III, and C1 for activated platelet integrin $\alpha_{IIb}\beta_3$.[7]

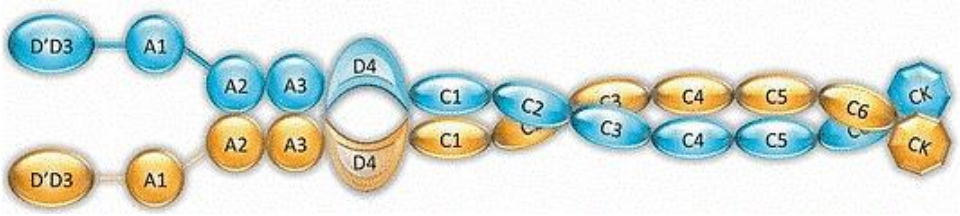

Figure 1.2. Structure of von Willebrand factor (vWF). vWF monomers assemble into dimers intertwined between the A3-D4 domains and the C-terminal CK (cysteine knot) domain, as observed via eletron microscopy. Figure adapted from Denis *et al*., *J Thromb Haemost*, 2012 Dec;10(12):2428-37. Reprinted with permission.

Following vessel damage, vWF is exposed from damaged endothelial cells and the ECM, upon which it unfolds, binds exposed ECM collagen, and polymerizes into larger vWF multimers in a shear-dependent manner.[8] Both vWF strings and vWF-collagen interactions form a platform for platelets to tether and roll. Platelets bind vWF via the platelet receptor glycoprotein (GP)Ibα, a part of the GPIb-IX-V complex, which is constitutively active to enable fast platelet binding. Importantly, platelet adhesion to vWF allows platelets to bind other ECM proteins and arrest at the site of injury. Deficiencies in vWF production or function (quantitative or qualitative defects, respectively) result in the common, autosomal dominant bleeding disorder von Willebrand disease (vWD), a heterogeneous disease of varying bleeding severity.[9]

1.2.2 *Collagen*

Collagen is a triple helical protein with at least 28 subtypes classified by their structural form, with the highly networked type IV being the predominant form exposed in superficial injuries and the fibrillar types I and III present in deeper layers of the ECM being exposed in more serious injuries.[5] Exposed collagen binds the vWF domains A1 (collagen type IV) and A3 (collagen types I and III), forming a platform for platelets to bind. Platelet binding to collagen occurs via the platelet GPVI receptor binding glycine-proline-hydroxyproline (GPO) sequences on collagen.[10] Platelet

integrin $\alpha_2\beta_1$ facilitates additional firm adhesion to collagen, acting as a secondary reinforcement of the platelet-collagen interaction and enhancing signaling through GPVI.[11]

1.2.3 *Laminin*

Laminin is a heterotrimeric glycoprotein of α, β, and γ chains that is found in the ECM. Laminins of type α4 and α5 (specifically, α4β1γ1, α5β1γ1, and α5β2γ1) have been shown to bind platelet integrin $\alpha_6\beta_1$.[12] This laminin-integrin $\alpha_6\beta_1$ interaction is a prerequisite for subsequent binding of laminin to GPVI. Laminin then activates GPVI signaling pathways similar to those of collagen.[13]

1.2.4 *Other ECM proteins*

Other platelet integrins facilitate binding to numerous ECM proteins. Specifically, integrin $\alpha_5\beta_1$ binds fibronectin and fibrillin, and integrin $\alpha_v\beta_3$ binds ECM proteins containing an arginine-glycine-asparagine (RGD) motif, which includes fibrinogen, fibronectin, fibrillin, osteopontin, vitronectin, nidogens, thrombospondin, and vWF.[5, 14] While integrin $\alpha_{IIb}\beta_3$ also binds fibrinogen, this does not occur until after binding by vWF and collagen facilitates intracellular signaling to activate the integrin. Overall, interactions between ECM proteins and platelet integrins facilitate additional platelet adhesion to the site of injury before further platelet signaling can occur.

1.3 Platelet adhesion receptor-mediated signaling

Platelet adhesion at the site of injury activates platelet receptors to initiate intracellular signaling cascades (Figure 1.3).[15] This includes the vWF receptor GPIb-IX-V, collagen receptor GPVI, podoplanin receptor CLEC-2, and fibrinogen receptor integrin $\alpha_{IIb}\beta_3$. Several of these receptors also associate with Fc receptors (FcRs), immune cell receptors that bind the unchanging (Fc) region of

immunoglobulins (Igs) and other ligands and propagate signaling through an immunoreceptor, tyrosine-based activation motif (ITAM). Together, these adhesion receptors activate signaling cascades involving protein kinases, phospholipases, and adaptor proteins that lead to platelet activation.

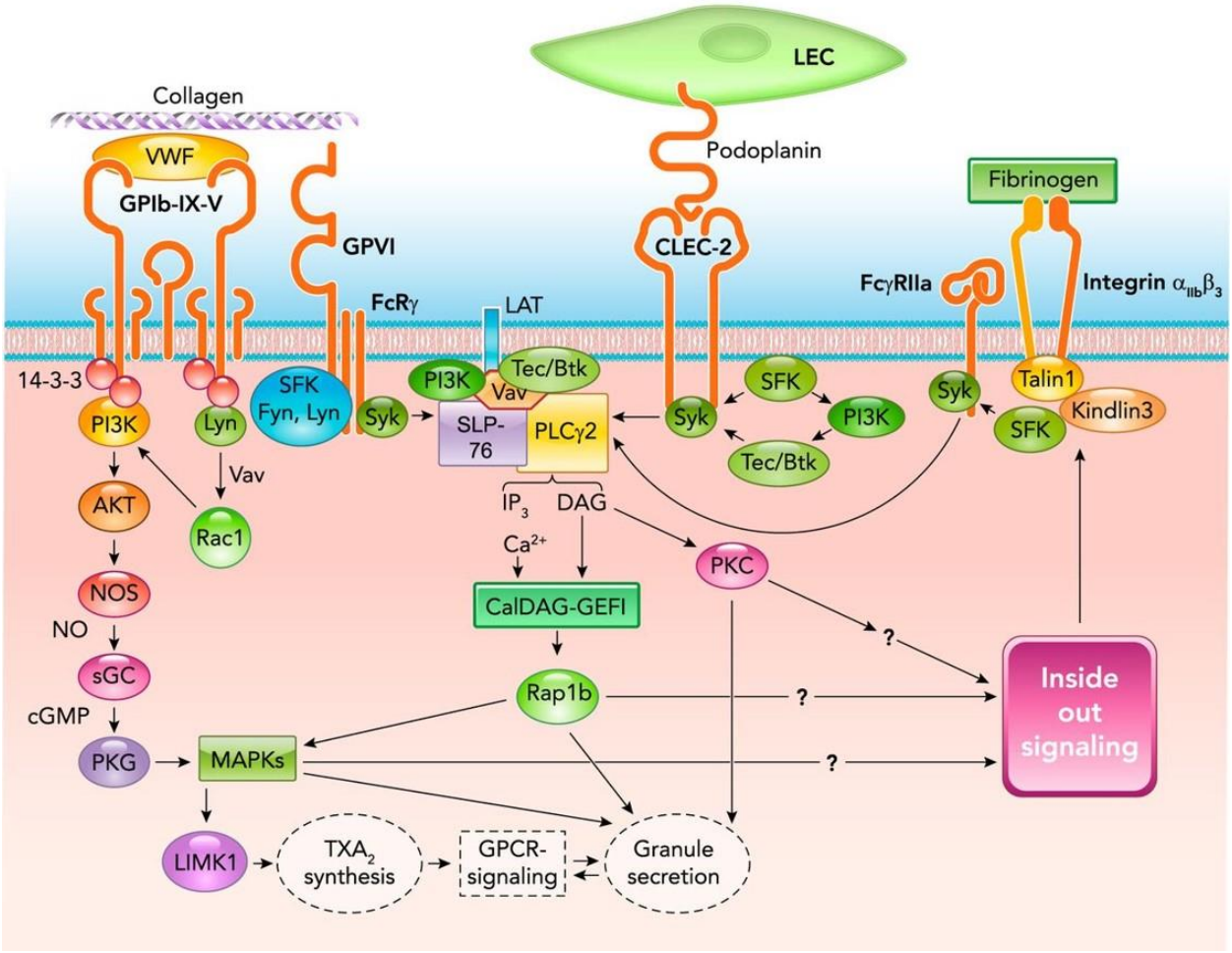

Figure 1.3. Platelet signaling. Platelet signaling occurs following binding of vWF to GPIb-IX-V, collagen to GPVI/FcRγ, podoplanin to CLEC-2, and fibrinogen to integrin $\alpha_{IIb}\beta_3$. Platelet signaling involves protein tyrosine kinases, adapter proteins, and phospholipases to result in platelet granule secretion, thromboxane (TxA_2) synthesis, and activation of integrin $\alpha_{IIb}\beta_3$. Figure adapted from Estevez B. and Du X., *Physiology (Bethesda)*, 2017 Mar;32(2):162-177. Permission is not required by the publisher for this type of use.

1.3.1 *GPIb-IX-V*

GPIb-IX-V is a leucine-rich glycoprotein complex that is exclusive to platelets and megakaryocytes, comprised of a total of 9 units: one outer GPIX unit, two GPIbβ units, one GPIbα, one central GPV, followed by another GPIbα, two GPIbβ units, and a GPIX (Figure 1.4).[16] Each unit is a transmembrane protein containing a leucine-rich repeat (LRR) domain, ectodomain, transmembrane helix, and short cytoplasmic tail.[17] The GPIbα LRR domain binds to ligands, primarily vWF but also other coagulation factors, P-selectin, integrins, and bacteria. Ligand binding initiates associations between the cytoplasmic tail and the cytoskeletal protein filamin A, the scaffolding protein 14-3-3ς, the messenger protein calmodulin, and phosphoinositide 3-kinase (PI3-kinase), initiating downstream signaling leading to platelet activation.[18] FcR γ-chain also associates with the GPIbα cytoplasmic tail and transmits signals similar to those of the GPVI FcR γ-chains, though more weakly.[19] Mutations in the structure of GPIbα, GPIbβ, and/or GPIX result in a rare autosomal recessive bleeding disorder called Bernard-Soulier syndrome (BSS), resulting in macrothrombocytopenia or abnormally large platelets combined with low platelet count.[20]

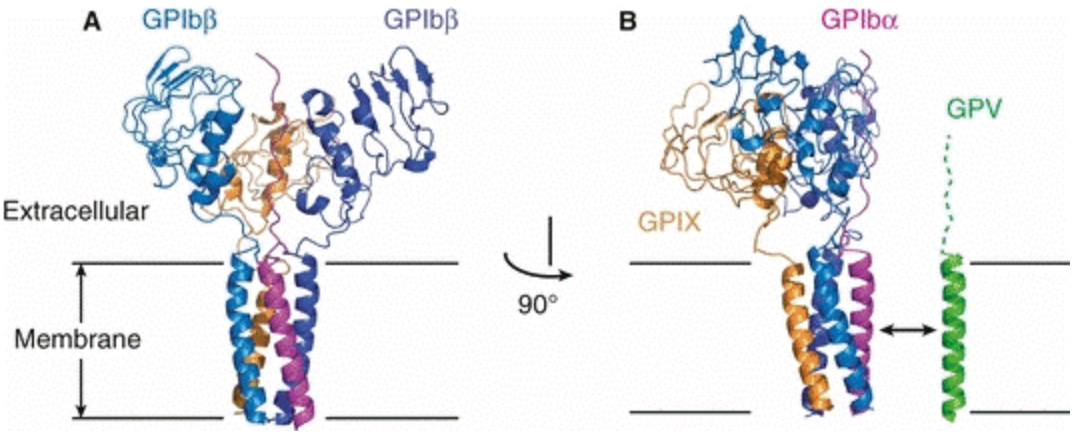

Figure 1.4. GPIb-IX-V receptor structure. Ribbon diagram showing the GPIb-IX-V receptor complex at 90° rotated angles (A and B), comprising one subunit of GPIX, two GPIbβ dimers, one GPIbα, and a central GPV. Another GPIbα, GPIbβ dimer, and GPIX are clustered on the opposite side of GPV (not shown), for a total of nine subunits. Figure adapted from Mo *et al*., *J Thromb Haemost*. 2012 Sep;10(9):1875-86. Reprinted with permission.

1.3.2 *GPVI*

Closely related to the Fcα receptor found on leukocytes, the platelet GPVI receptor is 339 amino acids long with two extracellular immunoglobulin (Ig) domains that bind collagen or laminin, followed by a mucin-like stalk, a transmembrane domain, and a cytosolic tail (Figure 1.5).[21, 22] Each GPVI monomer associates with a homodimer of FcR γ-chains via a salt bridge with an arginine (R) in the membrane and additional covalent interactions with the first 6 juxtamembrane amino acids in the cytosolic tail. The cytosolic tail contains a proline-rich domain (PRD) that binds the Src homology 3 (SH3) domain of Src-family kinases Lyn and Fyn, which remain constitutively bound to enable fast activation.[10] The FcR γ-chains each contain an ITAM with two YxxL (tyrosine _ _ leucine) sequences required for signal transduction.

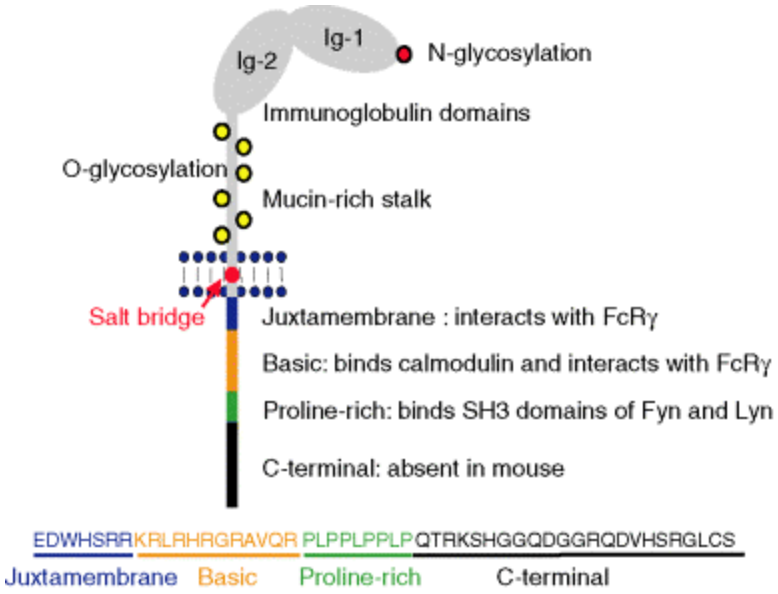

Figure 1.5. GPVI and FcR γ-chain structure. The GPVI receptor contains two extracellular Ig domains for ligand binding followed by a mucin-like stalk containing o-glycosylation sites. GPVI is coupled to a dimer of FcR γ-chains (not shown) through a salt bridge at an arginine residue in the membrane as well as additional interactions with the juxtamembrane and basic regions of the GPVI tail. The proline-rich region on the tail contains binding sites for the SH3 domains of Fyn and Lyn, which phosphorylate the FcR γ-chain ITAMs to initiate downstream signaling. Figure adapted from Watson *et al*., *J Thromb Haemost*, 2005 Aug;3(8):1752-62. Reprinted with permission.

Collagen binding to GPVI induces cross-linking of multiple GPVI receptors, which brings Lyn and Fyn into closer association with the FcR γ-chains, where they can phosphorylate the ITAM tyrosines (Figure 1.6).[19] Once phosphorylated, the ITAMs allow docking of the tyrosine kinase Syk. This results in assembly of a signaling complex (signalosome) containing PI3-kinase, the Rho guanine nucleotide exchange factors (RhoGEFs) Vav1/2/3, Tec and Btk kinases, SH2-containing leukocyte protein of 76 kDa (SLP-76), phospholipase Cγ2 (PLCγ2), and others. PLCγ2 is an important effector protein that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) in the platelet membrane into the second messengers inositol 1,4,5-trisphosphosphate (IP3) and 1,2-diacylglycerol (DAG).[23] IP3 facilitates release of calcium stores, and DAG activates protein kinase C (PKC), while calcium and DAG together activate the GTPase Rap1.[24] PKC facilitates platelet granule release, while Rap1

promotes "inside-out" activation of integrin $\alpha_{IIb}\beta_3$ to an open conformation that allows fibrinogen binding and platelet-platelet adhesion.

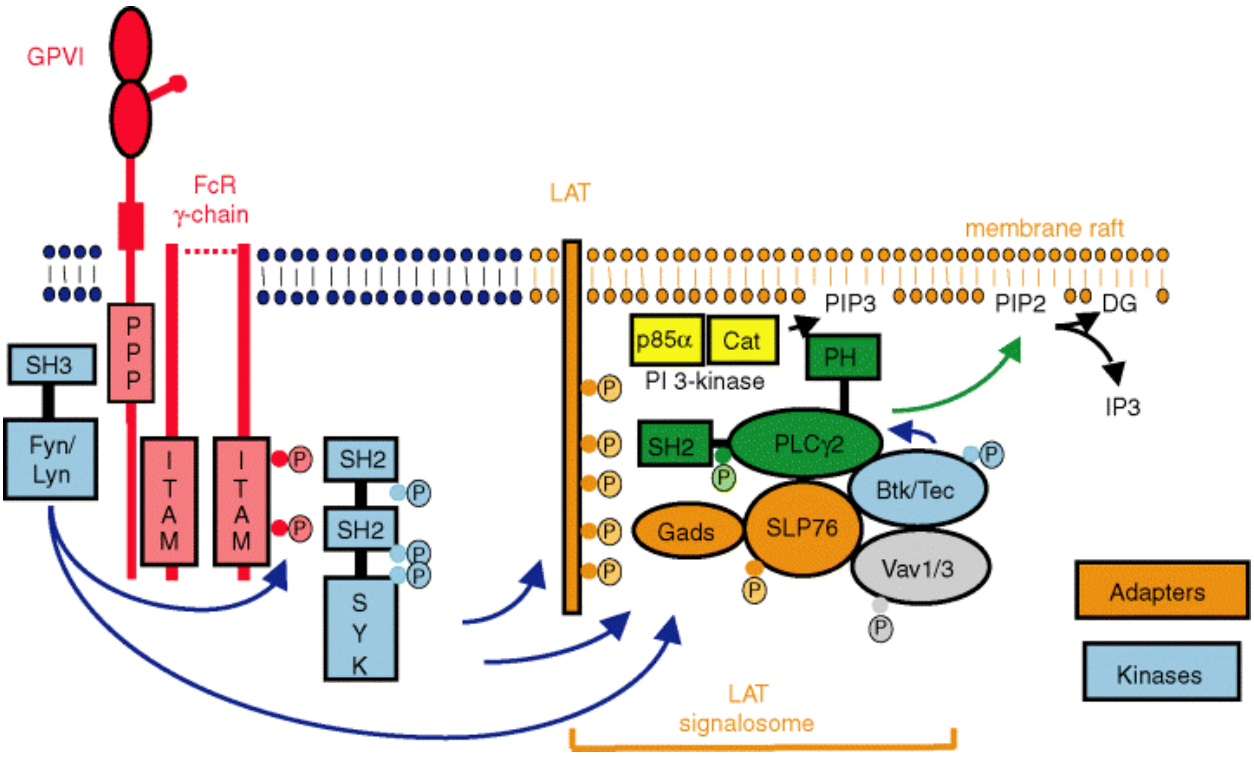

Figure 1.6. GPVI signaling cascade. Following ligand binding and GPVI receptor crosslinking, the associated FcR γ-chain dimers are phosphorylated by Fyn/Lin kinases, leading to phosphorylation of Syk, which generates the LAT signolosome, resulting in activation of PLCγ2 and hydrolysis of PIP2. Figure adapted from Watson *et al*., *J Thromb Haemost*, 2005 Aug;3(8):1752-62. Reprinted with permission.

1.3.3 *CLEC-2*

Platelets also highly express C-type lectin receptor-2 (CLEC-2), which activates platelets upon binding the glycoprotein podoplanin expressed on lymphatic endothelial cells and subendothelial pericytes.[25] CLEC-2 expresses a single YxxL motif known as a hemi-ITAM in its cytoplasmic domain and is able to dimerize upon ligand binding to initiate a signaling cascade similar to that of GVPI.[26] However, unlike GPVI, the CLEC-2 ITAM is phosphorylated by both Src and Syk kinases, and CLEC-2 is more dependent on ADP and TxA_2 signaling and cytoskeletal

rearrangements, which occur later in platelet activation. This suggests CLEC-2 is less critical to hemostasis than GPVI, although CLEC-2 has recently been found to contribute to lymphatics management and thrombus stability.[27]

1.3.4 *FcγRIIA*

FcγRIIA (CD32) is a type of Fcγ receptor expressed on platelets and macrophages and only present in higher primates. FcγRIIA contains two extracellular Ig domains and an intracellular ITAM with two YxxL sequences separated by 12 amino acids. FcγRIIA binds IgG-bearing immune complexes and IgG-opsonized pathogens and thus is involved in immune responses to pathogens but also autoimmune complications such as heparin-induced thrombocytopenia.[28] Similar to GPVI/FcR γ-chain activation, ligand binding to FcγRIIA causes receptor crosslinking, bringing the associated Src-family kinase Lyn into close proximity to phosphorylate neighboring FcγRIIA ITAM tyrosines, which then provide a docking site for Syk. Activated Syk phosphorylates targets such as PI3-kinase and LAT, leading to PLCγ2 activation.[29] FcγRIIA can also amplify the activation of nearby receptors, including integrin $\alpha_{IIb}\beta_3$, GPIb-IX-V, and G-protein coupled receptors, likely through the action of SFKs or Syk.[28, 30]

1.3.5 *Integrin $\alpha_{IIb}\beta_3$*

Integrin $\alpha_{IIb}\beta_3$ is specific to platelets and highly expressed at over 80,000 copies per platelet.[31] Integrins are transmembrane α/β heterodimers that are classified by the type of β subunit, with platelets expressing three β_1 members ($\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) and two β_3 members ($\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$). All mediate adhesion to ECM proteins, but $\alpha_{IIb}\beta_3$ alone plays a critical role in platelet aggregation, such that abnormalities in $\alpha_{IIb}\beta_3$ result in a rare autosomal recessive bleeding disorder called Glanzmann's thrombasthenia.[32] Each subunit of $\alpha_{IIb}\beta_3$ consists of a long extracellular region, a transmembrane

helix, and a short cytoplasmic tail. Resting integrin $\alpha_{IIb}\beta_3$ maintains a bent extracellular conformation that minimizes ligand binding. “Inside-out” signaling from platelet receptors such as GPVI activates the GTPase Rap1, which recruits the cytoskeletal proteins talin and kindlin to bind the β_3 cytosolic tail and initiate integrin conformational change. Once the integrins shift to an extended form, ligands such as circulating fibrinogen can bind at the interface between the N-terminal β-propeller and β_A domains of α_{IIb} and β_3, respectively.[33] This initiates receptor clustering and “outside-in” signaling near the cytosolic tails of α_{IIb} and β_3. Receptor clustering initiates activation of Src, which recruits Syk to the integrin β_3 tail, leading to assembly of a signalosome similar to that of GPVI but without the involvement of LAT (Figure 1.7). In addition, $\alpha_{IIb}\beta_3$ clustering activates signaling through the G-protein G_{13} and calcium- and integrin-binding protein 1 (CIB1), the latter of which activates focal adhesion kinase (FAK) to phosphorylate the actin-binding protein α-actinin, causing actin filament cross-linking that leads to platelet spreading.[34]

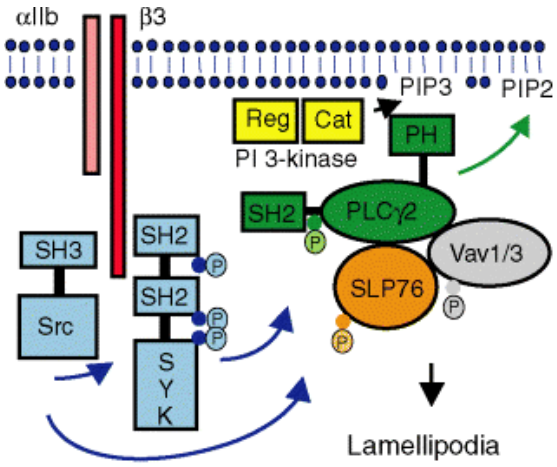

Figure 1.7. Integrin $\alpha_{IIb}\beta_3$ outside-in signaling. Binding of fibrinogen to $\alpha_{IIb}\beta_3$ initiates signaling through Src and Syk, leading to signalosome assembly that results in calcium release, granule secretion, and cytoskeletal remodeling. Figure adapted from Watson, *et al.*, *J Thromb Haemost*, 2005 Aug;3(8):1752-62. Reprinted with permission.

1.4 Platelet GPCR signaling

Many of the soluble agonists released by platelets bind specific G-protein coupled receptors on neighboring platelets, including ADP to $P2Y_{12}$ and $P2Y_1$ receptors, serotonin to $5\text{-}HT_{2A}$ receptors, epinephrine to α_{2A} adrenergic receptors, TxA_2 to TP receptors, and PGE_2 to EP receptors, initiating a wave of G-protein signaling and activation.[35] Subsequently, the trimeric G-protein αβγ complex becomes activated through exchange of GDP for GTP, and this Gα-GTP subunit disassociates from the Gβγ subunit to activate downstream targets.[36] The Gβγ subunit regulates the inactive, GDP-bound state but can also activate phosphoinositide 3-kinases (PI3Ks) involved in platelet activation and spreading.[37] The Gα subunit takes different forms, which in platelets include G_q, $G_{12/13}$, G_i, and G_s. Through G-protein signaling events, GPCR activation facilitates platelet granule release and spreading (Figure 1.8). We will begin with a description of signaling pathways for each Gα subtype before discussing GPCRs of particular clinical interest, $P2Y_{12}$ and PAR1/4.

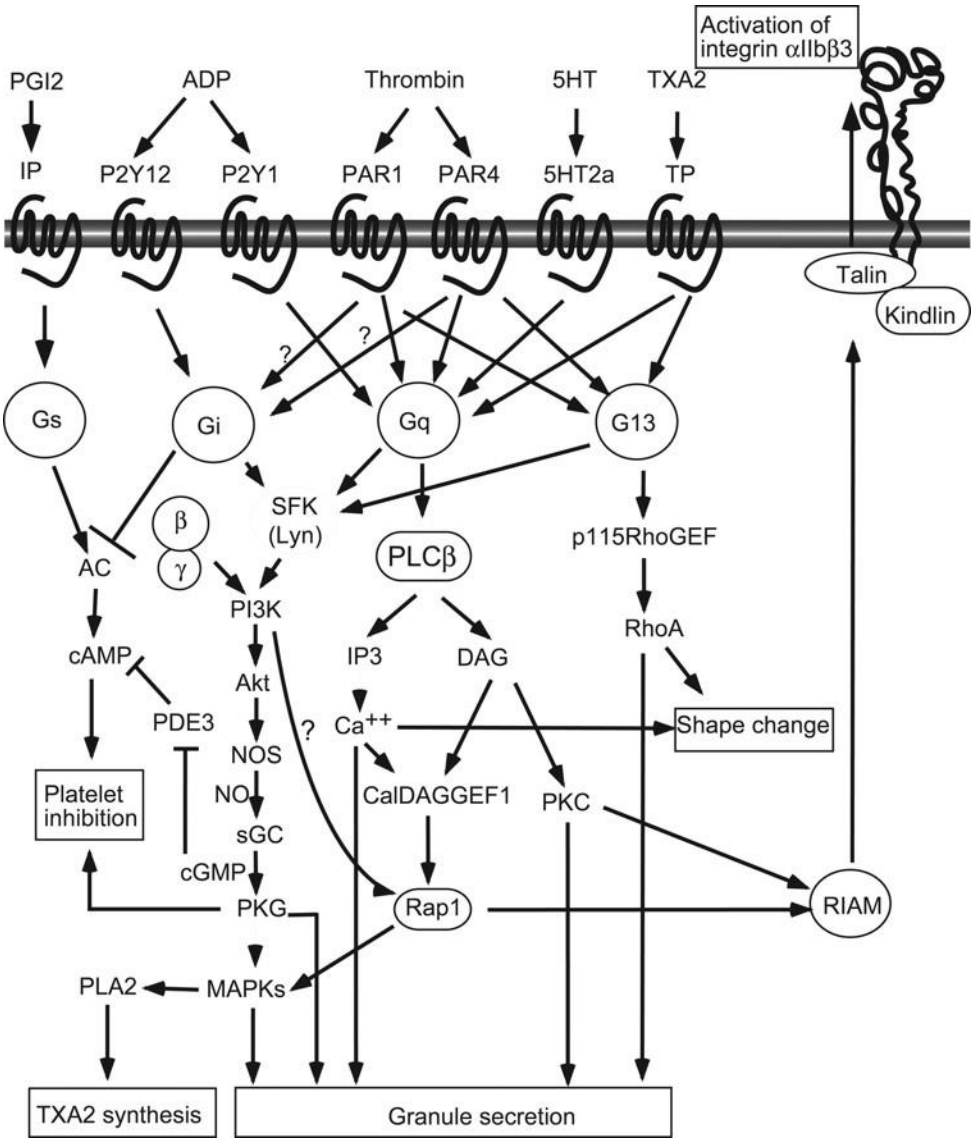

Figure 1.8. GPCR signaling in platelets. Agonists activate GPCRs, causing release and activation of their associated Gβγ subunit and Gα subunits G_s, G_q, $G_{12/13}$, or G_i, which activate downstream targets to cause granule secretion, TxA_2 synthesis, and integrin activation. Figure adapted from Du, *et al*., *Arterioscler Thromb Vasc Biol*, 2010 Dec;30(12):2341-9. Reprinted with permission.

1.4.1 *G_s signaling*

G_s is coupled to the IP receptor, which binds prostacyclin (PGI_2) released by endothelial cells to inhibit platelet activation. G_s activates the enzyme adenylyl cyclase, which catalyzes synthesis of cyclic AMP (cAMP) through the removal of a pyrophosphate unit from ATP. cAMP activates protein

kinase A (PKA), which phosphorylates multiple inhibitory proteins and decreases intracellular calcium levels, preventing platelet granule secretion.[38]

1.4.2 *G_i signaling*

G_i is coupled to the $P2Y_{12}$ and PAR1 receptors. In opposition to G_s, G_i inhibits adenylyl cyclase activity, thus preventing synthesis of cyclic AMP and allowing platelet activation to occur. G_i also activates Lyn, which activates PI3Kγ in coordination with the G-protein βγ subunit.[39] PI3Kγ activates the mitogen-activated protein kinase (MAPK) pathway to cause granule secretion and also activates Rap1b to cause inside-out activation of integrin $\alpha_{IIb}\beta_3$.[37]

1.4.3 *G_q signaling*

G_q is coupled to PAR1/4, $P2Y_{12}$, TP, and $5\text{-}HT_{2A}$ receptors. Upon activation, G_q causes activation of the phospholipase C isoform PLCβ, which, similar to the role of PLCγ in tyrosine kinase signaling pathways, causes generation of IP3 and DAG, leading to release of calcium stores and granule secretion.[40]

1.4.4 *$G_{12/13}$ signaling*

G_{12} and G_{13} are coupled to PAR1/4 and TP receptors but also integrin $\alpha_{IIb}\beta_3$.[41] Activated (GTP-loaded) G_{12} or G_{13} enables the associated Rho guanine-nucleotide exchange factor (RhoGEF) family member p115RhoGEF to transfer a GTP to the Rho GTPase RhoA, which activates it.[42] RhoA activates downstream effectors to promote platelet shape change and granule secretion.

1.4.5 *$P2Y_1$ and $P2Y_{12}$*

The P2Y receptors are GPCRs that bind purine and pyrimidine nucleotides, with platelets expressing two types, $P2Y_1$ and $P2Y_{12}$. These bind adenosine diphosphate (ADP) released from platelet dense granules. $P2Y_1$ is coupled to G_q, while $P2Y_{12}$ is coupled to G_i. $P2Y_1$ is widely distributed on other cells and tissues, while $P2Y_{12}$ expression is mostly limited to platelets, vascular smooth muscle cells, and brain microglial cells, making $P2Y_{12}$ a better target for antiplatelet drugs.[43] As a result, several classes of $P2Y_{12}$ inhibitors have been developed, including thienopyridines such as clopidogrel and ATP analogs such as ticagrelor.[44]

1.4.6 *PAR1 and PAR4*

Human and non-human primate platelets express protease-activated receptors 1 and 4 (PAR1/4), GPCRs that are activated through N-terminal cleavage by proteases such as thrombin, cathepsin G, plasmin, and trypsin. Cleavage of the N-terminal domain generates a tethered ligand that binds the receptor and initiates signaling through G_q, $G_{12/13}$, and G_i. Studies of calcium flux have shown that PAR1 activation initiates a short, transient calcium signal, while PAR4 activation causes a more prolonged signal, suggesting PAR4 plays a distinct role to support the later phases of platelet aggregation.[45] Deficiencies in PAR1 or PAR4 are not known, but mutations in PAR4 have recently been discovered that are associated with several-fold increases in platelet aggregation; these polymorphisms are more prevalent in Black Americans, sub-Saharan Africans, and Papua New Guineans.[46] Antagonists against PAR4 are currently in development and have shown promising results in animal studies.[47]

1.5 Molecular chaperones in platelets

While a number of more classical signaling proteins, including specific kinases, phospholipases, and small GTPases are widely studied in platelets, other biological mediators of platelet physiology may play critical roles in platelet function, such as the numerous protein chaperones that are abundantly expressed in platelets. A number of steps in platelet activation require protein quality control that may be regulated by chaperones, including intracellular signaling and the cytoskeletal remodeling events that generate filopodia and lamellipodia to enable platelet attachment and spreading.[48]

Sections 1.5 and 1.11.2 are adapted from a book chapter accepted for publication in *Heat Shock Protein 70 in Biology and Medicine*, Springer Publishing, 2017.

1.5.1 *Protein disulfide isomerases*

Some chaperone proteins, particularly the disulfide isomerases such as PDI and ERp57, are emerging as important regulators of platelet action. These oxidoreductase chaperones regulate disulfide rearrangements to facilitate surface integrin conformational changes required for platelet-platelet interactions in thrombus formation. Members of the PDI family, including PDI, ERp5, ERp57, and Ero1α bind integrin $\alpha_{IIb}\beta_3$ and are required for $\alpha_{IIb}\beta_3$ activation.[49-52] These oxidoreductase chaperones physically interact with $\alpha_{IIb}\beta_3$ on the platelet surface to facilitate conformational changes required for activation.[49-51, 53-56]

1.5.2 *Heat shock proteins*

The heat shock protein family comprises molecular chaperones ranging in size from 10 to over 100 kDa. Several heat shock protein family members and associated proteins have been identified in

platelets, including family members of Hsp27, Hsp60, Hsp70, and Hsp90.[57-63] Quantitative proteomics analyses of human and mouse platelets have reported that the heat shock cognate 71 protein (HSP7C, HSPA8) is one of the most abundant proteins in platelets (abundance ranking #89 in human; #26 in mouse).[64, 65] Recent *in vitro* studies demonstrate that extracellular Hsp72 can modulate platelet function to support platelet aggregation.[66] Heat shock proteins in platelets have also been reported to be covalently modified by protein phosphorylation, suggesting involvement in signaling.[62, 67-69] Interestingly, Hsp70, like oxidoreductases, can be secreted from platelets, potentially playing roles in integrin conformational change and subsequent platelet activation. Studies in other cellular systems demonstrate that Hsp70 family members associate with PDI, Erp57, or integrins to fulfill chaperone activities and promote integrin activation.[55, 70, 71] Work in other biological systems demonstrates a role for Hsp70 in assembly of protein complexes near the intracellular membrane.[72, 73] Intriguingly, a recent study by Durrant *et al*. finds that Hsp90-α associates with the PI3K signalosome, which lies downstream of and proximal to the LAT signalosome in the regulation of platelet granule secretion and integrin activation.[74] Hsp70 has also recently been found to associate with the CLEC-14a receptor to promote angiogenesis, hinting at potential roles for Hsp70 in modulating CLEC-2 receptor activities in platelets, proximal to tyrosine kinase and PLC signaling.[75]

In addition to targeting platelet receptors such as integrins, some studies suggest extracellular chaperones mediate the organization and assembly of platelet receptor ligands such as fibrinogen/fibrin, collagen and other extracellular matrix components.[76] For example, interactions between Grp75 and the fibrinogen binding extracellular protein fibulin-1C are hypothesized to have roles in incorporating fibrin into blood clots.[77, 78] Activated platelets also express surface Hsp47, a chaperone that associates with collagen, and this interaction is required for platelet activation by collagen.[79] Overall, roles for extracellular chaperone activities in physiological processes such as

platelet activation are multifaceted and only just beginning to be explored.[80-82] A scheme of proposed Hsp70 activities in platelets is shown in Figure 1.9.

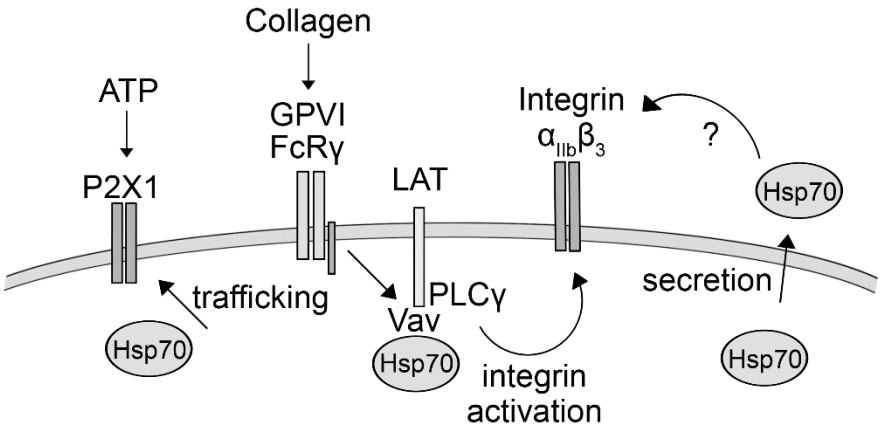

Figure 1.9. Roles for Hsp70 in platelets. Platelet Hsp70 facilitates trafficking of the P2X1 receptor to the membrane and assembly of the LAT signolosome leading to integrin $\alpha_{IIb}\beta_3$ activation. Hsp70 may also be secreted from platelets to coordinate integrin $\alpha_{IIb}\beta_3$ conformational change on the platelet surface.

1.6 Platelet granule secretion

Platelet activation causes release of internal stores from alpha and dense granules and lysosomes, which release a host of contents to attract and activate neighboring platelets (Figure 1.10).[83] Platelet dense (δ-)granules release small molecules and charged ions such as adenosine di- and tri-phosphate (ADP and ATP), epinephrine, serotonin, Ca^{2+}, and polyphosphate. Platelet alpha (α-)granules release numerous proteins, including the coagulation proteins fibronectin, factor V, and vWF, the inflammatory chemokines platelet factor 4 (PF4 or CXCL4) and CXCL7, prostaglandin E_2 (PGE_2) and the growth factors IGF-1 and TGFβ.[84] Additionally, the membrane of alpha granules contains the cell adhesion molecule P-selectin, which becomes translocated to the platelet surface upon granule release and binds P-selectin glycoprotein ligand-1 (PSGL-1) on circulating leukocytes or endothelial cells. Platelet activation also initiates conversion of arachidonic acid from the platelet membrane into the prostaglandins thromboxane (TxA_2) and, in lesser amounts, prostaglandin E_2

(PGE_2) by cyclooxygenase (COX) enzymes, and these prostaglandins diffuse through the platelet membrane to activate neighboring platelets. Finally, platelet lysosomes or λ-granules release degradative proteases and glycosidases involved in clot retraction and wound healing.

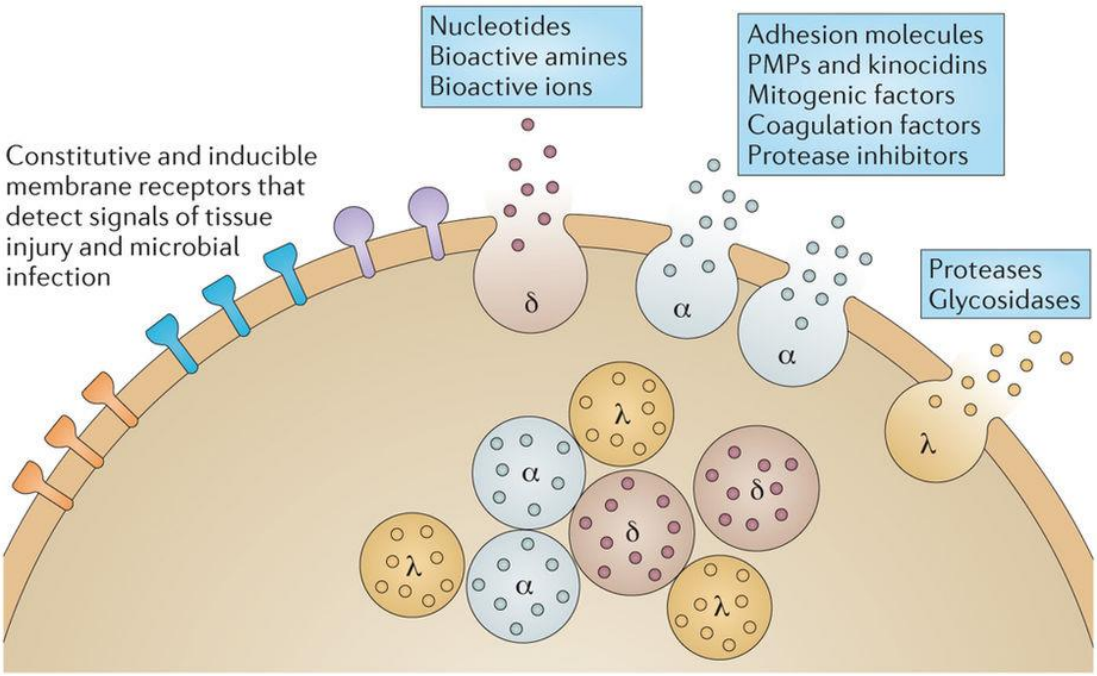

Figure 1.10. Platelet granule release. Activated platelets release granule contents from alpha (α-), dense (δ-), and lysosomal (λ-)granules. Figure adapted from Yeaman M.R., *Nat Rev Microbiol*. 2014 Jun;12(6):426-37. Reprinted with permission.

1.7 Platelet aggregation and spreading

During platelet activation, intracellular signaling culminates in Rap1-GTP-interacting adapter molecule (RIAM) interacting with cytoskeletal proteins at the cytoplasmic tail of integrin $\alpha_{IIb}\beta_3$ to cause integrin activation. This "inside out" activation enables $\alpha_{IIb}\beta_3$ to bind circulating fibrinogen and other ECM proteins that act as a bridge for platelet-platelet aggregates. Meanwhile, signaling cascades downstream of receptor activation converge on activation of PI3-kinase (PI3K) and Rho GTPase family members RhoA, Cdc42, and Rac1.[42] This leads to platelet spreading via actin

polymerization and remodeling, forming protrusions of filopodia and sheet-like lamellipodia (Figure 1.11) as well as longer membrane tethers that form under high shear conditions to enable platelet adhesion and aggregation.[85] At the same time, some platelets form large, balloon-like shapes known as procoagulant platelets, which create a "cap and balloon" morphology in which the densely packed caps create a platform for reactions involved in blood coagulation.[86]

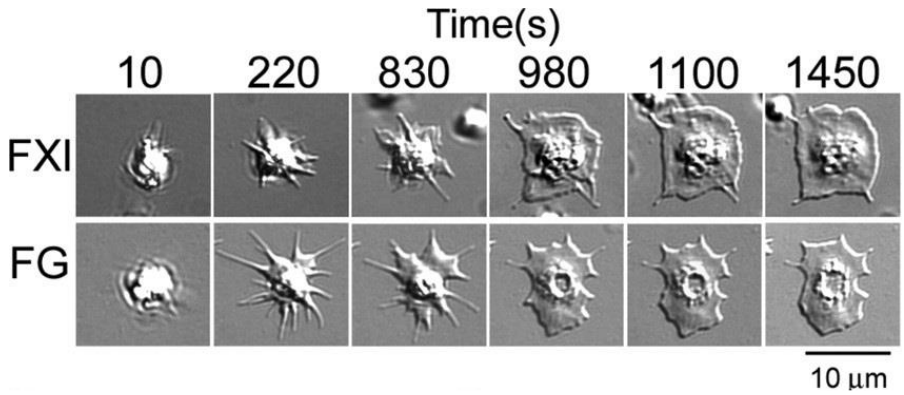

Figure 1.11. Platelet spreading. Differential interference contrast (DIC) microscopy of platelets spreading on surfaces of coagulation factor XI (FXI) or fibrinogen (FG) over a time course. Platelets initially form long, spindly filopodia and then sheet-like lamellipodia. Figure adapted from White-Adams, *et al*., *Arterioscler Thromb Vasc Biol*, 2009 Oct;29(10):1602-7. Reprinted with permission.

1.8 Coagulation

While platelets perform their activation sequence, the process of blood coagulation occurs in parallel to support hemostatic plug formation; this can be divided into initiation, amplification, and propagation phases (Figure 1.12).[87] In the initiation phase of coagulation, exposed tissue factor (TF) on subendothelial cells such as smooth muscle cells, pericytes, and fibroblasts binds and activates blood coagulation factor (F)VII to FVIIa, and this surface-bound TF/FVIIa complex captures and activates FIX to FIXa and FX to FXa.[88] On neighboring TF-expressing cells, the newly generated FXa associates with its cofactor FVa. Next, in the amplification phase, traces of circulating thrombin facilitate conversion of FV to FVa, FVIII to FVIIIa, and FIX to FIXa. Finally,

in the propagation phase, activated platelets express surface phosphatydylserine (PS) through a membrane flipping mechanism, which provides a surface for the assembly and activation of these blood coagulation factors.[89] On this PS surface, FIXa associates with its cofactor FVIIIa to form the tenase complex, which activates FX to FXa. Also on PS-expressing platelets, FVa and FXIa form the prothrombinase complex, which activates prothrombin (FII) to thrombin (FIIa). Thrombin further activates platelets by enzymatically cleaving the platelet protease activated receptors (PARs), PAR1 and PAR4, leading to additional platelet PS exposure. In the final step, thrombin cleaves fibrinogen into fibrin and activates FXIII to FXIIIa, which crosslinks the fibrin strands into a meshwork, binding together the hemostatic plug.

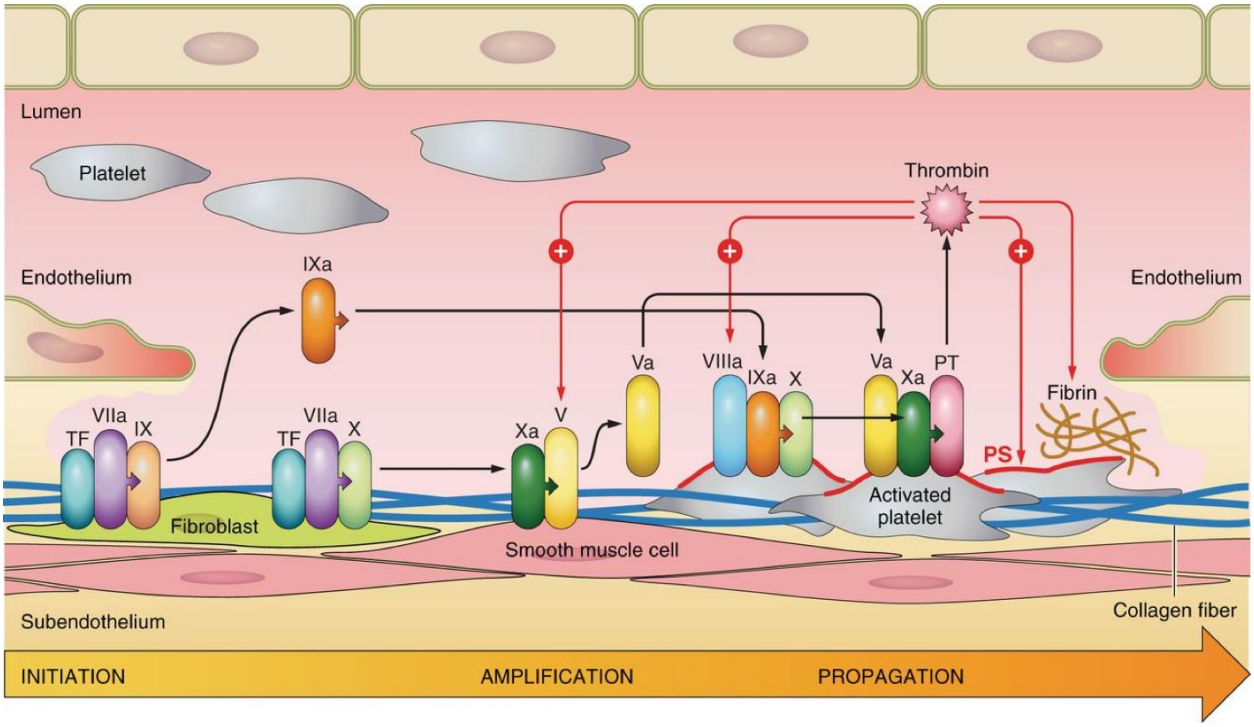

Figure 1.12. Blood coagulation. Coagulation factors assemble following injury to a blood vessel, progressing through phases of initiation, amplification, and propagation, from left to right. Figure adapted from Bevers E.M. and Williamson P.L., *Physiol Rev*, 2016 Apr;96(2):605-45. Permission is not required by the publisher for this type of use.

1.9 Inflammation

In addition to preserving hemostasis, platelets can promote inflammation, which is a multifaceted response of the innate immune system, also known as the non-specific immune system, to defend against microbial infection or damage.[90] Inflammation involves recruitment of leukocytes, which are white blood cells such as neutrophils, monocytes, and macrophages, and generation of symptoms such as pain, redness, and swelling. Platelets contribute to the inflammatory response through α-granule release of chemoattractants such as platelet derived growth factor (PDGF) and platelet factor 4 (PF4 or CXCL4), which recruit leukocytes.[83] Platelets also synthesize the vasoconstrictor thromboxane A_2 (TxA_2) and the proinflammatory cytokines interleukin (IL)-1α and IL-1β, which upregulate expression of endothelial adhesion molecules ICAM-1 and VCAM-1 and promote neutrophil migration. A list of inflammatory mediators released by platelets is shown in Table 1.1.[90]

Table 1.1. Inflammatory mediators released by platelets.

Molecule	Family	Location
IL-1β	Cytokine	Synthesized
Thromboxane A2	Eicosanoid	Synthesized
PF4/CXCL4	Chemokine	α- granules
β-thromboglobulin (CXCL7/ NAP-2)	Chemokine	α- granules
RANTES (CCL5)	Chemokine	α- granules
CD40L	Cytokine	α- granules
PDGF	Growth factor	α- granules
TGF-β	Growth factor	α- granules
TNF-α	Cytokine	α- granules
IL-1α	Cytokine	α- granules
GRO-α (CXCL1)	Cytokine	α- granules
ENA-78 (CXCL5)	Cytokine	α- granules
SDF-1 (CXCL12)	Cytokine	α- granules
MIP-1α (CCL3)	Chemokine	α- granules
MCP-3 (CCL7)	Chemokine	α- granules
NAP-2 (CXCL7)	Chemokine	α- granules
TARC (CCL17)	Chemokine	α- granules
Interleukin-8 (CXCL8)	Chemokine	α- granules
Polyphosphates	Phosphates	Dense granules
ATP	Nucleotide	Dense granules
Serotonin	Monoamine	Dense granules
Glutamate	Amino Acid	Dense granules

In addition, platelets bind to inflamed endothelial cells through platelet $\alpha_{IIb}\beta_3$, GPVI, P-selectin, GPIbα, and CD40L via a number of endothelial adhesive molecules, leading to platelet activation and amplifying inflammation (Figure 1.13).[91] Similarly, platelets bind to leukocytes through platelet P-selectin, GPIbα, and CD40L binding to leukocyte PSGL-1, Mac-1 (integrin $\alpha_M\beta_2$), and CD40, respectively.[92] The effects of platelet-leukocyte interactions can be bi-directional, with both prothrombotic and proinflammatory results, from platelet activation and generation of fibrin to neutrophil cytokine release and formation microbicidal structures called neutrophil extracellular traps (NETs).

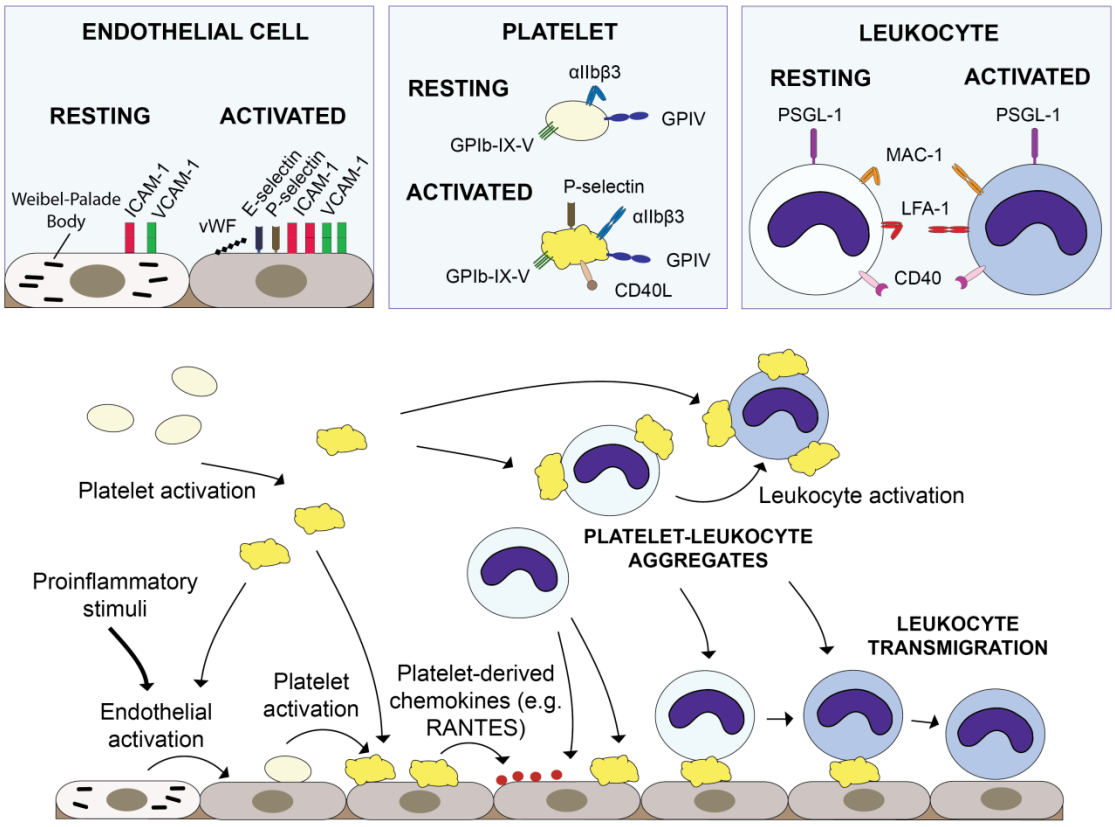

Figure 1.13. Platelets in inflammation. Platelets interact with endothelial cells and leukocytes in inflammation. Figure adapted from Mònica Arman, Holly Payne, Tatyana Ponomaryov and Alexander Brill (2015). Role of Platelets in Inflammation, The Non-Thrombotic Role of Platelets in Health and Disease, Dr. Steve W. Kerrigan (Ed.), InTech, DOI: 10.5772/60536. Available from: https://www.intechopen.com/books/the-non-thrombotic-role-of-platelets-in-health-and-disease/role-of-platelets-in-inflammation. This is an open-access book under CC BY 3.0 license.

1.10 Thrombosis

While platelets play a central role in maintaining a healthy vascular system, they can also become aberrantly activated and contribute to disease states. Thrombosis is the development of a blood clot that impedes blood flow, and it is the underlying cause of ischemic heart disease and stroke, causing one in four deaths worldwide.[93] Thrombosis can be a primary outcome or a secondary outcome in diseases such as cancer, cardiovascular disease, and inflammatory disease. Factors predisposing to thrombosis include stasis of blood flow, endothelial injury, and hypercoagulability, known as

Virchow's triad.[94] Platelets play a key role in the development atherothrombosis by aggregating at the site of atherosclerotic plaque rupture and releasing growth factors, proinflammatory cytokines, and ligands for leukocyte binding.[95] As a result, a number of antiplatelet therapies have been developed to target platelets in atherothrombosis, such as the COX inhibitor aspirin, the $P2Y_{12}$ inhibitors clopidogrel and ticagrelor, the integrin $\alpha_{IIb}\beta_3$ inhibitor ebtifibatide, and the PAR1 inhibitor vorapaxar.[96] However, all current antiplatelet therapies increase bleeding risk, prompting the need for continuing studies of platelet function to find better drug targets.

1.11 Emerging platelet therapies

In this section, we will detail three emerging strategies for targeting platelets to improve disease outcomes while decreasing bleeding risk, Btk inhibitors, Hsp70 modulators, and PAR4 inhibitors.

1.11.1 *Btk inhibitors*

The protein kinase Btk is involved in signal complex formation following activation of GPIb-IX-V, GPVI, CLEC-2, or FcγRIIa receptors on platelets, but it also plays important roles in signal transduction in B cells through the B cell antigen receptor (BCR). Drugs that inhibit Btk have become an important therapy in B cell malignancies, with the first-in-class Btk inhibitor ibrutinib being used for treatment of chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Waldenström's macroglobulinemia.[97-99] However, studies have shown that ibrutinib also targets other signaling molecules associated with Btk such as Tec kinase; in mice, deletion of both Tec and Btk confers defects in platelet signaling and aggregation.[100] A subset of patients taking ibrutinib develop bleeding side effects, necessitating the development of more selective Btk inhibitors such as acalabrutinib, which has shown great promise in a clinical trial for CLL.[101, 102] On the other hand, the antiplatelet effects seen with Btk inhibitors suggest they may be useful treatments for

thrombotic diseases, providing a new application for this already validated class of drugs. In sum, Btk inhibitors are important therapies against B cell malignancies with emerging potential as antithrombotic drugs as well.

1.11.2 *Heat shock protein modulators*

Meanwhile, heat shock proteins in platelets may also be targets in diseases of thrombosis and inflammation. Studies suggest Hsp70 may also be protective in cerebrovascular atherosclerosis; levels of circulating Hsp70 are lower in atherosclerosis patients than controls.[103] Allende *et al*. has reported that Hsp70 expression is highly downregulated in atrial fibrillation and stroke patients and that mice lacking Hsp70 develop thromboses more readily.[104, 105] Pharmacological Hsp70 induction in mice delays thrombus formation without altering aggregation or increasing bleeding.[105, 106] Intracellular Hsp70 also plays a cardioprotective role in complex with integrin-linked kinase (ILK) in cardiomyocytes.[107] In platelets, ILK promotes integrin $\alpha_{IIb}\beta_3$ activation,[108] yet a link between ILK and Hsp70 has not yet been investigated. Hsp70 family member Grp78 also plays an atheroprotective role by binding and inactivating tissue factor on the platelet surface.[58] Overall, these studies demonstrate numerous antithrombotic and pro-angiogenic effects of pharmacological Hsp70 induction.

Hsp70 family members in platelets may also be a target for inflammatory diseases.[109] High levels of circulating Hsp70 are a biomarker for cellular injury, oxidative stress, or inflammation in many disease contexts.[110-113] Hsp70 binds misfolded peptide segments during cellular stress, and these Hsp70-peptide complexes bind CD40 receptors on immune cells, facilitating peptide uptake and immune priming.[114] Extracellular Hsp70 is also associated with the onset and progression of inflammation in diabetes, while intracellular Hsp70 activity is protective.[115-117] Extracellular

Hsp70 has also been shown to bind the receptor for advanced glycation endproducts (RAGE) in cancer cells, activating inflammatory signaling pathways and cytokine secretion.[118] Circulating Hsp70 may have similar roles in stimulating CD40, RAGE and other immune receptors on platelets to prime thromboinflammatory disease states. Platelets are known to express receptors for heat shock protein Grp94 that neutralize Grp94 upon platelet activation and markedly diminish the immune response by dendritic cells, demonstrating an anti-inflammatory effect of Grp94 via platelets.[119] Platelets also express toll-like receptors (TLRs) upon activation, but whether platelets express Hsp70 receptors and whether pharmacological inhibitors of extracellular Hsp70 would help dampen inflammation remains an intriguing question.[120, 121] Meanwhile, administration of recombinant Hsp70 has been proposed to help treatment of sepsis by counteracting inflammatory responses.[122] Pharmacological Hsp90 inhibition may also have anti-inflammatory properties via Hsp70, as the Hsp90 inhibitor 17-DMAG improves outcomes of rats in sepsis by induction of Hsp70.[123] Overall, a better understanding of the interplay of platelets with Hsp70 and its co-chaperones will inform therapeutic strategies to modulate Hsp70 in thrombotic and inflammatory diseases.[124]

1.11.3 *PAR4 inhibitors*

While inhibitors of the ADP receptor $P2Y_{12}$ are currently in use for thrombotic diseases despite continued bleeding risks, an emerging alternative is to target the thrombin receptors PAR1 and PAR4. Vorapaxar was the first selective PAR1 antagonist developed, with the goal of reducing thrombotic cardiovascular events without bleeding. However, clinical trials demonstrated that patients on vorapaxar exhibited bleeding side effects, including more serious bleeding such as intracranial hemorrhaging.[125] As a result, attention has shifted to the PAR4 receptor as a potential target, given both *in vitro* studies and animal models showing PAR4 contributes to thrombosis, inflammation, and pain.[126, 127] Trials of a novel PAR4 antagonist in nonhuman primates suggest

a reduction in thrombosis without bleeding side effects, prompting a clinical trial in humans for recurrent stroke.[47, 128] Given the expression of PAR4 on monocytes and the activity of blood proteases such as neutrophil cathepsin G in cleaving PAR4, PAR4 inhibitors may also have applications in inflammatory diseases. Thus, PAR4 inhibitors are a fertile area of research as potential novel treatments for thrombotic and inflammatory diseases.

1.12 Thesis Overview

In this thesis, we examine specific molecular targets in platelets and their downstream impacts on platelet signaling and functional outcomes such as platelet aggregation, spreading, and platelet-leukocyte interactions. The following studies investigate the effect of Btk inhibitors on platelet function, the role of Hsp70 in tyrosine kinase-mediated platelet signaling, and the role of PAR4 activity in platelet granule release and platelet-leukocyte interactions.

In Chapter 3, we characterize the functional effects of Btk inhibitors on platelets, utilizing compounds that are analogues of ibrutinib, a treatment for B cell malignancies such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). Given the prevalence of bleeding side effects in patients taking ibrutinib, we investigated the effect of Btk inhibition on platelet function. Btk inhibitors impaired phosphorylation of Btk, Lyn, and other targets downstream of GPVI activation, resulting in diminished platelet spreading on fibrinogen, P-selectin exposure, and aggregate formation under shear flow. In addition, short-term *in vivo* oral dosing studies demonstrated that Btk inhibition demolished platelet aggregation but did not impact plasma clotting times or bleeding times during the short time frame of the study. This study demonstrates that Btk inhibitors analogous to ibrutinib significantly impair platelet function and have off-target effects on

signaling downstream of GPVI. Ultimately, these results have provided rationale for the development of more specific Btk inhibitors such as acalabrutinib.

Next, Chapter 4 details studies of the role of heat shock protein 70 (Hsp70) in coordinating platelet signaling and activation. Using well-characterized, small-molecule inhibitors of Hsp70 activity, we demonstrate that inhibition of Hsp70 impairs platelet secretion, spreading, and aggregation downstream of GPVI activation as well as whole blood aggregate formation under shear. Furthermore, biochemical studies show that Hsp70 is required for assembly of the LAT signalosome, a critical intermediate step between GPVI activation and functional outcomes such as integrin $\alpha_{IIb}\beta_3$ activation and granule secretion. These results identify a previously undiscovered function of heat shock protein 70 in platelet signaling and point to potential antiplatelet effects of Hsp inhibitors in development for cancer and other diseases.

Studies in Chapter 5 investigate the role of the platelet PAR4 receptor in promoting platelet dense granule release and platelet-leukocyte interactions. Platelet PAR4 is an emerging drug target for thrombotic diseases, yet the role of PAR4 activity in functional outcomes such as platelet dense granule release and platelet-leukocyte interactions remains unknown. We utilized PAR4 antagonists derived from BMS-986120, a PAR4 antagonist tested in recent human and non-human primate studies. PAR4 inhibition impaired platelet dense granule secretion by the blood proteases thrombin, cathepsin G, or plasmin and impaired platelet-leukocyte interactions in either a purified system or whole blood. These results demonstrate that PAR4 plays important roles in facilitating dense granule release and platelet-leukocyte interactions, suggesting PAR4 is a target not only in thrombotic disease but also in diseases of inflammation in which platelet-leukocyte interactions drive pathogenesis.

Finally, Chapter 6 presents conclusions from these studies and proposes future work to extend these results beyond platelet function to interactions between platelets, leukocytes, and endothelial cells. The overall goal of the presented work is to elucidate novel mechanisms of platelet activation while examining clinically relevant targets for the treatment of diseases of thrombosis and inflammation.

Chapter 2. Materials and Methods

2.1 Ethical Considerations

For all studies utilizing human blood, consent was obtained from healthy volunteers in accordance with an Oregon Health & Science University (OHSU) IRB-approved protocol. For all studies with baboons (*Papio anubis*), the animals were housed and cared for at the OHSU Oregon National Primate Research Center (ONPRC), a Category I facility, and all experiments were approved by the OHSU West Campus Animal Care and Use Committee according to the *Guide for the Care and Use of Laboratory Animals* by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (ISBN 0-309-05377-3, 1996). The Laboratory Animal Care and Use Program at the ONPRC is fully accredited by the American Association for Accreditation of Laboratory Animal Care and has an approved assurance (no. A3304-01) for the care and use of animals from the Office for Protection from Research Risks at the National Institutes of Health.

2.2 Common reagents

Collagen was from Chrono-Log (Havertown, PA). Collagen-related peptide (CRP) was from R. Farndale (Cambridge University, UK). Hanks' Balanced Salt Solution (HBSS) was from Corning cellgro (Manassas, VA, USA). Polymorphprep was from Axis-Shield (Oslo, Norway). PGI_2 was from Cayman Chemical (Ann Arbor, MI, USA). TRAP-6 (SFLLRN-NH_2) was obtained from Tocris (Bristol, UK). PPACK (D-Phe-Pro-Arg-chloromethylketone) was from Santa Cruz (Dallas, TX, USA). PAR4 activating peptide (AYPGKF-NH_2) was from Abgent (San Diego, CA, USA). Human fibrinogen was from Enzyme Research (South Bend, IN). Human α-thrombin and human plasmin

were from Haematologic Technologies (Essex Junction, VT, USA). Bovine thrombin, fatty-acid free bovine serum albumin (BSA), and all other reagents were from Sigma, unless specified otherwise.

2.3 Platelet and neutrophil isolation

2.3.1 *Platelet preparation*

Venous blood was obtained from healthy volunteers in accordance with an Oregon Health & Science University (OHSU) IRB-approved protocol. For washed platelet preparation, blood was drawn into 3.8% trisodium citrate 9:1 (v:v), and acid-citrate dextrose (ACD) was added at 1:10 (v:v). Platelet-rich plasma (PRP) was isolated by centrifugation at 200 *g* for 20 minutes, and platelets were separated from PRP at 1000 *g* for 10 minutes in the presence of prostacyclin (0.1 μg/ml). Platelets were resuspended in modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.3, supplemented with 5 mM glucose), washed by centrifugation at 1000 *g* for 10 minutes, and resuspended again in modified HEPES/Tyrode buffer to the specified platelet count.

2.3.2 *Granulocyte preparation*

Human granulocytes were isolated as previously described,[129] with minor modifications. Venous blood was drawn in accordance with an OHSU IRB-approved protocol at 7:1 (v:v) into citrate phosphate dextrose (CPD). Blood was layered onto an equal volume of Polymorphprep and centrifuged at 500 *g* for 45 min, and the middle layer containing granulocytes was removed and washed in Hanks' Balanced Salt Solution (HBSS) at 400 *g* for 10 minutes at 19 °C. The pellet was resuspended in cold sterile H_2O for 30 seconds to lyse remaining red blood cells, followed by dilution in 10× PIPES buffer (250 mM PIPES, 1.1 mM $CaCl_2$, 50 mM KCl, pH 7.4) and HBSS

buffer and centrifugation at 400 *g* for 10 minutes at 19 °C. The granulocyte pellet was resuspended in HBSS buffer to the specified cell count.

2.4 *In vitro* platelet studies

2.4.1 *Platelet spreading*

Glass coverslips were coated with proteins of interest, surface blocked with denatured bovine serum albumin (BSA), and incubated with washed platelets at 37 °C to allow platelets to adhere. Platelets were fixed, permeabilized, and stained with fluorescent antibodies of interest. Glass coverslips were mounted with Fluoromount G and stored at 4 °C before imaging and analysis by DIC and immunofluorescence microscopy.

2.4.2 *Platelet aggregation*

As previously described,[130] aggregation studies were performed using 300 µl of platelets pre-treated with inhibitors. Platelet aggregation was initiated by addition of 3 µl of agonist and followed by continuous stirring at 1200 rpm at 37 °C in a PAP-4 aggregometer. Aggregation was recorded as increase in light transmission, which was quantified as the vertical change from baseline, normalized to vehicle control.

2.4.3 *Platelet dense granule release*

Platelet dense granule secretion was measured as luminescence in an ATP-luciferin-luciferase reaction, as previously described.[182] Washed platelets were incubated in a white, flat bottom Corning 96-well plate with inhibitor or vehicle for 15 minutes at 37 °C with orbital shaking. Agonists

were added and incubated for 10 minutes. Finally, Chronolume detection agent was added and luminescence measured on an Infinite M200 spectrophotometer (TECAN, Switzerland).

2.5 Flow cytometry

Samples were pretreated with inhibitors before stimulation with agonists for 20 minutes in the presence of fluorescently-conjugated surface markers. Samples were diluted in buffer and analyzed by flow cytometry on a BD FACSCanto II or BD LSR II (Beckton Dickinson). The cell population of interest was identified by logarithmic forward and side scatter along with surface marker expression, if applicable. Expression of surface markers was recorded as the geometric mean fluorescence or as percent of cells above a specified fluorescence threshold.

2.6 Flow chamber studies

Glass capillary tubes were coated with proteins of interest and surface blocked with denatured BSA. Sodium citrate-anticoagulated whole blood was pre-treated with inhibitors as indicated and perfused through capillary tubes at 37 °C to form platelet aggregates, as previously described.[131] Aggregates were fixed, imaged using DIC microscopy, and analyzed as described below.

2.7 Microscopy

2.7.1 *General protocol*

Imaging was performed on a Zeiss Axiovert fluorescence microscope (Axio Imager; Carl Zeiss, Gittingen, Germany) using Köhler-illuminated Nomarski DIC optics with Zeiss EC Plan-Neofluar 40× or 63× magnification oil immersion lenses. Images were captured on a Zeiss Axiocam MRm camera using Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.).

2.7.2 *Image analysis*

The surface area of platelets or platelet aggregates was computed by manually outlining and quantifying platelet aggregates in ImageJ software, as previously described.[131]

Chapter 3. Oral administration of Bruton's tyrosine kinase inhibitors impairs GPVI-mediated platelet function

Rachel A. Rigg, Joseph E. Aslan, Laura D. Healy, Michael Wallisch, Cassandra P. Loren, Jiaqing Pang, Monica T. Hinds, András Gruber, and Owen J. T. McCarty

3.1 Abstract

The Tec family kinase Bruton's Tyrosine Kinase (Btk) plays an important signaling role downstream of immunoreceptor tyrosine-based activation motifs (ITAMs) in hematopoietic cells. Mutations in Btk are involved in impaired B-cell maturation in X-linked agammaglobulinemia (XLA), and Btk has been investigated for its role in platelet activation via activation of the effector protein PLCγ2 downstream of the platelet membrane glycoprotein VI (GPVI). Because of its role in hematopoietic cell signaling, Btk has become a target in the treatment of chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), with the covalent Btk inhibitor ibrutinib recently receiving FDA approval for these conditions. Antihemostatic events have been reported in some patients taking ibrutinib, though the mechanism of these events remains unknown. In this study, we sought to determine the effects of Btk inhibition on platelet function in a series of *in vitro* and *in vivo* models of platelet activation, spreading, and aggregation. Our results show that inhibition of Btk with either an ibrutinib analog or a novel Btk inhibitor, BTKI-43607, decreased human platelet activation, phosphorylation of Btk, P-selectin exposure, spreading on fibrinogen, and aggregation under shear flow conditions. Short-term *in vivo* studies showed that Btk inhibitors caused abrogation of platelet aggregation but without measurable effect on bleeding or plasma clotting times. Taken together, our results suggest that inhibition of Btk significantly decreased GPVI-mediated platelet activation,

spreading, and aggregation *in vitro* and *in vivo*; however, prolonged bleeding was not observed in a model of bleeding.

3.2 Introduction

Studies in this chapter were designed to determine the functional effects of Btk inhibition on platelets, given the emergence of mild bleeding symptoms in patients taking the Btk inhibitor ibrutinib. Here we show that Btk inhibition impairs GPVI-mediated tyrosine phosphorylation of multiple targets, platelet spreading, activation, and aggregation *in vitro* and that oral dosing with Btk inhibitors impairs platelet aggregation *in vivo*. This provides a clear rationale for the bleeding symptoms seen in the clinic and an impetus for the development of more specific Btk inhibitors in the future.

3.3 Background

Bruton's Tyrosine Kinase (Btk) is a member of the Tec family of non-receptor tyrosine kinases that is involved in signaling downstream of immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors in hematopoietic cells, including B cells, monocytes, neutrophils, natural killer cells, and platelets.[132-134] Btk was identified in 1993 as the cause of X-linked agammaglobulinemia (XLA), an immunodeficiency disease in which mutations in Btk are associated with a lack of B-cell maturation and a resulting low level (<2%) of circulating B cells.[132] Patients

with XLA show impaired platelet aggregation in response to both collagen and collagen-related peptide (CRP), a membrane glycoprotein VI (GPVI) agonist, along with decreased activation of the downstream effector protein phospholipase γ2 (PLCγ2), suggesting an important role for Btk in signaling in platelets.[21, 135]

Blood platelets are rapidly recruited to exposed extracellular matrix proteins such as collagen at sites of vascular injury. Initial platelet recruitment to the injury site occurs after circulating von Willebrand factor (vWF) undergoes a conformational change upon binding exposed collagen, followed by platelet receptor GPIb binding to vWF and platelet integrin $\alpha_2\beta_1$ binding to collagen.[10] Additional platelet adhesion to collagen and subsequent platelet activation is mediated by the platelet receptor GPVI, which is non-covalently associated with a disulfide-linked homodimer of Fc receptor (FcR) γ chains, each of which contains an ITAM unit.[21] Upon binding exposed collagen, GPVI forms crosslinks that enable the GPVI-bound Src kinases Fyn and Lyn to phosphorylate two tyrosines on the FcRγ ITAMs.[136] The tyrosine kinase Syk then binds the phosphorylated ITAMs, where it undergoes phosphorylation by the Src kinases and autophosphorylation.[21] Activation of Syk initiates assembly and activation of a signalosome including the transmembrane adapter protein LAT, the cytosolic adapter proteins SLP-76 and Gads, and the Tec kinases Btk and Tec in complex with the effector protein PLCγ2.[21] From this signaling complex, Btk is phosphorylated by Syk and Lyn and autophosphorylated and proceeds to phosphorylate PLCγ2, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), causing platelet activation through release of intracellular Ca^{2+} stores, activation of protein kinase C (PKC), synthesis of thromboxane A_2 (TXA_2), and subsequent platelet granule secretion.[21, 24]

Several other pathways of platelet activation converge on PLCγ2 and thus may require Btk for activation. Outside-in signaling of the fibrinogen receptor $\alpha_{IIb}\beta_3$ upon binding fibrinogen leads to activation of the Gα protein G13, which initiates c-Src activation of Syk, which activates PI 3-kinase and Btk to phosphorylate PLCγ2.[24] Binding of agonists such as thrombin and TXA_2 to GPCRs that activate G13 also initiates this signaling pathway.[24] Additionally, shear-dependent binding of the platelet membrane complex GPIb-IX-V to vWF recruits Lyn to activate PI 3-kinase, again leading to Btk and PLCγ2 activation.[137] Several of these pathways are interdependent and self-amplifying to promote platelet aggregation and formation of a stable thrombus.

Supporting its role in B-cell receptor signaling, Btk has been found to be elevated in B cell malignancies such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), and after several successful clinical trials, the covalent Btk inhibitor ibrutinib was recently approved by the United States Food and Drug Administration for treatment of CLL and MCL.[97, 98, 138-142] A mild bleeding diathesis has been reported in up to 50% of patients taking ibrutinib, with grade 3 or higher bleeding events occurring in up to 6% of these patients.[97, 98, 141] Several recent studies suggest these antihemostatic events may be due to the inhibitory effect of ibrutinib on platelet function.[143, 144] In order to further define the role of Btk inhibition on platelet function, this study investigated the effect of irreversible Btk inhibition using two ibrutinib analogs on platelet activation, spreading, and aggregation.

3.4 Materials and Methods

3.4.1 *Reagents*

Collagen was from Chrono-Log (Havertown, PA). Collagen-related peptide (CRP) was from R. Farndale (Cambridge University, UK). Human fibrinogen was from Enzyme Research (South Bend,

IN). FITC-conjugated Annexin V was from Life Technologies, and CD62E-FITC antibody was from Acris Antibodies. Antibodies for Western blotting experiments were from Santa Cruz (pLyn), Cell Signaling (pBtk and pSyk), and Millipore (4G10). Bovine thrombin, fatty-acid free BSA, and all other reagents were from Sigma or previously described sources.[130, 145]

The irreversible Btk inhibitors, BTKI-43607 and BTKI-43761, were provided by Pharmacyclics (Sunnyvale, CA), dissolved in dimethylsulfoxide at 10 mM, and stored at −20 °C until use. BTKI-43607 and BTKI-43761 are analogs of ibrutinib, and form a covalent bond with a cysteine residue on Btk. The molecular specificity of BTKI-43607 and BTKI-43761 interaction with Btk was evaluated using the Kinase Profiler (Millipore). The IC50 and selectivity of BTKI-43761 are reported in Table 3.1.

Table 3.1. IC_{50} values of the ibrutinib analog BTKI-43761.

	BTKI-43761	
Kinase	**IC_{50} (nM)**	**Selectivity for Btk**
Btk	0.39	1.0
ErbB4/HER4	0.64	1.6
Blk	0.94	2.4
Bmx/Etk	1.10	2.8
Fgr	2.86	7.3
Txk	2.87	7.4
Lck	3.49	9.0
Yes/YES1	3.94	10
Tec	5.49	14
Csk	6.17	16
EGFR	7.80	20
Brk	10.1	26
Itk	11.7	30
Hck	17.0	44
ErbB2/HER2	21.6	55
JAK3	21.9	56

3.4.2 *In vitro platelet studies*

To prepare washed human platelets, venous blood was drawn from healthy volunteers by venipuncture into sodium citrate in accordance with an Oregon Health & Science University IRB-approved protocol. The blood was centrifuged at 200 × g for 20 minutes to obtain platelet-rich plasma (PRP). Platelets were isolated from PRP via centrifugation at 1000 × g for 10 minutes in the presence of prostacyclin (0.1 μg/ml). The platelets were then resuspended in modified HEPES/Tyrode buffer and washed once via centrifugation at 1000 × g for 10 minutes. Washed platelets were resuspended in modified HEPES/Tyrode buffer to the desired concentration. Static adhesion assays, Western blots, and flow cytometry experiments were performed as previously described.[130, 145]

3.4.3 *Platelet aggregation*

Platelet aggregation studies were performed using 300 μl of platelets (2×10^8/ml) treated with inhibitors for 10 minutes. Platelet aggregation was triggered by CRP (3 μg/ml) or thrombin (0.1 U/ml) and monitored under continuous stirring at 1200 rpm at 37°C by measuring changes in light transmission using a PAP-4 aggregometer, as previously described.[130]

3.4.4 *Platelet aggregate formation under flow*

Sodium citrate-anticoagulated blood was treated with inhibitors as indicated and perfused at 2200 s^{-1} at 37°C through glass capillary tubes coated with collagen (100 μg/ml) and surface blocked with denatured BSA to form platelet aggregates, as previously described.[131] Imaging of aggregate formation was performed using Köhler-illuminated Nomarski DIC optics with a Zeiss 400 × 0.75 NE

EC Plan Neofluar lens on a Zeiss Axiocam MRm camera and Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.). Aggregate formation was computed by manually outlining and quantifying platelet aggregates, as previously described.[131]

3.4.5 *Non-human primate studies*

Non-human primate, male baboons (Papio anubis) were cared for and housed at the Oregon National Primate Research Center (ONPRC) at Oregon Health & Science University. All the experiments described here were reviewed and approved (approval numbers IS00002496 and IS00002092) by the Oregon Health & Science University West Campus Institutional Animal Care and Use Committee according to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (International Standard Book, Number 0-309-05377-3, 1996). The ONPRC is a Category I facility. The Laboratory Animal Care and Use Program at the ONPRC is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and has an approved Assurance (#A3304-01) for the care and use of animals on file with the Office for Protection from Research Risks at the National Institutes of Health (NIH).

In order to determine the effect of Btk inhibitors on platelet function *in vivo*, BTKI-43607 and BTKI-43761 were orally administered to individual non-human primates (n = 2) for 3 days at a dose of 10 mg/kg per day, followed by a 5 day rest period. This dose was selected to test the maximal response and potential bleeding risk of these new ibrutinib analogues within the dose range of 1.25-12.5 mg/kg/day used in clinical studies of ibrutinib.[146] At regular intervals, blood was drawn into sodium citrate and platelet-rich plasma (PRP) obtained via centrifugation of whole blood at 200 × g for 8 minutes. Supernatant was removed, and platelet poor plasma (PPP) was obtained by further

centrifugation of remaining blood at 5000 × g for 5 minutes. Platelets were counted using a Hemavet HV950 multispecies hematology system. Platelet count in PRP was further adjusted to 2×10^8/ml with PPP. Platelet aggregations were performed using the agonist CRP (1 and 0.5 μg/ml) in a Chrono-Log aggregometer.

Next, a longer time-course experiment was performed in which BTKI-43607 and BTKI-43761 were orally administered daily to individual non-human primates (n = 2) for 10 days at 10 mg/kg per day. Blood was withdrawn at regular intervals and processed as before for platelet aggregation studies. Tests of prothrombin time (PT) and activated partial thromboplastin time (APTT) were also performed on blood samples. To test the effect of the Btk inhibitors on bleeding, a standard template skin bleeding time (BT) assessment was performed using an FDA-approved incision device (Surgicutt; International Technidyne, Edison, NJ) at baseline and within 3 hours of each treatment. Additionally, tourniquet test (capillary resistance test, CRT) studies designed to detect abnormalities in capillary walls or thrombocytopenia were performed. The bleeding assay is an indicator of overall hemostatic response and was performed in light of the fact that bleeding side effects have been seen in patients taking ibrutinib.

3.4.6 *Statistical Analysis*

For flow chamber experiments, data were fitted to the quasi-binomial distribution with the identity link function. For static adhesion and flow cytometry experiments, two-way ANOVA (treatment and donor as factors) was performed, followed by post-hoc analysis with Tukey's test. For all tests, $P < 0.05$ was considered statistically significant. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria).

3.5 Results

3.5.1 *Effect of Btk inhibitors on tyrosine phosphorylation in human platelets*

Btk has been shown to play a role in the regulation of tyrosine kinase activation downstream of the ITAM-coupled receptor GPVI in human platelets.[21] As seen in Figure 3.1, Btk was rapidly phosphorylated following stimulation of purified platelets with the GPVI agonist collagen related peptide (CRP) in a concentration- and time-dependent manner (Panels A and B, respectively). We next designed experiments to validate the effects of Btk inhibition on signaling downstream of GPVI. Pretreatment of platelets with the selective Src-family kinase inhibitor PP2 abrogated Btk phosphorylation in response to 1 µg/ml CRP (Figure 3.1C). Pretreatment of platelets with the ibrutinib analogs BTKI-43761 or BTKI-43607 also inhibited Btk phosphorylation in response to 1 µg/ml CRP (Figure 3.1C). Interestingly, a reduction in Lyn phosphorylation was observed in the presence of BTKI-43761. The level of Syk phosphorylation was unaffected by pretreatment of platelets with 1 µM BTKI-43607 or BTKI-43761, while Syk phosphorylation was abrogated in the presence of PP2 (data not shown).

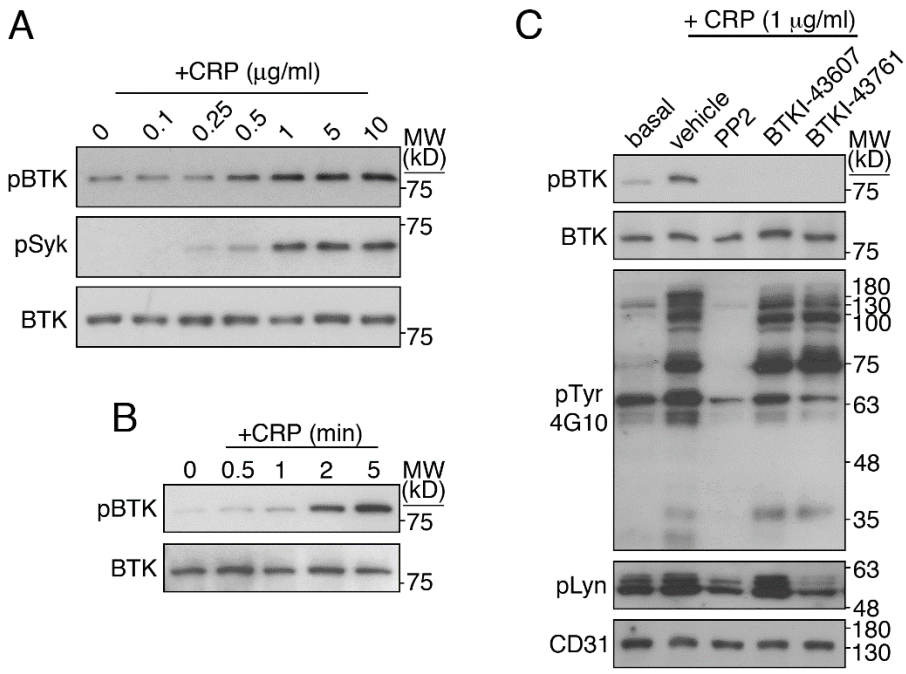

Figure 3.1. Tyrosine kinase phosphorylation downstream of GPVI following Btk inhibition. (A) Washed human platelets (5×10^8/ml) were stimulated with varying concentrations of CRP and subsequently lysed and blotted for Btk, phospho-Btk, and phospho-Syk. (B) Washed human platelets were stimulated with 1 μg/ml CRP for increasing amounts of time prior to being lysed and blotted for Btk and phospho-Btk. (C) Washed human platelets were treated with vehicle (DMSO, 0.1%), BTKI-43607 (5 μM), or BTKI-43761 (5 μM), and stimulated with 1 μg/ml CRP for 5 min. Platelets were subsequently lysed and blotted for phosphotyrosine moieties with 4G10 antisera as well as phospho-Btk and phospho-Lyn. The surface marker CD31 served as a loading control. Data are representative of 3-5 experiments.

3.5.2 *Effect of Btk inhibitors on human platelet P-selectin membrane exposure*

Platelet activation results in surface exposure of the transmembrane protein P-selectin, which is stored in the membrane of platelet alpha granules and is translocated to the platelet surface upon alpha granule release.[147] We next examined the effect of Btk inhibition on P-selectin exposure in response to platelet activation with the GPVI agonist CRP. As shown in Figure 3.2, pretreatment of purified platelets with 1, 3 and 10 μM BTKI-43607 or BTKI-43761 eliminated P-selectin exposure in response to stimulation with 10 μg/ml CRP, as determined by measuring platelet staining with an anti-P-selectin (CD62P) antibody using flow cytometry. A similar degree of inhibition was observed

in the presence of the Src kinase inhibitor, PP2. In contrast, P-selectin exposure in response to platelet activation with the G-protein-coupled agonist thrombin was unaffected by platelet treatment with 1 or 3 μM BTKI-43607 or BTKI-43761, while a partial inhibition was observed for 10 μM BTKI-43761.

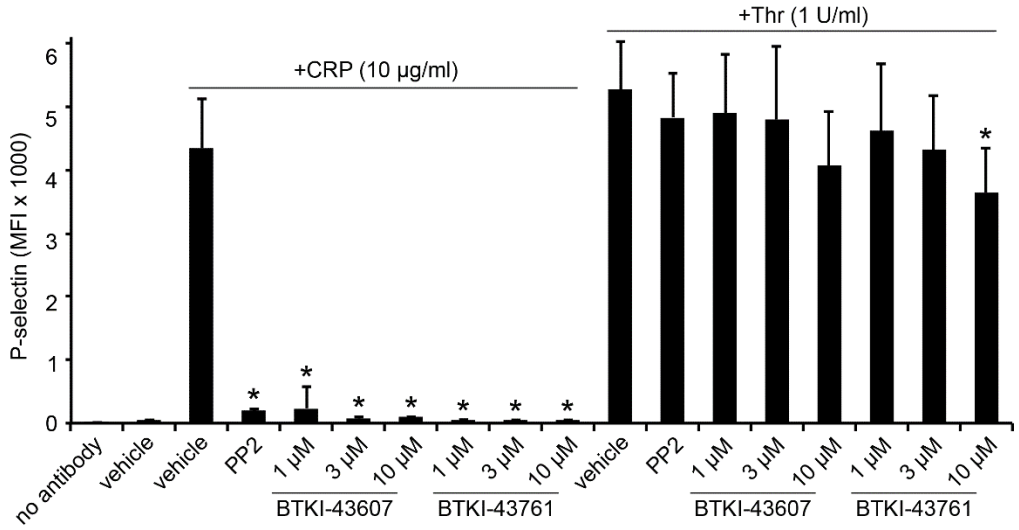

Figure 3.2. Platelet P-selectin exposure following Btk inhibition. Platelet surface P-selectin expression analyzed by flow cytometry following treatment with vehicle (DMSO, 0.1%), BTKI-43607 or BTKI-43761 prior to stimulation with CRP (10 μg/ml) or thrombin (1 U/ml) as indicated. * signifies $p < 0.05$ with respect to vehicle; error bars represent S.E.M.; $n = 4$.

3.5.3 *Effect of Btk inhibitors on human platelet spreading on fibrinogen and collagen surfaces*

We next examined the role of Btk on the ability of platelets to spread on surfaces of CRP, a GPVI-agonist, fibrinogen, which supports platelet spreading via the integrin $\alpha_{IIb}\beta_3$, or collagen, which supports platelet activation downstream of GPVI and adhesion and spreading via the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$.[148] As seen in Figure 3.3, treatment of platelets with 10 μM BTKI-43761 inhibited platelet spreading on CRP and on fibrinogen and collagen in the presence of the ADP scavenger apyrase, while BTKI-43607 failed to significantly inhibit platelet spreading on any of the three surfaces. Platelet spreading on both CRP and collagen was inhibited by the Src kinase inhibitor PP2.

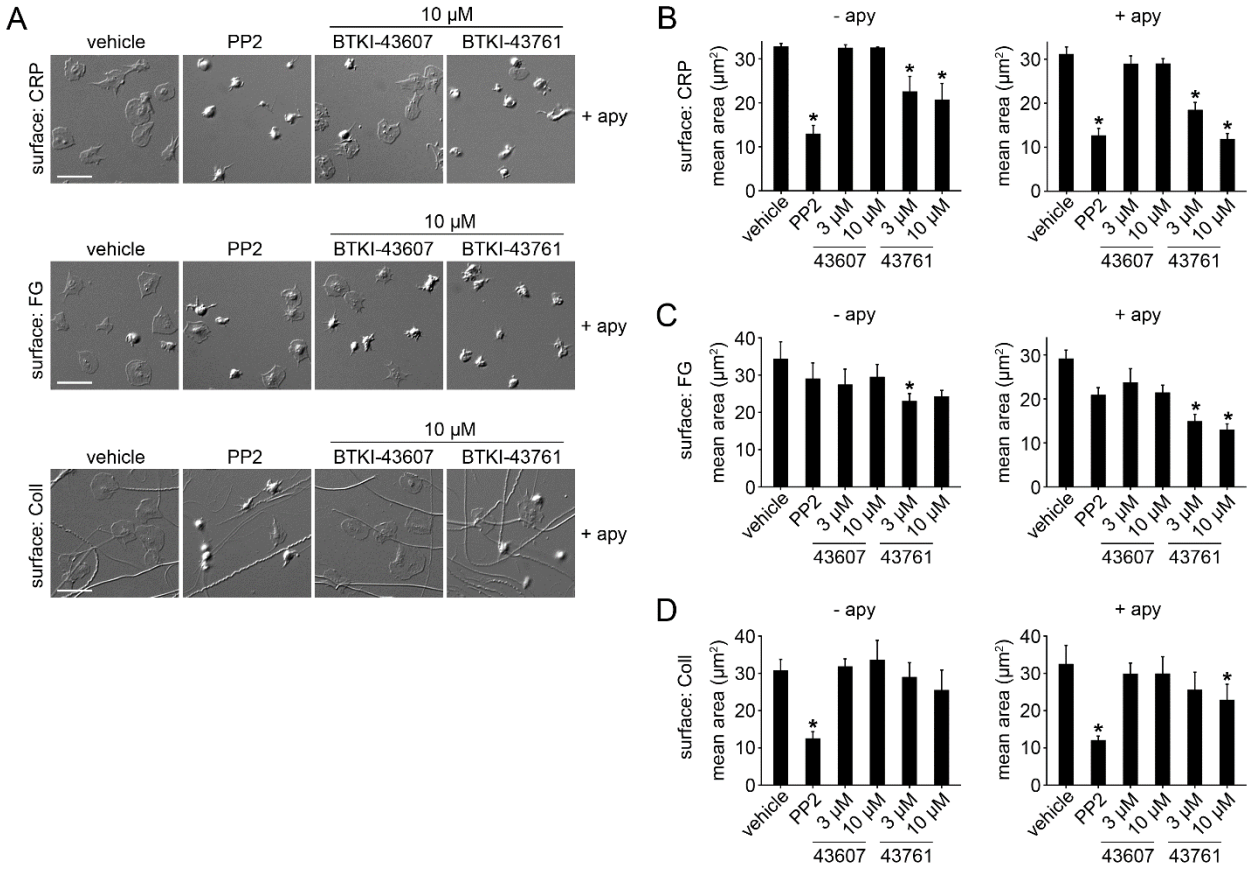

Figure 3.3. Platelet spreading following Btk inhibition. Replicate samples of washed human platelets (2×10^7/ml) treated for 10 minutes with vehicle (DMSO, 0.1%), the Src kinase inhibitor PP2 (10 µM), or BTKI-43607 or BTKI-43761 (3 or 10 µM) were allowed to spread on immobilized collagen-related peptide (CRP, 50 µg/ml), fibrinogen (FG, 50 µg/ml) or collagen (100 µg/ml) at 37 °C in the absence or presence of the ADP scavenger apyrase (apy). After 45 min, platelets were fixed, mounted onto slides, and (A) visualized with DIC microscopy. Scale bar = 10 µm. (B-D) The surface area of spread platelets was quantified and reported as mean ± S.E.M.; $n = 3$; * signifies $p < 0.05$ with respect to vehicle.

3.5.4 *Effect of Btk inhibitors on human platelet aggregate formation under flow*

We next examined the role of Btk on platelet adhesion and aggregate formation on collagen under shear flow conditions. Sodium citrate-anticoagulated whole human blood was pretreated with either vehicle (DMSO), BTKI-43607 or BTKI-43761 prior to being perfused over a surface of fibrillar collagen at a shear rate of 2200 sec^{-1} (representative shear of stenosed arteries) for 4 minutes, fixed, and visualized. The degree of surface area coverage was analyzed for at least 3 fields of view for 3

independent experiments. As seen in Figure 3.4, pretreatment of whole blood with 1 μM BTKI-43607 or BTKI-43761 significantly reduced the degree of platelet aggregate formation on collagen under shear, as measured by the extent of surface coverage of platelet aggregates, with a reduction of 43% and 17% compared to control, respectively.

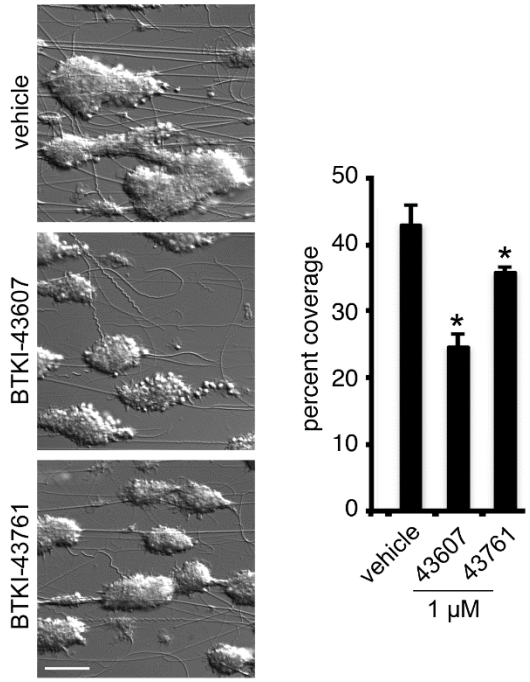

Figure 3.4. Platelet aggregate formation under shear following Btk inhibition. Whole blood was treated with vehicle (DMSO, 0.1%), BTKI-43607 (1 μM) or BTKI-43761 (1 μM) and perfused at 2200 s^{-1} through glass capillary tubes coated with collagen (100 μg/ml) and surface blocked with denatured BSA at 37 °C to form platelet aggregates. (A) Aggregates were visualized with DIC microscopy. Scale bar = 10 μm. (B) The percent surface area covered by aggregates was computed by outlining and quantifying platelet aggregates over 3 fields of view for each condition. * signifies $p < 0.05$ with respect to vehicle; error bars represent S.E.M.; n = 3-4.

3.5.5 *Effect of Btk inhibitors on non-human primate platelet aggregation*

We next investigated the *in vivo* effect of Btk inhibition on non-human primate platelet aggregation. The ibrutinib analogs BTKI-43607 or BTKI-43761 were orally administered to non-human primates for 3 days at a dose of 10 mg/kg per day, followed by a 5 day rest period. Our data demonstrate that

non-human primate platelets exhibited robust aggregation in response to 1 µg/ml CRP prior to administration of BTKI-43607 and BTKI-43761 (Figure 3.5; baseline) along with platelet shape change in response to 0.5 µg/ml CRP (data not shown). A dramatic inhibition of CRP-induced aggregation was observed as early as one hour following administration of either BTKI-43607 or BTKI-43761 (Figure 3.5; Day 0). Platelet aggregation was abrogated within four hours after administration of either BTKI-43607 or BTKI-43761 (Day 0), and platelets failed to aggregate in response to CRP during the three days that the non-human primates received either BTKI-43607 or BTKI-43761 (Day 0-2). While minimal aggregation was observed following two days of rest (Day 4), platelet aggregation was nearly restored to baseline levels by Day 7.

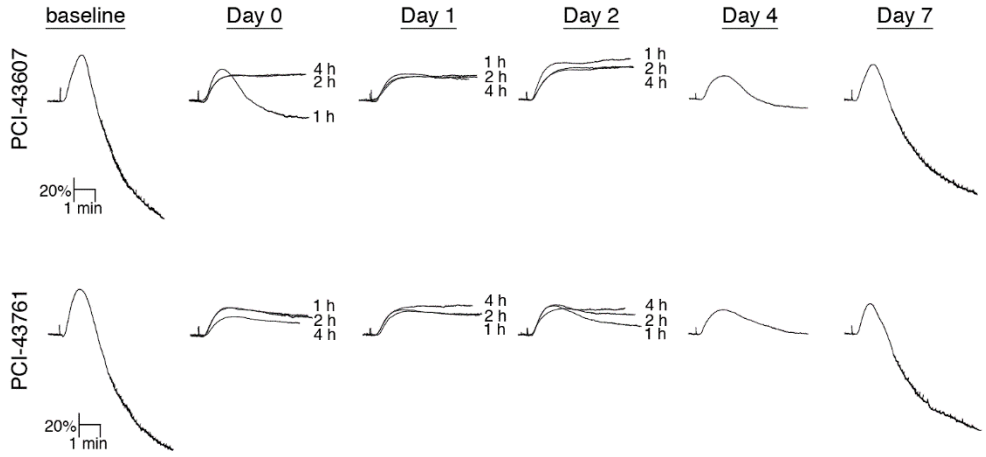

Figure 3.5. Aggregation of non-human primate PRP in the short-term oral dosing study. PRP from non-human primates was stimulated with CRP (1 µg/ml) and analyzed by Born aggregometry. Representative traces are shown for PRP from non-human primates (*n* = 2) dosed with BTKI-43607 or BTKI-43761 from the short-term study.

We next performed a time-course experiment in which BTKI-43607 was orally administered daily to the non-human primates for 10 days (10 mg/kg per day), followed by an 11-day non-dosing period, followed by administration of BTKI-43761 for 10 days (10 mg/kg per day). Our data show that prior to administration of either BTKI-43607 or BTKI-43761, a robust degree of platelet aggregation in

PRP was observed in response to 1 µg/ml CRP (Figure 3.6 and Table 3.2; Day -3), and platelet shape change was observed in response to 0.5 µg/ml CRP (data not shown). Platelet aggregation and shape change was eliminated in response to 1 µg/ml or 0.5 µg/ml CRP, respectively, during the entire time course of treatment with either BTKI-43607 or BTKI-43761 (Figure 3.6, Table 3.2**,** and data not shown; Day 0, 2, 4, and 9).

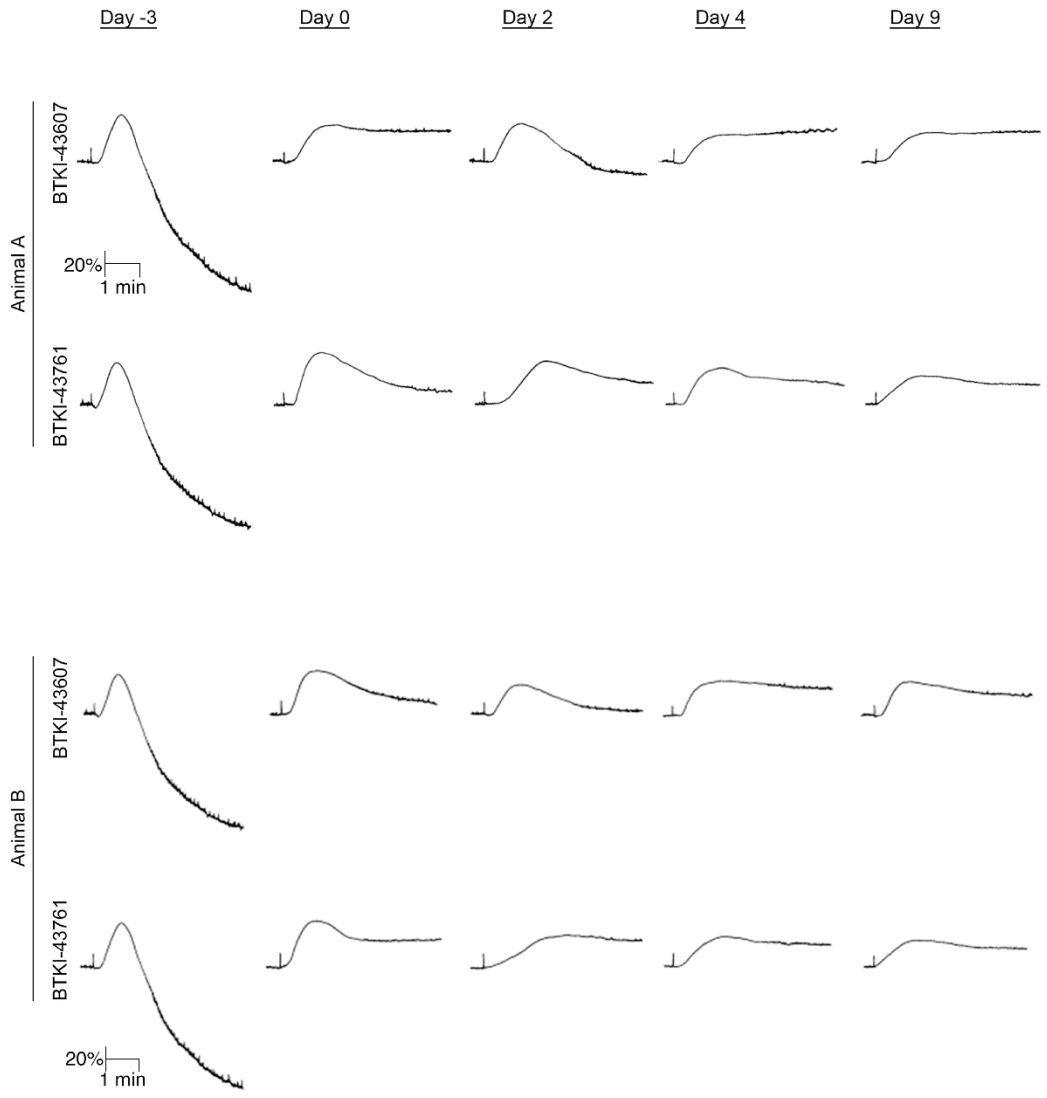

Figure 3.6. Aggregation of non-human primate PRP in the long-term oral dosing study. PRP from non-human primates was stimulated with CRP (1 µg/ml) and analyzed by Born aggregometry. Traces are shown for non-human primate A and B dosed with BTKI-43607 or BTKI-43761 from the long-term study.

Table 3.2. Average vertical drop and percent aggregation of non-human primate PRP in the long-term oral dosing study. Change in optical density from aggregation traces was recorded as vertical drop to quantify the extent of the inhibition of platelet aggregation. PRP was stimulated with CRP (1 μg/ml) from non-human primates dosed with the ibrutinib analogs BTKI-43607 or BTKI-43761 in the long-term study (n = 2).

Drug	Day	Vertical Drop (cm)	S.E.M.	Percent Aggregation	S.E.M.
BTKI-43607	-3	3.85	0.05	100.0	0.0
	0	-0.95	0.15	-24.6	3.6
	2	-0.15	0.15	-3.9	3.9
	4	-1.00	0.10	-26.0	2.9
	9	-1.00	0.10	-25.9	2.3
BTKI-43761	-3	3.85	0.05	100.0	0.0
	0	-1.00	0.20	-25.9	4.9
	2	-1.05	0.15	-27.3	4.3
	4	-0.85	0.05	-22.1	1.0
	9	-0.45	0.15	-11.6	3.7

3.5.6 *Effect of Btk inhibitors on bleeding times, capillary fragility, and coagulation in vivo*

To test the potential effect of Btk inhibitors on bleeding, a standard template skin bleeding time (BT) assessment was performed using an FDA-approved incision device (Surgicutt; International Technidyne, Edison, NJ) at baseline and within 3 hours of receiving either BTKI-43761 or BTKI-43607. Our data show that neither BTKI-43761 nor BTKI-43607 significantly increased template bleeding times during the 10-day dosing time course (Table 3.3). Moreover, tourniquet test (CRT) studies designed to detect abnormalities in capillary walls or thrombocytopenia were negative. In comparison, studies have shown that treatment of the non-human primates with 1006 μg/kg low molecular weight heparin increased template bleeding times by 1.46-fold over baseline.[100]

Table 3.3. PT, APTT, bleeding time, and capillary resistance test results from non-human primates in the long-term oral dosing study.

Non-Human Primate A					
Drug	**Dosing Day**	**PT (s)**	**APTT (s)**	**Bleeding Time (min)**	**CRT**
-	-3	13.3	39.4	3	Negative
BTKI-43607 (10 mg/kg)	0	12.6	41.3	3	Negative
	2	12.5	38.8	3	Negative
	4	13.0	40.7	4	Negative
	9	13.8	38.8	3	Negative
BTKI-43761 (10 mg/kg)	0	12.8	37.5	3	Negative
	2	12.8	39.8	3	Negative
	4	12.6	38.7	4	Negative
	9	12.8	40.5	3	Negative
Non-Human Primate B					
Drug	**Dosing Day**	**PT (s)**	**APTT (s)**	**Bleeding Time (min)**	**CRT**
-	-3	13.3	38.1	3	Negative
BTKI-43607 (10 mg/kg)	0	12.9	39.6	3	Negative
	2	12.5	39.6	3.5	Negative
	4	12.9	38.5	4	Negative
	9	12.4	38.3	3	Negative
BTKI-43761 (10 mg/kg)	0	13.2	37.9	3	Negative
	2	12.9	40.0	3.5	Negative
	4	13.1	38.3	3.5	Negative
	9	13.0	38.6	3.5	Negative

The effect of Btk inhibitors on coagulation *in vivo* was evaluated by measuring prothrombin time (PT) and activated partial thromboplastin time (APTT). Our data show that neither BTKI-43761 nor BTKI-43607 significantly increased either plasma PT or APTT during the 10-day dosing time course (Table 3.3). In comparison, studies have shown that treatment of the non-human primates with 1300 μg/kg low molecular weight heparin increased APTT by 1.9-fold over baseline.[100]

3.6 Discussion

This study examined the role of the Btk in human platelet function *in vitro* and *in vivo* and non-human primate platelet function *in vivo*. The data demonstrate that inhibition of Btk reduced GPVI-mediated platelet activation, Btk phosphorylation, spreading on fibrinogen, and aggregation under shear flow. First, we confirmed that inhibition of Btk reduced Btk phosphorylation in CRP-stimulated human platelets while having no effect on Syk phosphorylation compared to the positive control PP2, confirming that Btk acts downstream of Syk in the GPVI-ITAM signaling cascade. However, BTKI-43761 was also shown to partially inhibit Lyn phosphorylation, suggesting that this ibrutinib analog may have off-target effects on Src, Syk or Tec family kinases. For instance, kinases with a cysteine in the active site, such as Tec, represent possible targets for covalent binding with ibrutinib analogs. Moreover, our kinase screen demonstrated that BTKI-43761 may partially inhibit Fgr, although recent work has shown that Fgr only plays a minor role in platelet signal transduction.[136]

Studies of platelet activation by flow cytometry showed the inhibition of Btk eliminated P-selectin exposure in CRP-stimulated human platelets, supporting the notion that Btk plays a major role in platelet α-granule secretion via the GPVI pathway. These results are supported by early findings that the platelets of Btk-deficient XLA patients inhibited aggregation by CRP or, to a lesser extent, collagen but not thrombin.[135]

To explore platelet activation on immobilized surfaces or fibrinogen or collagen, static adhesion studies were performed. Platelet lamellipodia formation was significantly reduced on CRP, fibrinogen and collagen surfaces in the presence of 10 μM BKT-43761 and the ADP scavenger apyrase, suggesting that inhibition of Btk has an inhibitory effect on GPVI- and $\alpha_{IIb}\beta_3$ outside-in

signaling, perhaps by inhibiting Btk activation of PLCγ2 downstream of ITAM signaling initiated by GPVI or activated $\alpha_{IIb}\beta_3$, respectively.[24] Conversely, when ADP signaling was not blocked, the inhibition of platelet spreading was on fibrinogen and collagen by 10 μM BKT-43761 was reversed, suggesting that platelet activation through GPVI and integrin signaling coupled with ADP signaling via $P2Y_{12}/P2Y_1$ receptors was able to overcome Btk inhibition under static conditions. This highlights some redundancy within the ITAM- and GPCR-signaling pathways, which has been documented in knock-out mouse studies showing that following platelet activation by collagen, that Tec kinase could partially compensate for Btk deletion under static conditions.[100] These findings are in accord with the recent work by Bye *et al*. demonstrating that ibrutinib inhibited platelet adhesion and spreading on CRP but not collagen under static conditions.[149]

Platelet recruitment and aggregation under shear is mediated by the transient binding of VWF to platelet GPIb-IX-V, enabling the more durable adhesion of $\alpha_2\beta_1$ to collagen and subsequent activation of platelets via GPVI-mediated signaling, followed by platelet $\alpha_{IIb}\beta_3$-mediated aggregation. Our data show that inhibition of Btk partially reduced platelet aggregate formation on collagen under arterial rates of shear. Studies in Btk knock-out mice have similarly found a VWF- and GPIb-dependent role for Btk signaling in collagen-induced platelet activation under arterial shear,[137] supporting the findings of the current study which utilized human platelets. These results are supported by recent investigations in which the platelet-rich plasma of patients taking the Btk inhibitor ibrutinib exhibited significant reductions in collagen-mediated adhesion and aggregation under arterial shear compared to controls,[143, 144] and studies showing an additive effect of the Btk inhibitor ibrutinib and the $P2Y_{12}$ inhibitor, cangrelor, in thrombus formation under shear flow.[149]

Finally, a short-term study in non-human primates of the ibrutinib analogs BTKI-43607 and BTKI-43761 revealed a robust inhibition of GVPI agonist-induced aggregation for either inhibitor (10

mg/kg) by 4 hours after administration. The impaired aggregation began to recover two days after dosing ceased, with full recovery of aggregation after 5 dose-free days. These data demonstrated that both ibrutinib analogs exhibit strong inhibition of aggregation. A longer trial with non-human primates showed that both BTKI-43607 and BTKI-43761 consistently inhibited GVPI agonist-induced platelet aggregation during a 10-day dosing period. Tests for bleeding, capillary abnormalities, and thrombocytopenia were negative throughout the dosing period for these ibrutinib analogs, revealing no measurable impairment of hemostasis during the study. These findings are in line with findings from the ibrutinib clinical trials, which reported predominantly low-grade bleeding events in humans.[97, 98]

In conclusion, this study demonstrated that inhibition of Btk significantly decreased GPVI-mediated platelet activation, spreading, and aggregation *in vitro* and *in vivo*, which may affect hemostasis in patients. However, bleeding side-effects were not observed in a non-human primate model of template bleeding in this study.

Chapter 4. Heat shock protein 70 (Hsp70) regulates platelet integrin activation, granule secretion and aggregation

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4.1 Abstract

Molecular chaperones that support protein quality control, including heat shock protein 70 (Hsp70), participate in diverse aspects of cellular and physiological function. Recent studies have reported roles for specific chaperone activities in blood platelets in maintaining hemostasis; however, the functions of Hsp70 in platelet physiology remain uninvestigated. Here we characterize roles for Hsp70 activity in platelet activation and function. *In vitro* biochemical, microscopy, flow cytometry and aggregometry assays of platelet function as well as *ex vivo* analyses of platelet aggregate formation in whole blood under shear were carried out under Hsp70-inhibited conditions. Inhibition of platelet Hsp70 blocked platelet aggregation and granule secretion in response to collagen-related peptide (CRP), which engages the ITAM-bearing collagen receptor GPVI/FcRγ complex. Hsp70 inhibition also reduced platelet $\alpha_{IIb}\beta_3$ activation downstream of GPVI, as Hsp70-inhibited platelets showed reduced PAC-1 and fibrinogen binding. *Ex vivo*, pharmacological inhibition of Hsp70 in whole human blood prevented the formation of platelet aggregates on collagen under shear. Biochemical studies supported a role for Hsp70 in maintaining the assembly of the LAT signalosome, which couples GPVI-initiated signaling to integrin activation, secretion and platelet function. Together, our results suggest that Hsp70 regulates platelet activation and function by supporting LAT-associated signaling events downstream of platelet GPVI engagement, suggesting a role for Hsp70 in the intracellular organization of signaling systems that mediate platelet secretion,

"inside-out" activation of platelet integrin $\alpha_{IIb}\beta_3$, platelet-platelet aggregation, and ultimately hemostatic plug and thrombus formation.

4.2 Introduction

Studies in this chapter were designed to investigate the role of heat shock protein 70 in platelet function, given emerging evidence for the importance of other chaperone systems in platelet integrin activation. Here we demonstrate for the first time that Hsp70 coordinates assembly of the LAT signalosome downstream of GPVI activation, a key intermediate step in platelet granule secretion and integrin activation. This reveals a new biological mechanism by which molecular chaperones can modulate platelet function. Furthermore, these results suggest that inhibition of Hsp70 may result in defects in platelet aggregation, of concern for the development of Hsp70 antagonists in cancer and other diseases.

4.3 Background

Heat shock protein 70 (Hsp70) is an ATP-powered, 70 kDa molecular chaperone that regulates a myriad of protein quality control processes, including the folding of nascent polypeptides, the trafficking proteins across membranes, the prevention of protein aggregation and protein complex assembly and disassembly.[150-152] Hsp70 is an abundantly expressed and highly-conserved protein with 50% sequence homology between mammals and prokaryotes, highlighting the importance of

Hsp family members in protein homeostasis throughout evolution.[153] In human cells, the significance and complexity of Hsp70 function is evident in the functions of the 13 members of the human Hsp70 family, including the cytosolic, stress-inducible Hsp70 (or Hsp72) and its constitutively expressed cognate Hsc70, the ER-localized Grp78 (or BiP) and the mitochondrial form mtHsp70 (or Grp75/mortalin).[154] These Hsp70 family members regulate diverse cellular processes through an association with over 50 co-chaperones, and the arrangement of co-chaperones with Hsp70 varies across different contexts, allowing for a precise combinatorial control over the activity and specificity of Hsp70.[154, 155]

Platelets serve as the cellular guardians of vascular integrity. Upon exposure to extracellular matrix proteins such as collagen, platelets undergo a carefully orchestrated activation program, resulting in platelet shape changes, secretion, filopodia formation and ultimately platelet-platelet aggregation.[42, 145] Platelet-platelet aggregation is largely mediated by the activation of integrin $\alpha_{IIb}\beta_3$, which undergoes conformational changes in response to platelet stimulation to bind soluble fibrinogen, bridging platelets to one another to form hemostatic plugs.[156] Chaperone proteins such as protein disulfide isomerase (PDI) Ero1α, ERp57, and ERp5 have emerging roles in regulating the activities of platelets other hematopoietic cells.[49, 51, 53, 55] However, while both Hsp70 and Hsp90 family members have been identified in platelets,[57-60] specific roles for Hsp70 activity in platelet function and aggregate formation have not been addressed.

Here we investigate the role of Hsp70 in platelet function. We find that platelets express abundant Hsp70 protein and that Hsp70 localizes throughout platelets, while partially colocalizing with other chaperones such as Hsp90 and PDI. *In vitro* and *ex vivo* assays of platelet physiological function point to a role for Hsp70 upstream of "inside-out" activation of integrin $\alpha_{IIb}\beta_3$, granule secretion and platelet aggregation as well as aggregate formation under shear. Analyses of the intracellular

signaling events of platelet activation suggest that Hsp70 has a minimal role in the activation of signaling events that mediate platelet activation, but support a role for Hsp70 in regulating the assembly of LAT signalosome to ensure a proper coordination of platelet signaling, activation and function. Together, our results support roles for Hsp70 chaperone activity in platelet function and aggregation through the regulating the organization of the molecular events of platelet activation upstream of platelet $\alpha_{IIb}\beta_3$ activation and secretion.

4.4 Materials and Methods

4.4.1 *Reagents*

All reagents were from Sigma except as noted. VER 155008 and TRAP6 were from Tocris Bioscience, and MKT-077 was from Sigma-Aldrich. Collagen was from Chrono-Log (Havertown, PA). Collagen-related peptide (CRP) was from R. Farndale (Cambridge University, UK). Human fibrinogen was from Enzyme Research. For flow cytometry experiments, CD62E/CD62P-FITC antibody was from Acris Antibodies, PAC-1-FITC antibody was from Beckton Dickinson, Oregon Green-labeled human FG (OG488-FG) was from Invitrogen, and CD61-PE antibody was from R&D Systems. Src pTyr416 (#2101), LAT pTyr171 (#3581), LAT (#9166) and Akt pSer473 (#9271) antibodies were from Cell Signaling, Inc. Hsp70 (sc-24), Hsp90 (sc-101494) and PDI (sc-20132) antibodies were from Santa Cruz Biotechnology. Tubulin (T6199) antibody was from Sigma and 4G10 antibody was from EMD Millipore.

4.4.2 *In vitro platelet studies*

To prepare washed human platelets, venous blood was drawn from a rotating pool of 18 healthy volunteers by venipuncture into sodium citrate (1:9 v/v) in accordance with an Oregon Health &

Science University IRB-approved protocol, as previously described.[145] Blood was centrifuged at 200 × *g* for 20 minutes to obtain platelet rich plasma (PRP), and platelets were isolated from PRP by centrifugation at 1000 × *g* for 10 minutes in the presence of prostacyclin (0.1 μg/ml). Platelets were resuspended in modified HEPES/Tyrode buffer and washed once via centrifugation at 1000 × *g* for 10 minutes. Washed platelets were resuspended in modified HEPES/Tyrode buffer to the indicated concentration.

4.4.3 *Platelet aggregation*

Aggregation studies were performed using 300 μl platelets (2×10^8/ml) pre-treated with inhibitors for 10 minutes. Platelet aggregation was initiated by CRP (1 or 3 μg/ml) and monitored under continuous stirring at 1200 rpm at 37 °C by measuring changes in light transmission using a PAP-4 aggregometer, as previously described.[130]

4.4.4 *Flow cytometry*

Washed human platelets (2×10^7/ml) were pretreated with inhibitors for 10 minutes before stimulation with CRP (10 μg/ml) or thrombin (1 U/ml) for 20 minutes in the presence of CD62P-FITC, PAC-1-FITC, OG488-FG, or CD61-PE. Samples were diluted in HEPES/Tyrode buffer and analyzed by flow cytometry on BD FACSCanto II (Beckton Dickinson). Platelets were gated by forward and side scatter, and activation marker expression was recorded as geometric mean fluorescence. Results were analyzed in Flowing Software.

4.4.5 *Platelet aggregate formation under flow*

Sodium citrate-anticoagulated blood was pre-treated with inhibitors or antibodies for 10 minutes and perfused at 2200 s^{-1} and 37°C through glass capillary tubes coated with collagen (100 μg/ml) and surface blocked with denatured BSA to form platelet aggregates, as previously described.[131] Imaging of aggregates was performed using Köhler-illuminated Nomarski DIC optics with a Zeiss 40 × 0.75 NE EC Plan Neofluar lens on a Zeiss Axiocam MRm camera and Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.). Aggregate surface area was computed by manually outlining and quantifying platelet aggregates, as previously described.[131]

4.4.6 *Hsp70 signaling and interaction studies*

For Hsp70 protein association studies, Hsp70-GST and GST proteins were expressed *in vitro* from plasmid encoding GST-tagged human HSPA1A (Genecopoeia, Inc.) or pGEX, as previously described.[157] LAT immunoprecipiations and Western blotting were carried out as previously described[130]

4.4.7 *Statistical Analysis*

Data were analyzed by two-way ANOVA (date and treatment as factors), and post-hoc analysis was performed via Tukey's honest significant difference (HSD) test. $P < 0.05$ was considered statistically significant for all tests. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria).

4.5 Results

4.5.1 *Hsp70 expression and localization in platelets*

To investigate a role for Hsp70 in platelet physiological function, we first examined the relative expression of Hsp70 and Hsp90 proteins in human platelets. Human platelet lysates were separated by gel electrophoresis, transferred to nitrocellulose and analyzed for Hsp70 and Hsp90 expression by Western blot. As seen in Figure 4.1A, human platelets contain abundant Hsp70 protein at levels similar to MDA-MB-231 breast cancer cells. Platelets also express Hsp90 protein; however, the ratio of Hsp90 to Hsp70 proteins in platelets was less than in nucleated MDA-MB-231 cells. In eukaryotic cells, Hsp proteins regulate a multitude of functions associated with the maintenance of protein quality control in the endoplasmic reticulum and cytoplasm as well as at specific intracellular membrane systems.[158] Accordingly, we next examined the intracellular localization of Hsp70 and Hsp90 proteins in human platelets adherent to a surface of fibrinogen by fluorescence microscopy. As seen in Figure 4.1B, Hsp70 localized in punctate pattern throughout the platelet cytoplasm, as revealed by co-staining of platelets for Hsp70 and actin. Co-staining of platelet Hsp70 together with Hsp90 revealed partial co-localization of these Hsp chaperone proteins in the organelle- and vesicle-rich platelet granulomere while Hsp70 showed a more diffuse localization throughout the platelet cytoplasm as well as the platelet plasma membrane apart from Hsp90 (Figure 4.1C). Co-staining of Hsp70 together with protein disulfide isomerase (PDI), an endoplasmic reticulum (ER)-localized protein chaperone which is enriched in the platelet dense tubular system (DTS),[159] demonstrated minimal co-localization with Hsp70, suggesting that chaperone functions for Hsp70 and PDI are differentially compartmentalized in platelets (Figure 4.1D).

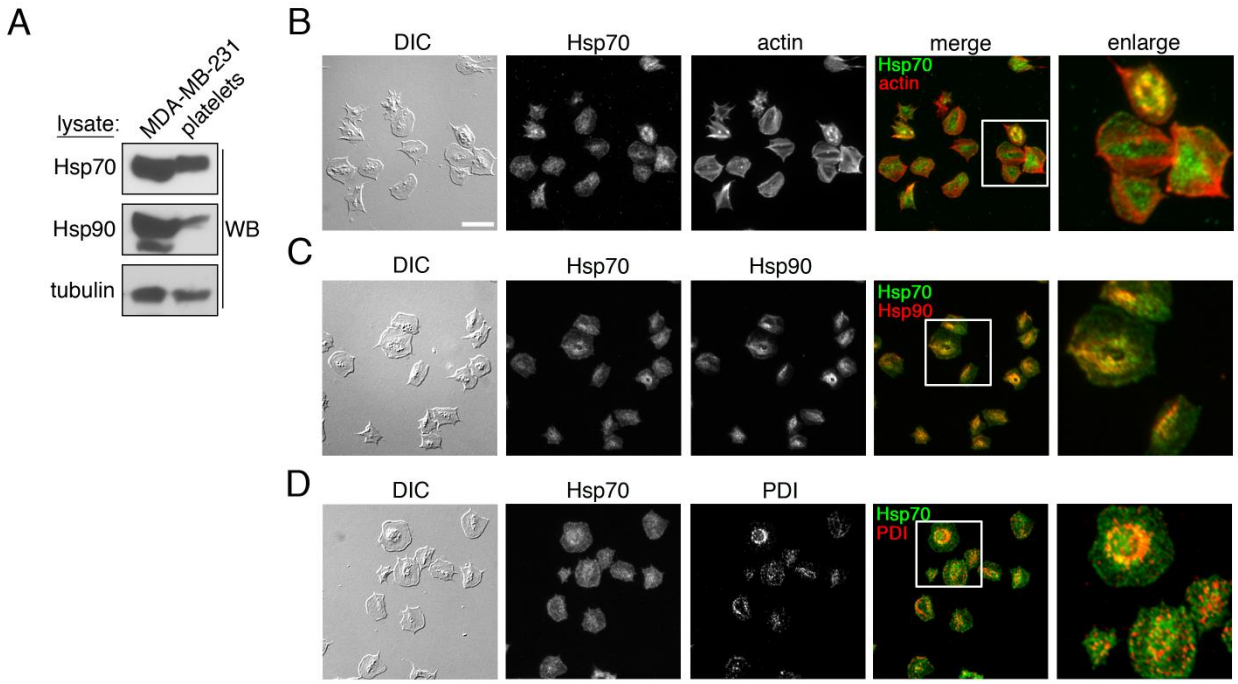

Figure 4.1. Hsp70 expression and localization in human platelets. (A) Purified human platelets (1×10^9/ml) and MDA-MB-231 breast cancer cells were lysed directly into Laemmli sample buffer, separated by SDS-PAGE and analyzed for Hsp70 and Hsp90 protein expression by Western blot (WB). Samples were normalized to total α-tubulin content to control for protein loading. **(B)** Replicate samples of washed human platelets (2×10^7/ml) were spread on a surface of fibrinogen before fixation, staining and visualization by differential interference contrast (DIC) and fluorescence microscopy for expression and colocalization of Hsp70 (green) and actin (red), **(C)** Hsp70 (green) and Hsp90 (red) and **(D)** Hsp70 (green) and PDI (red). Scale bar = 10 μm.

4.5.2 *Hsp70 inhibition reduces platelet aggregation and P-selectin exposure*

Upon stimulation with the glycoprotein (GP)VI agonist collagen-related peptide (CRP), platelet Src kinase activation promotes the assembly of the LAT signalosome at the platelet plasma membrane to support intracellular calcium signaling events as well as "inside-out" mediated conformational changes of integrin $\alpha_{IIb}\beta_3$ to, in turn, drive fibrinogen binding, granule secretion and platelet-platelet aggregation.[21] To test the hypothesis that Hsp70 activity has roles in the platelet activation program, we first examined the ability of platelets to aggregate in response to CRP under Hsp70-inhibited conditions. To inhibit Hsp70 activities, we took advantage of two separate pharmacological

inhibitors of Hsp70, VER-155008 and MKT-077 (referred to as VER and MKT hereafter). VER is an adenosine derivative that interacts directly with the ATP binding pocket (NBD) of Hsp, Hsc70, and Grp78 [160-162]. MKT is a rhodacyanine dye that binds mitochondrial Hsp70 (mtHsp70 or mortalin), Hsc70, and Hsp70 at an allosteric site near the ATP binding site (NBD), preventing allosteric communication between the Hsp70 NBD and SBD.[163, 164] As seen in Figure 4.2A, aggregation of washed human platelets in response to stimulation with 1 µg/ml CRP was strongly inhibited by 20 µM MKT or VER (>80% inhibition relative to control); aggregation in response to 3 µg/ml CRP was also inhibited by 20 µM MKT or VER. Conversely, aggregation in response to the thrombin receptor-activating peptide-6 (TRAP-6), which promotes platelet activation through protease activated receptor (PAR)-coupled G_q and G_{13} pathways independent of ITAM receptor and LAT signalosome-mediated signaling, was not significantly inhibited by 20 µM MKT or VER.

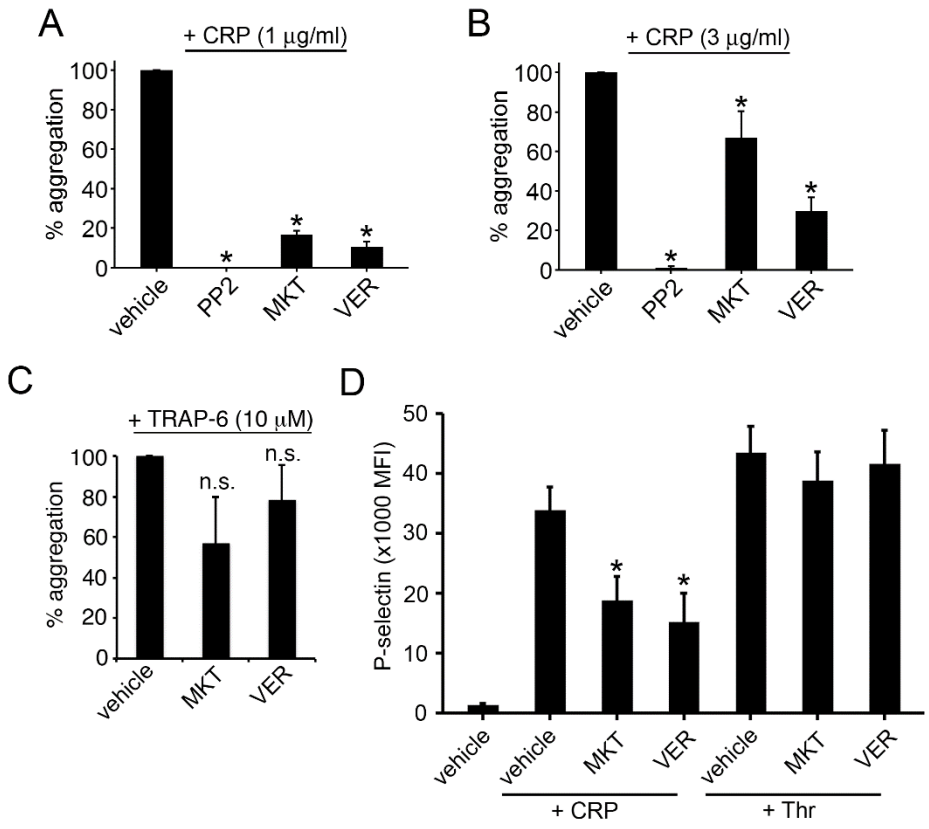

Figure 4.2. Hsp70 inhibitors block platelet aggregation and P-selectin exposure. Replicate samples of washed human platelets (2 × 108/ml, n = 3-5), were pretreated with the Src kinase inhibitor PP2 (20 μM), the Hsp70 inhibitors MKT-077 (20 μM) or VER-155008 (20 μM), or vehicle alone (0.1% DMSO) prior to stimulation with (A) 1 μg/ml collagen-related peptide (CRP) or (B) 3 μg/ml CRP and analysis for platelet aggregation by Born aggregometry. Changes in optical density were recorded as a vertical drop and lag times to quantify the extent of platelet aggregation. * indicates $p < 0.05$. (C) Replicate samples of washed human platelets (2 × 108/ml, n = 3-7), were pretreated with the Src kinase inhibitor PP2 (20 μM), the Hsp70 inhibitors MKT-077 (20 μM) or VER-155008 (20 μM) or vehicle alone (0.1% DMSO) prior to stimulation with 10 μg/ml CRP or 1 U/ml thrombin and analysis for P-selectin (CD62P) surface exposure by flow cytometry. * indicates $p < 0.05$.

Platelet activation by CRP initiates the secretion of P-selectin from platelet alpha granules to support platelet aggregate growth and stability through interactions with integrin $\alpha_{IIb}\beta_3$ that also allow for fibrinogen binding and platelet-platelet aggregation.[165] To examine the role of Hsp70 in platelet granule secretion and P-selectin exposure by flow cytometry, washed human platelets were treated with Hsp70 inhibitors before stimulation with CRP or thrombin and labeling with antibodies against

P-selectin (CD62P). As seen in Figure 4.2C, MKT or VER (20 μM) both significantly decreased P-selectin exposure upon stimulation by CRP (55.7 ± 1.8% and 45.1 ± 4.2% of control, respectively) but not by thrombin (89.1 ± 11.2% and 95.4 ± 13.0% of control, respectively).

4.5.3 *Hsp70 inhibition blocks platelet integrin $\alpha_{IIb}\beta_3$ activation*

Next, to specifically examine "inside-out" integrin $\alpha_{IIb}\beta_3$ activation following Hsp70 inhibition, Hsp70-inhibited platelets were stimulated with CRP before incubation with fluorescently labeled PAC-1 antibodies that bind specifically to activated integrin $\alpha_{IIb}\beta_3$. As seen in Figure 4.3A, MKT or VER (20 μM) decreased the activation of integrin $\alpha_{IIb}\beta_3$ upon CRP stimulation as indicated by decreased detection of PAC-1 (67.1 ± 7.8% and 7.2 ± 0.7% of control, respectively). Hsp70 inhibition and loss of $\alpha_{IIb}\beta_3$ activation was associated with a reduced capacity of stimulated platelets to bind to soluble fibrinogen, as measured by flow cytometry analysis of Hsp70-inhibited platelets capacity to bind OG488-labeled fibrinogen (31.7 ± 8.7% and 27.5 ± 6.1% of control, respectively; Figure 4.3B). The reduction in fibrinogen binding following Hsp70 inhibition was not likely the result of a reduced integrin $\alpha_{IIb}\beta_3$ present on the surface of the platelet plasma membrane, as resting integrin β_3 (CD61) surface expression was not significantly changed by treatment with 20 μM MKT or VER (107.1 ± 7.1% and 104.5 ± 8.0% of control, respectively, $n = 4$), as determined by flow cytometry analysis.

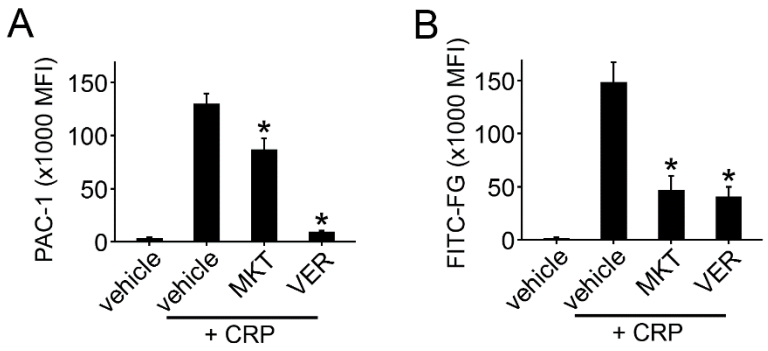

Figure 4.3. Inhibition of Hsp70 prevents platelet integrin $\alpha_{IIb}\beta_3$ activation and fibrinogen binding. Replicate samples of washed human platelets (2×10^8/ml, n = 5), were pretreated with the Src kinase inhibitor PP2 (20 μM), the Hsp70 inhibitors MKT-077 (20 μM) or VER-155008 (20 μM) or vehicle alone (0.1% DMSO) prior to stimulation with 10 μg/ml CRP and flow cytometric analysis for integrin $\alpha_{IIb}\beta_3$ activation as determined by **(A)** PAC-1 binding or **(B)** fluorescent fibrinogen binding.

4.5.4 *Inhibition of Hsp70 dampens platelet aggregate formation under shear*

In addition to supporting platelet aggregation in solution, GPVI signaling, integrin $\alpha_{IIb}\beta_3$ "inside-out" activation and fibrinogen binding serve roles in platelet aggregate growth upon surfaces of extracellular matrix proteins under conditions of physiological shear.[166] Accordingly, we next examined platelet aggregate formation on collagen under physiological shear using control and Hsp70-inhibited conditions. Whole, citrated human blood was pretreated with Hsp70 inhibitor before perfusion over a surface of collagen at an arterial shear rate of 2200 sec^{-1} for 5 min. Adherent cells were fixed, visualized and quantified for surface area. As seen in Figure 4.4, VER (40 μM), which is bioavailable *in vivo*,[161] reduced aggregate surface area to 42.7 ± 11.4% of control (0.1% DMSO). This *ex vivo* assay of platelet function supports a role for Hsp70 in whole blood in regulating integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregate formation under shear.

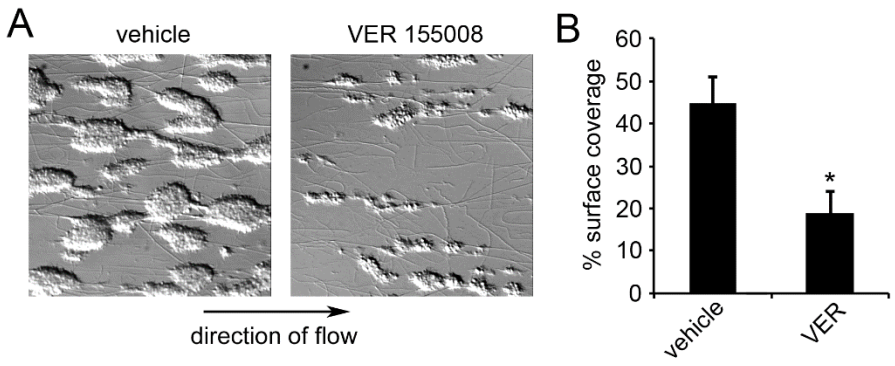

Figure 4.4. *Ex vivo* inhibition of Hsp70 prevents platelet aggregate formation under physiological shear. (A) Whole blood was treated with vehicle (DMSO, 0.1%) or VER-155008 (40 μM) and perfused at 2200 s^{-1} at 37°C through capillary tubes coated with collagen (100 μg/ml) to form platelet aggregates. **(B)** The percent surface area covered by aggregates was computed by outlining and quantifying platelet aggregates. * signifies $p < 0.05$ with respect to DMSO control; error bars represent S.E.M; $n = 3$; scale bar = 10 μm.

4.5.5 *Hsp70 interacts with and regulates components of the LAT signalosome*

The inhibition of integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation as well as P-selectin exposure in response to CRP but not PAR agonists under Hsp70-inhibited conditions suggested a role for Hsp70 in the intracellular signaling events that drive platelet activation downstream of GPVI engagement.[21] Accordingly, we next examined the activation of signaling pathways downstream of CRP stimulation in platelets under Hsp70-inhibited conditions. As seen in Figure 4.5A, under control conditions, stimulation of platelets with CRP promoted the activation of Src-mediated tyrosine kinase signaling events, as determined by Western blot for total phosphotyrosine proteins with 4G10 antisera as well as phosphorylated Src, phosphorylated LAT and PLCγ2 as well as Akt phosphorylation (Figure 4.5B). As expected, the activation of kinase signaling was abolished by preincubation of platelets with the Src kinase inhibitor PP2, which blocks the earliest steps of platelet activation initiated by CRP stimulation (Figure 4.5A, B). However, despite a decrease in platelet integrin activation, aggregation and P-selectin exposure following CRP stimulation (Figure 4.2-Figure 4.3), inhibition of Hsp70 did not significantly affect CRP-stimulated protein tyrosine

phosphorylation, Src kinase phosphorylation or Akt activation, while partially inhibiting PLCγ2 phosphorylation (Figure 4.5A, B).

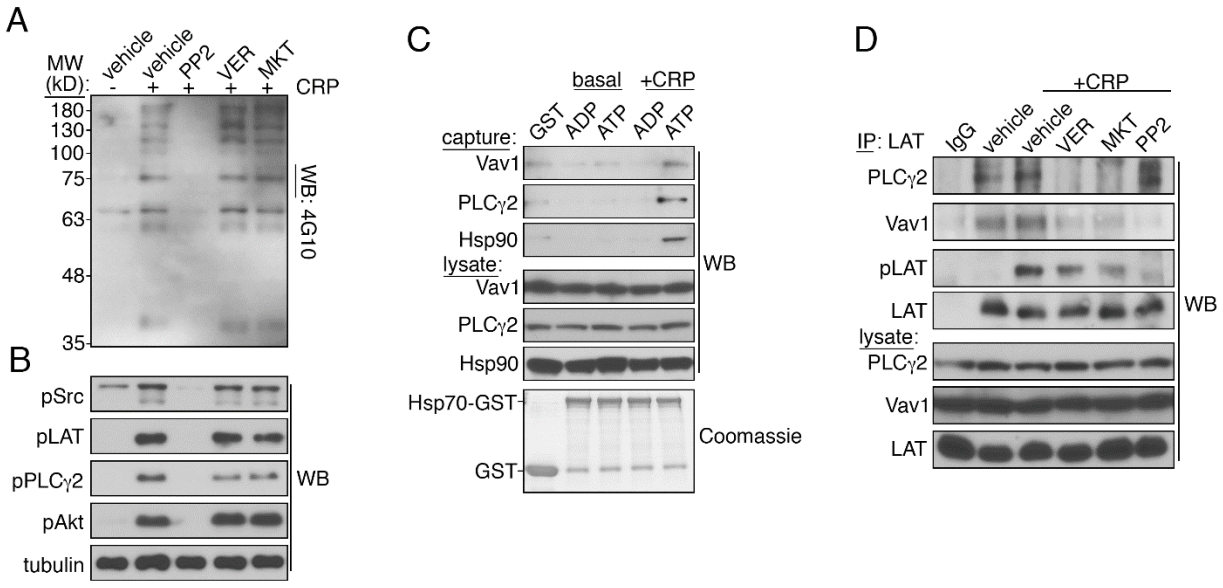

Figure 4.5. Hsp70 interacts with and regulates the organization of the LAT signalosome. (A) Replicate samples of washed human platelets (5×10^8/ml) were pretreated with the Src kinase inhibitor PP2 (20 μM), the Hsp70 inhibitors VER-155008 (20 μM) and MKT-077 (20 μM) or vehicle alone (0.1% DMSO) for 10 min prior to stimulation with CRP (10 μg/ml, 5 min). Protein tyrosine phosphorylation was analyzed by Western blot (WB) using 4G10 antisera. Protein molecular weight markers (MW) are shown. **(B)** Phosphorylation of Src kinase pTyr416 (pSrc), LAT pTyr171 (pLAT), PLCγ2 pTyr759 (pPLCγ2) and Akt pSer473 (pAkt) was also examined. α-tubulin levels serve as a control for equal protein loading. **(C)** Recombinant GST protein (1 μg) or Hsp70-GST protein (1 μg) pre-loaded with 10 μM ADP or 10 μM ATP was incubated with basal and CRP-stimulated platelet lysates prior to protein capture with glutathione sepharose and Western blot (WB) analysis for co-captured Vav1, PLCγ2 and Hsp90. Coomassie staining confirmed efficient pull-down of GST and Hsp70-GST proteins. **(D)** Platelets (5×10^8/ml) were pretreated with PP2 (20 μM), VER-155008 (20 μM), MKT-077 (20 μM) or vehicle alone (0.1% DMSO) prior to stimulation with CRP (10 μg/ml, 5 min) and lysis into IP buffer. LAT immunoprecipitates (IP) were examined for capture of total (LAT) and phosphorylated LAT (pLAT) protein as well as coprecipitating PLCγ2 and Vav1 by Western blot (WB). Representative signaling, protein capture and LAT immunoprecipitation results are representative of three experiments.

Like inhibition of Hsp70, genetic deletion of the Linker for Activation of T cells (LAT)—a scaffolding protein that organizes functional interactions amongst key mediators of platelet activation

downstream of ITAM receptor stimulation, including SLP-76, Vav1/3 and PLCγ2—has limited effects on tyrosine kinase activation and protein phosphorylation downstream of GPVI engagement.[167] LAT deletion also disrupts the organization of PLCγ2 mediated signaling requisite for platelet integrin activation, aggregation and granule secretion.[168, 169] Given the diverse roles for Hsp70 in protein complex assembly in a number of intracellular signaling processes,[73] particularly at the intracellular surface of the plasma membrane,[72] we next examined the ability of ADP- and ATP-loaded Hsp70 to interact with components of the LAT signalosome from resting and activated platelets *in vitro*. As seen in Figure 4.5C, under ATP-loaded conditions, immobilized GST-Hsp70 supported the capture of LAT signalosome components including Vav1 and PLCγ2, from CRP-stimulated platelet lysates (Figure 4.5C). As a positive control, ATP-loaded Hsp70 also supported the capture of Hsp90 from platelet lysates (Figure 4.5C).

Next, to examine roles for Hsp70 activity in LAT signalosome organization and platelet function, we immunoprecipitated endogenous LAT from resting and CRP-activated platelets under control and Hsp70-inhibited conditions and monitored the co-precipitation of LAT signalosome associated proteins (Figure 4.5D). Western blot analyses of LAT immunoprecipitates confirmed that LAT readily immunoprecipitated from platelet lysates (Figure 4.5D). In agreement with signaling studies (Figure 4.5A), stimulation of platelets with CRP prior to lysis and LAT immunoprecipitation upregulated the phosphorylation of immunocaptured LAT, which was inhibited in the presence of PP2 but not Hsp70 inhibitors VER and MKT (Figure 4.5D). Notably, PLCγ2, which co-precipitated with LAT under resting as well as CRP-stimulated conditions, did not associate with LAT in the presence of Hsp70 inhibitors VER or MKT. Hsp70 inhibition similarly prevented the detectable interaction of LAT with Vav1, which associates with the LAT signalosome to regulate PLCγ2 activation and platelet aggregation [170]. Together, these results suggest that in platelets, Hsp70

activity has a role in organizing and maintaining the assembly of the LAT signalosome independent of the activation of tyrosine kinases that signal the initiation and progression of the platelet activation program in response to CRP.

4.6 Discussion

Here we report a role for the molecular chaperone Hsp70 in platelet function. Physiological, biochemical and flow cytometry experiments highlighted a role for Hsp70 in platelet integrin $\alpha_{IIb}\beta_3$ activation, granule secretion and aggregation, as two distinct Hsp70 inhibitors both reduced CRP-induced PAC-1 or fluorescent fibrinogen binding as well as P-selectin surface exposure and platelet aggregation. Hsp70 inhibition also prevented platelet aggregate formation in an *ex vivo* model of blood flow under arterial shear, demonstrating an inhibitory effect under physiological shear in addition to static conditions. Together with intracellular signaling and protein complex studies of the LAT signalosome, our results suggest that following the activation of ITAM-bearing receptor systems in platelets, Hsp70 helps to organize intracellular molecular processes that orchestrate "inside-out" integrin activation as well as secretion events that drive platelet-platelet aggregation.

Previous studies have alluded to roles for Hsp70 as well as Hsp90 family members in platelet function. Phosphorylated Hsc70 associates with Hsp90 and protein phosphatase 1 (PP1) in resting platelets and dissociates from this complex upon platelet activation by collagen but not thrombin.[60] Inhibition of Hsp90 reduces the trafficking of the ATP-gated ion channel P2X1 to the platelet surface and also interferes with P2X1 calcium channel gating, reducing receptor responsiveness to ATP.[171] Grp94, the ER paralog of Hsp90, assists in folding and assembly of the platelet glycoprotein Ib-IX-V complex, the receptor for von Willebrand factor (vWF)-mediated platelet adhesion and activation.[59] In this study, we identified a role for Hsp70 in platelet activation and

aggregation associated with the "inside-out" activation of integrin $\alpha_{IIb}\beta_3$. While inhibition of Hsp70 did not limit the localization of integrin $\alpha_{IIb}\beta_3$ on the platelet surface, Hsp70 inhibition abrogated platelet functional responses associated with "inside-out" integrin $\alpha_{IIb}\beta_3$ activation, notably platelet aggregation as well as granule secretion (Figure 4.2) and platelet aggregate formation under physiological shear (Figure 4.4).

Despite the inability of platelets to fully activate integrin $\alpha_{IIb}\beta_3$ and aggregate in response to CRP stimulation under Hsp70-inhibited conditions (Figure 4.2 and Figure 4.3), the intracellular signaling systems that support these processes appear to activate normally (Figure 4.5A), suggesting potential organizational defects in the molecular events of platelet activation when Hsp70 is inhibited. As the effects of Hsp70 inhibition phenocopy those reported for genetic deletion of LAT in platelets,[167] we examined roles for Hsp70 in the assembly and organization of LAT signalosome. Following activation of ITAM-bearing receptors such as GPVI/FcRγ and CLEC-2, tyrosine phosphorylation of LAT organizes a complex of key mediators of platelet activation, most notably PLCγ2, as well as Vav1, SLP-76, Btk/Tec, Grb2 and Gads [172] to support platelet granule secretion, integrin activation and platelet aggregation.[167, 173] We found that recombinant, ATP-loaded Hsp70 captured Vav1 and PLCγ2 from CRP-stimulated platelet lysates (Figure 4.5C). Immunoprecipitation of endogenous LAT from resting and CRP-activated platelets revealed that Hsp70 inhibition blocked the association of PLCγ2 as well as Vav1 from LAT but had no effect on LAT phosphorylation (Figure 4.5D). To date, there have been no reports of chaperone activities in the regulation of LAT-associated signaling and cellular physiological function. However, Vav1, a component of the LAT signalosome, associates with and is regulated by Hsc70 in tumor cells.[174] Also, the Hsp70 co-chaperone BAG-3 helps to regulate phospholipase protein signaling downstream of EGF receptor activation.[175, 176] While BAG proteins have not yet been examined for roles in platelet

physiology, proteomics studies have reported the expression of a number of BAG family members in platelets.[64]

In addition to the heat shock protein family members, oxidoreductase chaperones have emerging roles in platelet activation and thrombotic function. Protein disulfide isomerase (PDI) and two of its homologs (ERp5 and ERp57) have been found to be released from endothelial cells and platelets, with endothelial PDI being essential for thrombus formation and platelet PDI essential for thrombus growth.[53] Extracellular PDI, ERp5, ERp57, and the endoplasmic reticulum oxidoreductase Ero1α have been found to regulate the active conformation of integrin $\alpha_{IIb}\beta_3$, and blocking these thiol isomerases inhibits activation of $\alpha_{IIb}\beta_3$.[49-52] Like the oxidoreductase chaperones PDI, ERp5, ERp57, and Ero1α, our results show a role for Hsp70 chaperone activity in integrin activation, suggesting that chaperones in addition to thiol isomerases may regulate platelet integrin conformational changes and activation. While our data support a role for intracellular Hsp70 in the regulation platelet secretion as well as integrin activation, we cannot exclude a role for extracellular Hsp70 in supporting platelet integrin activation, fibrinogen binding and thrombus formation. Indeed, while Hsp70 is primarily present intracellularly, under activating conditions Hsp70 can translocate to the cell surface and is also secreted from exosomes.[177, 178] Extracellular Hsp70 binds toll-like receptors (TLRs) and other surface receptors on neighboring macrophages, monocytes, neutrophils or dendritic cells, which initiates an inflammatory response through activation of the NFκB and MAP kinase (MAPK) pathways.[179-184] A hypothetical role for extracellular Hsp70 activity is also supported by work in other cell models showing that Hsp70 associates with integrins,[71] that the Hsp70 family member Grp78/BiP and PDI cooperate to carry out chaperone activities,[70] and that the cytosolic Hsp70 member Hsc70 associates with PDI and Erp57 in endothelial cell lipid rafts to facilitate integrin activation.[60, 70]

Apart from specific platelet-based functions, Hsp70 has hypothesized roles in cardiovascular health that may be linked to platelet physiology. Circulating Hsp70 has a role in vascular calcification in patients with atherosclerosis.[113] Cardioprotective roles have also been shown for circulating Hsp70, suggesting that higher levels of Hsp70 in hypertensive individuals can mitigate the development of atherosclerosis, potentially through inflammatory functions mediated by Hsp70.[185] Furthermore, intracellular Hsp70 has been shown to exert a cardioprotective effect through association with integrin-linked kinase (ILK) in cardiomyocytes.[107] While platelet ILK has a role in integrin $\alpha_{IIb}\beta_3$ activation and thrombus growth,[186] specific connections between ILK and Hsp70 in platelets have not yet been investigated. Surface Grp78 in platelets was also found to have an atheroprotective role through binding and inactivation of tissue factor.[58] The contrasting effects of circulating and intracellular Hsp70 suggest that Hsp70 plays diverse roles in circulatory cell function in a manner related to the cardiovascular and inflammatory state of the patient or experimental subject in question.

In conclusion, this study finds a role for Hsp70 activity in platelet hemostatic function associated with the regulation of integrin activation and granule secretion following stimulation of platelet ITAM receptors. Our data suggest that Hsp70 activity ensures the proper molecular organization of intracellular mediators of the platelet activation program around the LAT signalosome, including Vav1 and PLCγ2, which support activation of integrin $\alpha_{IIb}\beta_3$ and granule secretion to mediate platelet aggregation and aggregate formation under shear. Given the emerging roles of molecular chaperones in the regulation of cellular signaling pathways and cellular physiology, Hsp70 associated activities represent an interesting topic of investigation for future studies of physiological and pathological processes that impact platelet activation in normal physiology and disease states.

Chapter 5. Protease-activated receptor 4 (PAR4) activity promotes platelet granule release and platelet-leukocyte interactions

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5.1 Abstract

Human platelets express two protease-activated receptors, PAR1 (F2R) and PAR4 (F2RL3), which are activated by a number of serine proteases that are generated during pathological events and cause platelet activation. Recent interest has focused on PAR4 as a therapeutic target, given PAR4 seems to promote experimental thrombosis and procoagulant microparticle formation, without a broadly apparent role in hemostasis. However, it is not yet known whether PAR4 activity plays a role in platelet-leukocyte interactions, which are thought to contribute to both thrombosis and acute or chronic thrombo-inflammatory processes. We sought to determine whether PAR4 activity contributes to granule secretion from activated platelets and platelet-leukocyte interactions. We performed *in vitro* and *ex vivo* studies of platelet granule release and platelet-leukocyte interactions in the presence of PAR4 agonists including PAR4 activating peptide, thrombin, cathepsin G, and plasmin in combination with small-molecule PAR4 antagonists. Activation of human platelets with thrombin, cathepsin G, or plasmin potentiated platelet dense granule secretion that was specifically impaired by PAR4 inhibitors. Platelet-leukocyte interactions and platelet P-selectin exposure following stimulation with PAR4 agonists were also impaired by activated PAR4 inhibition in either a purified system or in whole blood. These results indicate PAR4-specific promotion of platelet granule release

and platelet-leukocyte aggregate formation and suggest that pharmacological control of PAR4 activity could potentially attenuate platelet granule release or platelet-leukocyte interaction-mediated pathological processes.

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5.2 Introduction

Studies in this chapter examine the activity of protease-activated receptor 4 (PAR4) in platelet granule release and platelet-leukocyte interactions. PAR4 is an emerging target in the treatment of thrombotic disease, with PAR4 antagonists showing efficacy in animal studies of thrombosis with minimal bleeding side effects. However, less is known about PAR4 activity in inflammation, which drives platelet-leukocyte interactions. Here we demonstrate that PAR4 inhibition impairs platelet granule release and platelet-leukocyte interactions in purified systems and in whole blood. These results show that PAR4 activity contributes to platelet functions beyond hemostasis, suggesting alternative applications for PAR4 antagonists in inflammatory disease treatment.

5.3 Background

Platelets become activated upon vessel injury or inflammation by serine proteases such as thrombin that cleave platelet protease-activated receptors (PARs) and initiate intracellular signaling pathways. Human platelets express PAR1 and PAR4, G-protein coupled receptors (GPCRs) that are activated by proteolytic cleavage of an N-terminal site to reveal a tethered ligand that binds the receptor itself and initiates intracellular G-protein signaling.[187] PAR1 and PAR4 activation of G proteins leads to

signaling cascades causing release of calcium stores, secretion of dense granule contents, and platelet shape change, culminating in platelet activation, adhesion, and aggregation.

Structural differences between PAR1 and PAR4 result in differing outputs in platelet function. PAR1 contains a negatively-charged N-terminal sequence that binds the anion-binding exosite I of thrombin,[188] which allosterically enhances thrombin's activity and enables it to activate both PAR1 and PAR4 while tethered to PAR1. PAR4 lacks this thrombin binding sequence, and higher concentrations of thrombin are required to activate PAR4 compared to PAR1.[189] Thrombin binds PAR1 transiently, causing robust platelet activation that is carefully constrained by rapid phosphorylation, internalization and degradation of the receptor.[190] PAR4 is also internalized to terminate its activity, but this internalization occurs via a different route than that of PAR1, in a manner hypothesized to enable prolonged signaling.[191] These differences in PAR4 result in a response to thrombin that is slower but more sustained over time, with varying functional effects, including described roles in enhancing clot stability and procoagulant microparticle release that suggest a more pro-thrombotic effect of platelet PAR4 activity.[126, 192] Moreover, platelet PAR4 plays a described role in the activation of PKC substrates, which are required for platelet dense granule release.[126] Release of platelet dense granule contents, which include a variety of biologically active molecules, is a physiologically important phenomenon,[84] but it has also been implicated in the pathomechanism of certain diseases.[193, 194]

PAR1 and PAR4 are cleaved by overlapping but distinct sets of proteases, leading to diverse functional outputs. PAR1 is known to be cleaved at its canonical N-terminal site (R^{41}/S^{42}) by thrombin, factor Xa, plasmin, and MMP1/13, and it also can be cleaved at different noncanonical sites by elastase, APC, and proteinase-3.[195] Meanwhile, PAR4 is known to be cleaved only at its canonical site (R^{47}/G^{48}) by thrombin, trypsin, tissue kallikrein, plasmin, and cathepsin G.[196-199]

Given the unique role of neutrophil cathepsin G in cleavage of PAR4, this suggests involvement of PAR4 in facilitating interactions between platelets and leukocytes, including neutrophils, a subset of granulocytes, and monocytes, a subset of peripheral blood mononuclear cells (PBMCs). Platelet-leukocyte interactions increase during pathological conditions such as atherosclerosis and may detrimentally affect disease outcomes.[2, 200]

In addition to observations that the neutrophil releasate cathepsin G activates platelets via PAR4 cleavage, studies have shown that PAR4 activity promotes leukocyte recruitment in animal models of inflammation and pain.[201] Current interest is focused on PAR4 as a potential target against thrombosis, with the PAR4 inhibitor BMS-986120 showing promise in animal models of thrombosis as well as a completed phase 2 clinical trial in combination with aspirin for the prevention of recurrent stroke.[47, 128] However, investigations into the role of PAR4 activity on human platelet-leukocyte interactions are lacking. Therefore, in this study, we investigated the effect of PAR4 activity on platelet dense granule release and platelet-leukocyte interactions to interrogate the potential role of platelet PAR4 in inflammation and innate immunity.

5.4 Materials and Methods

5.4.1 *Reagents*

Activated PAR4 antagonists were synthesized, characterized, dissolved in dimethyl sulfoxide (DMSO), and stored refrigerated as described previously.[202] Structure and characterization of PAR4 antagonists are described in Figure 5.1 and Figure 5.2. All other reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Hanks' Balanced Salt Solution (HBSS) was from Corning cellgro (Manassas, VA, USA). Polymorphprep was from Axis-Shield (Oslo, Norway). PGI_2 and the PAR1 inhibitor SCH 79797 were from Cayman Chemical (Ann Arbor, MI, USA).

Collagen-related peptide (CRP) was from R. Farndale (Cambridge University, UK). TRAP-6 (SFLLRN-NH_2) was obtained from Tocris (Bristol, UK). PPACK (D-Phe-Pro-Arg-chloromethylketone) and RBC Lysis Buffer were from Santa Cruz (Dallas, TX, USA). PAR4 activating peptide (AYPGKF-NH_2) was from Abgent (San Diego, CA, USA). Human α-thrombin and human plasmin were from Haematologic Technologies (Essex Junction, VT, USA). Human cathepsin G was from Innovative Research (Novi, MI, USA). For human flow cytometry studies, anti-CD66b-PE and anti-CD14-PE/Cy7 were from BD Biosciences (Franklin Lakes, NJ, USA), and anti-CD41-FITC was from Invitrogen (Carlsbad, CA, USA). For nonhuman primate flow cytometry studies, anti-CD41-FITC was from Invitrogen, and anti-CD62P-PE and anti-CD45-APC were from BD Biosciences. Chronolume detection agent was from Chrono-Log Corporation (Havertown, PA, USA).

5.4.2 *Platelet preparation*

Venous blood was obtained from healthy volunteers in accordance with an Oregon Health & Science University (OHSU) IRB-approved protocol. No demographic data was collected on volunteers. For washed platelet preparation, blood was drawn into 3.8% trisodium citrate 9:1 (v:v), and acid-citrate dextrose (ACD) was added at 1:10 (v:v). Platelet-rich plasma (PRP) was isolated by centrifugation at 200 *g* for 20 minutes, and platelets were separated from PRP at 1000 *g* for 10 minutes in the presence of prostacyclin (0.1 μg/ml). Platelets were resuspended in modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na_2HPO4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.3, supplemented with 5 mM glucose), washed by centrifugation at 1000 *g* for 10 minutes, and resuspended again in modified HEPES/Tyrode buffer to the specified platelet count. Of note, exogenous $CaCl_2$ was not added to any of the assays.

5.4.3 *Platelet dense granule secretion assay*

Platelet dense granule secretion was measured as luminescence in an ATP-luciferin-luciferase reaction, as previously described.[203] Briefly, washed platelets (20×10^7/ml; 70 µl) were incubated in a white, flat bottom Corning 96-well plate with 10 µl inhibitor or vehicle (1% DMSO) for 15 minutes at 37 °C with orbital shaking. Agonists (10 µl) were added and incubation continued for an additional 10 minutes. Finally, 10 µl Chronolume detection agent was added and luminescence measured on an Infinite M200 spectrophotometer (TECAN, Switzerland).

5.4.4 *Granulocyte preparation*

Human granulocytes were isolated as previously described,[129] with minor modifications. Venous blood was drawn in accordance with an OHSU IRB-approved protocol at 7:1 (v:v) into citrate phosphate dextrose (CPD). Blood was layered onto an equal volume of Polymorphprep and centrifuged at 500 *g* for 45 min, and the middle layer containing granulocytes was removed and washed in Hanks' Balanced Salt Solution (HBSS) at 400 *g* for 10 minutes at 19 °C. The pellet was resuspended in cold sterile H_2O for 30 seconds to lyse remaining red blood cells, followed by dilution in 10× PIPES buffer (250 mM PIPES, 1.1 mM $CaCl_2$, 50 mM KCl, pH 7.4) and HBSS buffer and centrifugation at 400 *g* for 10 minutes at 19 °C. The granulocyte pellet was resuspended in HBSS buffer to the specified cell count.

5.4.5 *Flow cytometry – platelets and granulocytes*

Isolated platelets and granulocytes from the same donor were prepared as described above and combined in equal volume to a final concentration of 2×10^6/ml granulocytes and 20×10^7/ml platelets (1:100) and incubated with inhibitor or vehicle (0.2% DMSO) for 15 minutes at 37 °C.

Treated cells (60 µl) were added to FACS tubes containing 20 µl antibody and agonist mixtures and incubated at room temperature 20 minutes. Antibody dilutions were 1:50 for anti-CD41, 1:80 for anti-CD62P, and 1:400 for anti-CD66b. Samples were fixed in BD Cytofix and modified HEPES/Tyrode buffer (1% PFA final) for 10 minutes before dilution to 300 µl in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA). Samples were collected for 30 seconds at medium flow rate in a BD FACSCantoII and analyzed on FlowJo software v. 10.2 (Ashland, OR).

5.4.6 *Flow cytometry – whole blood*

We developed a whole blood flow cytometry assay based on several approaches.[204, 205] Venous blood from healthy volunteers under an OHSU IRB-approved protocol was drawn 9:1 (v:v) into 3.8% trisodium citrate containing PPACK (50 µM final) and subsequently diluted 1:1 (v:v) in PBS. Blood was incubated with inhibitor or vehicle (0.2% DMSO) for 15 minutes at room temperature. Treated blood (60 µl) was added to FACS tubes containing 20 µl of antibody and agonist mixture and incubated 20 minutes at room temperature. Antibody dilutions were 1:80 for anti-CD41 and anti-CD62P, 1:200 for anti-CD66b, and 1:400 for anti-CD14. Samples were fixed and red blood cells lysed in a mixture of BD Cytofix (1% PFA final) and RBC Lysis Buffer (0.67× final) in PBS for 10 minutes before dilution to 300 µl in RBC lysis buffer in PBS (1× final). Samples were collected for 60 seconds at low flow rate in a BD FACSCantoII and analyzed on FlowJo software v. 10.2 (Ashland, OR).

5.4.7 *Electron microscopy*

Isolated platelets and granulocytes from the same donor were prepared as described above and combined in equal volume to a final concentration of 2×10^6/ml granulocytes and 20×10^7/ml platelets (1:100) and incubated with agonist for 10 minutes at 37 °C. The sample was then

centrifuged at 2500 *g* for 15 minutes, the pellet was fixed in 2.5% glutaraldehyde, and sections were prepared using standard methods in the OHSU Department of Pathology Electron Microscopy Research Laboratory, similar to previous studies demonstrating neutrophil-platelet adhesion.[206]

5.4.8 *Nonhuman primate studies*

Male baboons (*Papio anubis*) were housed and cared for at the OHSU Oregon National Primate Research Center (ONPRC), a Category I facility. All experiments described herein were approved by the OHSU West Campus Animal Care and Use Committee according to the *Guide for the Care and Use of Laboratory Animals* by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (ISBN 0-309-05377-3, 1996). The Laboratory Animal Care and Use Program at the ONPRC is fully accredited by the American Association for Accreditation of Laboratory Animal Care and has an approved assurance (no. A3304-01) for the care and use of animals from the Office for Protection from Research Risks at the National Institutes of Health.

For whole blood flow cytometry experiments in nonhuman primates, methods were similar to the human experiments, with a few modifications. Venous baboon blood was drawn 9:1 (v:v) into 3.8% trisodium citrate containing PPACK (50 μM final) and subsequently diluted 1:1 (v:v) in phosphate-buffered saline (PBS). Blood was incubated with inhibitor or vehicle (0.2% DMSO) for 15 minutes at room temperature. Treated blood (60 μl) was added to FACS tubes containing 20 μl of antibody mixture and incubated 20 minutes at room temperature. Antibody dilutions were 1:80 for anti-CD41, 1:50 for anti-CD62P and 1:300 for anti-CD45. Samples were fixed and red blood cells lysed in a mixture of BD Cytofix (1% PFA final) and RBC Lysis Buffer (0.67× final) in PBS for 10 minutes

before dilution to 300 µl in RBC lysis buffer in PBS (1× final). Samples were collected for 30 seconds at low flow rate in a BD LSR II and analyzed on FlowJo software v. 10.2 (Ashland, OR).

5.4.9 *Data analysis*

Data in bar graphs have been normalized to vehicle (with agonist) to facilitate comparison of inhibitor treatments across agonist conditions. For statistical analysis, a one-way ANOVA was employed with post-hoc analysis using the Dunnett test to compare treatments to vehicle. $P < 0.05$ was considered statistically significant. Analysis was performed in GraphPad Prism software v. 6 (La Jolla, CA).

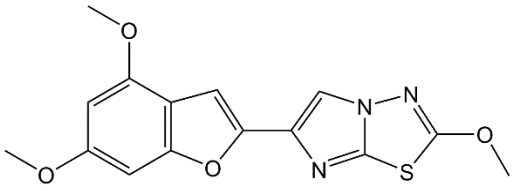
5.5 Results

5.5.1 *PAR4 activity potentiates platelet dense granule secretion*

Platelet dense granules store a number of immunomodulatory molecules such as ADP, serotonin, glutamate, and polyphosphates, and dense granule secretion has been implicated in the proinflammatory recruitment of leukocytes and vascular remodeling in the progression of atherosclerosis.[109, 193, 207] Dense granule secretion follows PAR1- and PAR4-mediated activation of protein kinase C (PKC),[208] but PAR4 has been shown to activate PKC substrates more robustly than PAR1, suggesting a greater role for PAR4 in dense granule secretion.[126] To test whether PAR4 plays a distinct role in platelet dense granule release, we performed a luminescent ATP-luciferin-luciferase assay on washed human platelets stimulated with PAR4 activating peptide (AP, AYPGKF-NH_2), α-thrombin, or two other activators of PAR4, neutrophil cathepsin G and plasmin, which are proteases involved in inflammation and fibrinolysis, respectively. For comparison, platelets were stimulated with the PAR1 activating peptide TRAP-6 or the platelet GPVI

receptor agonist CRP. In select experiments, platelets were pre-incubated with either the PAR1 inhibitor SCH 79797 (CAS # 1216720-69-2) or one of two small-molecule PAR4 antagonists, VU0652925, an analogue of BMS-986120, and VU0661245, an intermediate generated in an effort to define the minimum pharmacophore of VU0652925 (structures shown in Figure 5.1).[202]

VU0661245



VU0652925

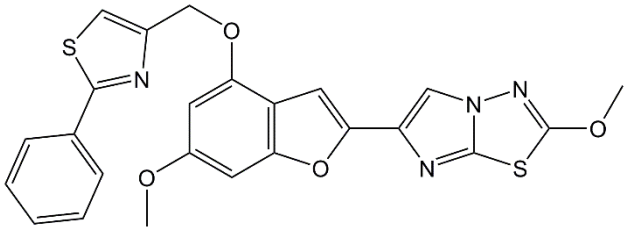


Figure 5.1. Structures of PAR4 antagonists VU0661245 and VU0652925.

Characterization studies show that both PAR4 antagonists inhibit PAR4 activity by either PAR-4 AP or the PAR4 tethered ligand generated by thrombin. However, at higher thrombin concentrations (316 nM γ-thrombin), VU0652925 inhibits platelet activation more potently than VU0661245, as shown in Figure 5.2.

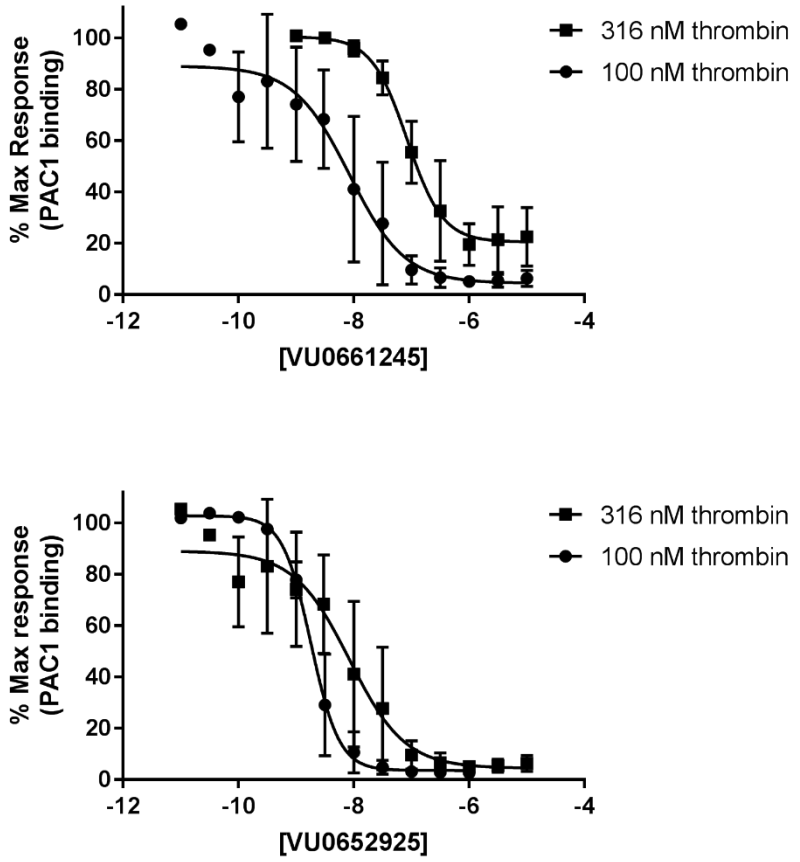


Figure 5.2. Inhibition of thrombin-mediated platelet activation by PAR4 antagonists VU0652925 and VU0661245. Platelets were pre-treated with increasing concentrations (shown in log scale) of VU0652925 or VU0661245 for 20 minutes before activation with 100 nM or 316 nM γ-thrombin. Platelet activation was measured as integrin $\alpha_{IIb}\beta_3$ inside-out activation (PAC1 expression) by flow cytometry. Data are normalized to vehicle control and displayed as means ± S.E.M.; *n* = 3.

In the dense granule release assay, either VU0652925 or VU0661245 blocked dense granule release by PAR4 AP, while the PAR1 inhibitor SCH 79797 had no effect (Figure 5.3A). Conversely, dense granule release by the PAR1 agonist TRAP-6 was blocked by SCH 79797 but not by either PAR4 inhibitor (Figure 5.3B). Dense granule release in response to a moderately high concentration of α-thrombin (5 nM, 0.7 U/ml) was partially blocked by the PAR4 inhibitor VU062925 but not by the PAR1 inhibitor SCH 79797, while VU0652925 and SCH 79797 combined together caused full blockage of dense granule release (Figure 5.3C). When platelets were stimulated by cathepsin G,

VU0652925 completely blocked dense granule secretion, while the PAR1 inhibitor SCH 79797 did not provide any inhibition either alone or in combination with either PAR4 inhibitor (Figure 5.3D). Upon stimulation with plasmin, either PAR4 inhibitor blocked dense granule release, while SCH 79797 had no effect, either alone or in combination with a PAR4 inhibitor (Figure 5.3E). This suggests these potentially inflammatory and profibrinolytic proteases, cathepsin G and plasmin, facilitate platelet dense granule release through cleavage of PAR4 but not PAR1. Finally, none of the PAR4 or PAR1 inhibitors blocked dense granule release by collagen-related peptide (CRP), suggesting no involvement in signaling downstream of the collagen receptor GPVI (Figure 5.3F).

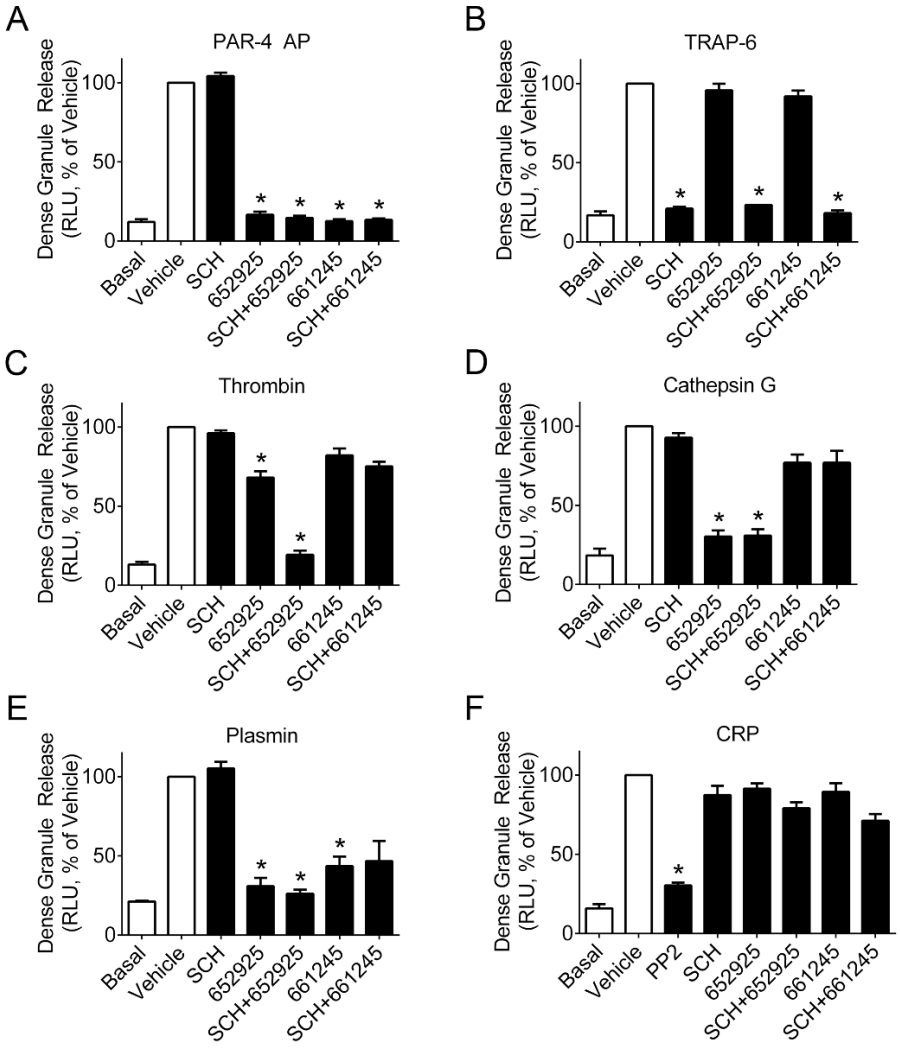


Figure 5.3. PAR4 inhibitors impair platelet dense granule release. Washed human platelets (20 × 10^7/ml) were pretreated with the PAR1 inhibitor SCH 79797 (3 μM), PAR4 inhibitors VU0652925 (10 μM) and VU0661245 (10 μM), Src kinase inhibitor PP2 (10 μM, only shown in CRP condition), or vehicle (0.2% DMSO), then stimulated with the agonists (A) PAR4 AP (activating peptide, 200 μM), (B) TRAP-6 (20 μM), (C) human α-thrombin (5 nM = 0.7 U/ml), (D) human cathepsin G (340 nM = 0.1 U/ml), (E) human plasmin (260 nM = 0.3 U/ml), or (F) CRP (10 μg/ml) and assessed for dense granule release by luminescent ATP assay. Basal = no agonist; n = 3-6 independent experiments; * designates $p < 0.05$ versus vehicle.

5.5.2 *PAR4 activity promotes platelet-granulocyte interactions in a purified system*

Platelet dense granule secretion has been linked to platelet-leukocyte interactions, which are a marker of inflammation and hypothesized to prime leukocyte-endothelial interactions underlying the progression of disease states such as atherosclerosis.[193, 200] Given our results demonstrating the role of PAR4 in facilitating dense granule release via plasmin and cathepsin G, we next investigated whether PAR4 also promotes platelet-leukocyte interactions. Isolated platelets and granulocytes from the same donor were combined, pre-incubated with inhibitors, and stimulated with agonists before analysis. Ultrastructural interactions between activated platelets and neutrophils were observed by electron microscopy (Figure 5.4).

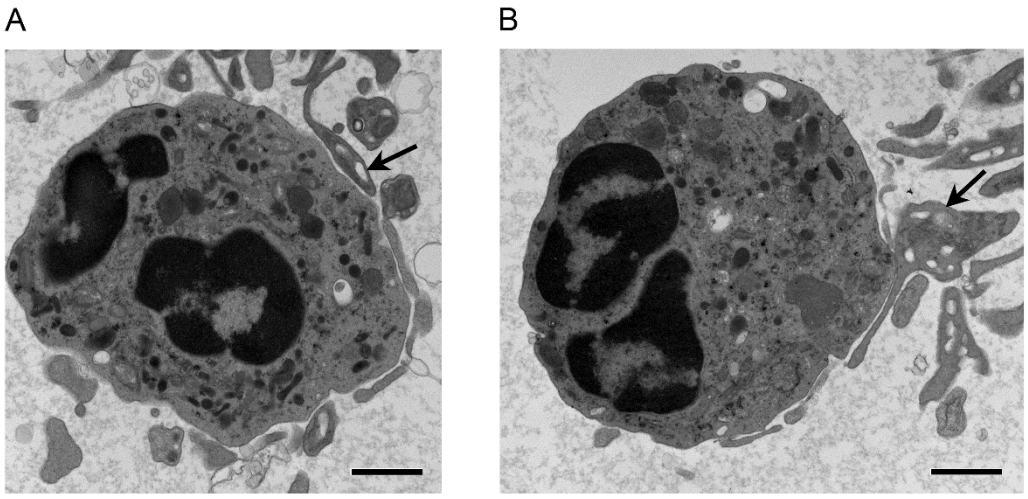


Figure 5.4. Ultrastructural analysis of granulocyte-platelet interactions from purified isolates. Washed human platelets (20×10^7/ml) and purified granulocytes (2×10^6/ml) from the same donor were combined and activated with human α-thrombin (5 nM = 0.7 U/ml) before preparation for electron microscopy. Granulocytes were identified by characteristic ultrastructural features. (A-B) Platelets (arrows) are shown interacting with one granulocyte in each image. Interactions show characteristic molding of surface morphology and cohesion. Scale bar for each panel = 1 μm.

Platelet-neutrophil interactions were analyzed via flow cytometry (Figure 5.5) as expression of the platelet marker CD41 in the granulocyte gate (Figure 5.5C). Platelet-granulocyte interactions were

diminished by PAR4 inhibitors upon stimulation with PAR4 AP (AYPGKF-NH_2), while SCH 79797 had no effect. Platelet-granulocyte interactions following platelet activation with TRAP-6 were blocked by the PAR1 inhibitor SCH 79797 but not either PAR4 inhibitor. Following stimulation by 5 nM α-thrombin, platelet-granulocyte interactions were not significantly impaired by PAR4 or PAR1 inhibitors. When stimulated by cathepsin G, platelet-granulocyte interactions did not increase above the baseline level; the presence of either PAR4 or PAR1 inhibitors had no effect on platelet-granulocyte interactions in the presence of cathepsin G. Meanwhile, platelet-granulocyte interactions stimulated by plasmin were blocked by VU0652925 but not VU0661245 or SCH 79797. Finally, PAR4 or PAR1 inhibitors did not impair platelet-granulocyte interactions induced by the GPVI agonist CRP. These results were recapitulated for the activation state of platelets bound to granulocytes, as quantified by P-selectin (CD62P) surface exposure in the granulocyte gate (Figure 5.5D), except for in the cathepsin G stimulated condition, in which VU0652925 impaired activated platelet-granulocyte interactions. Finally, platelet activation via α-granule release was measured as P-selectin (CD62P) exposure in the platelet gate (Figure 5.5E). P-selectin exposure following stimulation by PAR4 AP was fully blocked by PAR4 inhibitors, while SCH 79797 had no effect. Meanwhile, upon stimulation with TRAP-6, P-selectin surface exposure was blocked by SCH 79797 but not PAR4 inhibitors. P-selectin exposure induced by human α-thrombin was slightly impaired by VU0652925 but not by SCH 79797. Stimulation of platelet P-selectin exposure by cathepsin G was blocked by VU0652925 but not VU0661245 or the PAR1 inhibitor. Meanwhile, stimulation of platelet P-selectin exposure by plasmin was fully blocked by VU0652925 and partially blocked by VU0661245, while SCH 79797 had no effect. Neither PAR4 inhibitor nor SCH 79797 impaired P-selectin exposure stimulated by the GPVI agonist CRP.

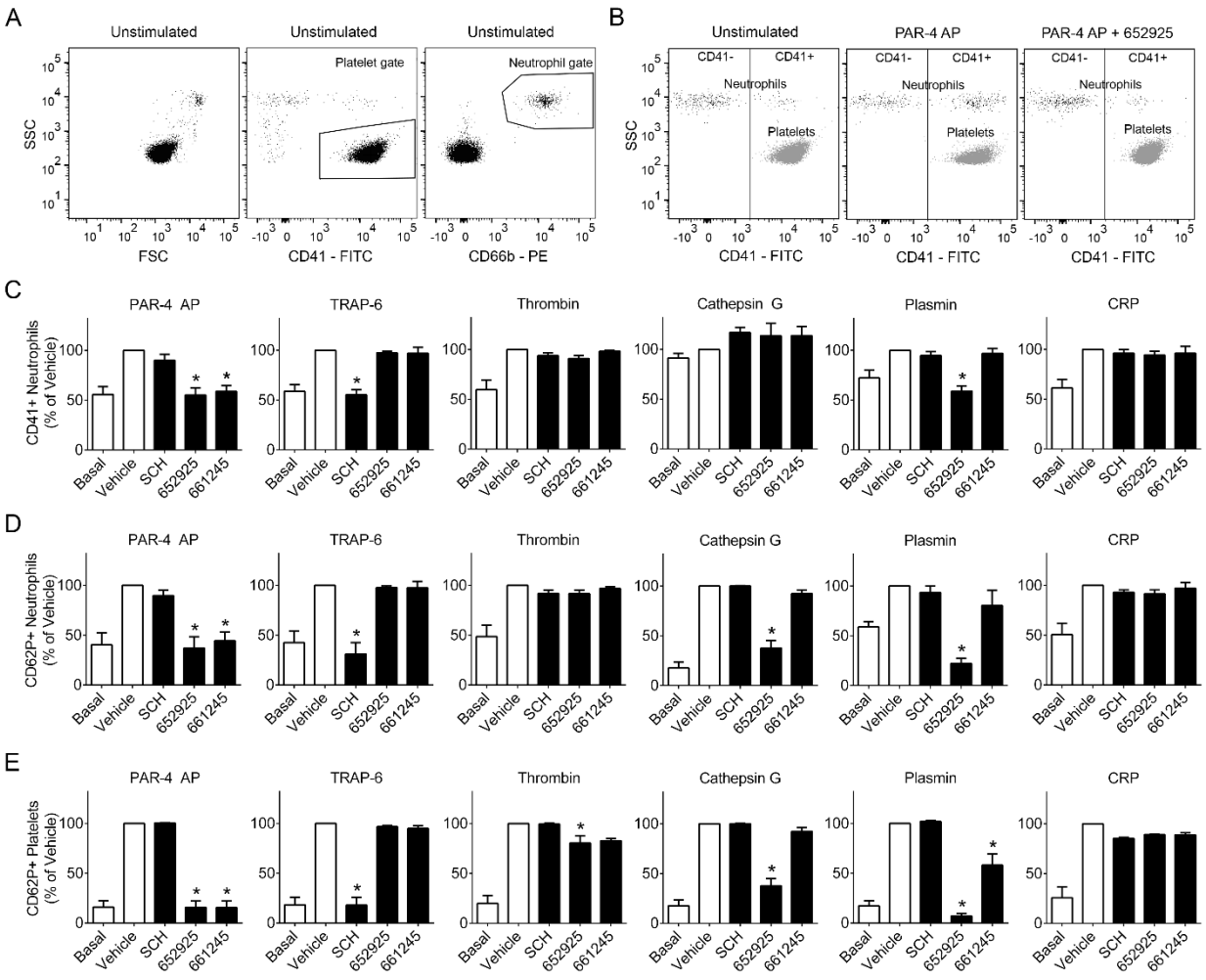


Figure 5.5. PAR4 inhibitors diminish platelet-granulocyte interactions and platelet alpha granule release. Washed human platelets (20×10^7/ml) and purified granulocytes (2×10^6/ml) from the same donor were combined and pretreated with the PAR1 inhibitor SCH 79797 (3 μM), PAR4 inhibitors VU0652925 (10 μM) and VU0661245 (10 μM), or vehicle (0.2% DMSO), stimulated with the agonists PAR4 AP (activating peptide, 200 μM), TRAP-6 (20 μM), human α-thrombin (5 nM = 0.7 U/ml), human cathepsin G (340 nM = 0.1 U/ml), human plasmin (260 nM = 0.3 U/ml), or CRP (10 μg/ml), and stained with markers for granulocytes (CD66b), platelets (CD41), and activated platelets (CD62P). (A) Samples were gated for platelets (CD41+) and granulocytes (CD66b+). (B) Granulocytes (CD66b+) were plotted against CD41 and a line drawn for the CD41+ region. Platelets (CD41+) are shown for comparison. Bar graphs designate (C) percent of CD41-positive granulocytes and (D) percent of activated (CD62P+) platelets per treatment. Basal = no agonist; $n = 3$ independent experiments; * = $p < 0.05$ vs. vehicle.

5.5.3 *PAR4 activity promotes platelet-leukocyte interactions in whole human blood*

To investigate platelet-leukocyte interactions in the context of whole blood, we developed and utilized a whole blood flow cytometry assay (Figure 5.6) using blood drawn directly into the protease inhibitor PPACK to prevent fibrin formation while allowing platelet activation and platelet-leukocyte interactions to occur. The addition of PPACK precluded the use of thrombin, cathepsin G, and plasmin as agonists; instead, the agonists PAR4 AP, TRAP-6, and CRP were utilized to validate the selectivity of the inhibitors in whole blood. Platelet-granulocyte or platelet-monocyte interactions were measured as expression of the platelet marker CD41 in the granulocyte or monocyte gate (Figure 5.6C and D, respectively). Upon stimulation with PAR4 AP (AYPGKF-NH_2), PAR4 inhibitors blocked interactions between platelets and granulocytes and platelets and monocytes, while no inhibition was seen with SCH 79797. Conversely, stimulation of platelet-granulocyte or platelet-monocyte interactions with TRAP-6 was blocked with SCH but not PAR4 inhibitors. Neither the PAR4 inhibitors nor SCH 79797 blocked platelet-granulocyte or platelet-monocyte interactions induced by the platelet GPVI agonist CRP. These results were recapitulated for the activation state of platelets bound to granulocytes or monocytes, measured as P-selectin (CD62P) surface exposure in the granulocyte or monocyte gate (Figure 5.6E and F, respectively). Lastly, platelet activation was measured as expression of P-selectin (CD62P) in the platelet gate (Figure 5.6G). For platelet P-selectin exposure, stimulation with PAR4 AP was also blocked by PAR4 inhibitors but not SCH 79797. TRAP-6-induced P-selectin exposure on platelets was blocked by SCH 79797 but not PAR4 inhibitors. CRP-stimulated P-selectin exposure on platelets was not impaired by either PAR4 inhibitor or the PAR1 inhibitor SCH 79797. These results demonstrate that both platelet-granulocyte interactions and platelet-monocyte interactions are stimulated by PAR4 activity and can be specifically blocked by PAR4 inhibition in a whole blood setting.

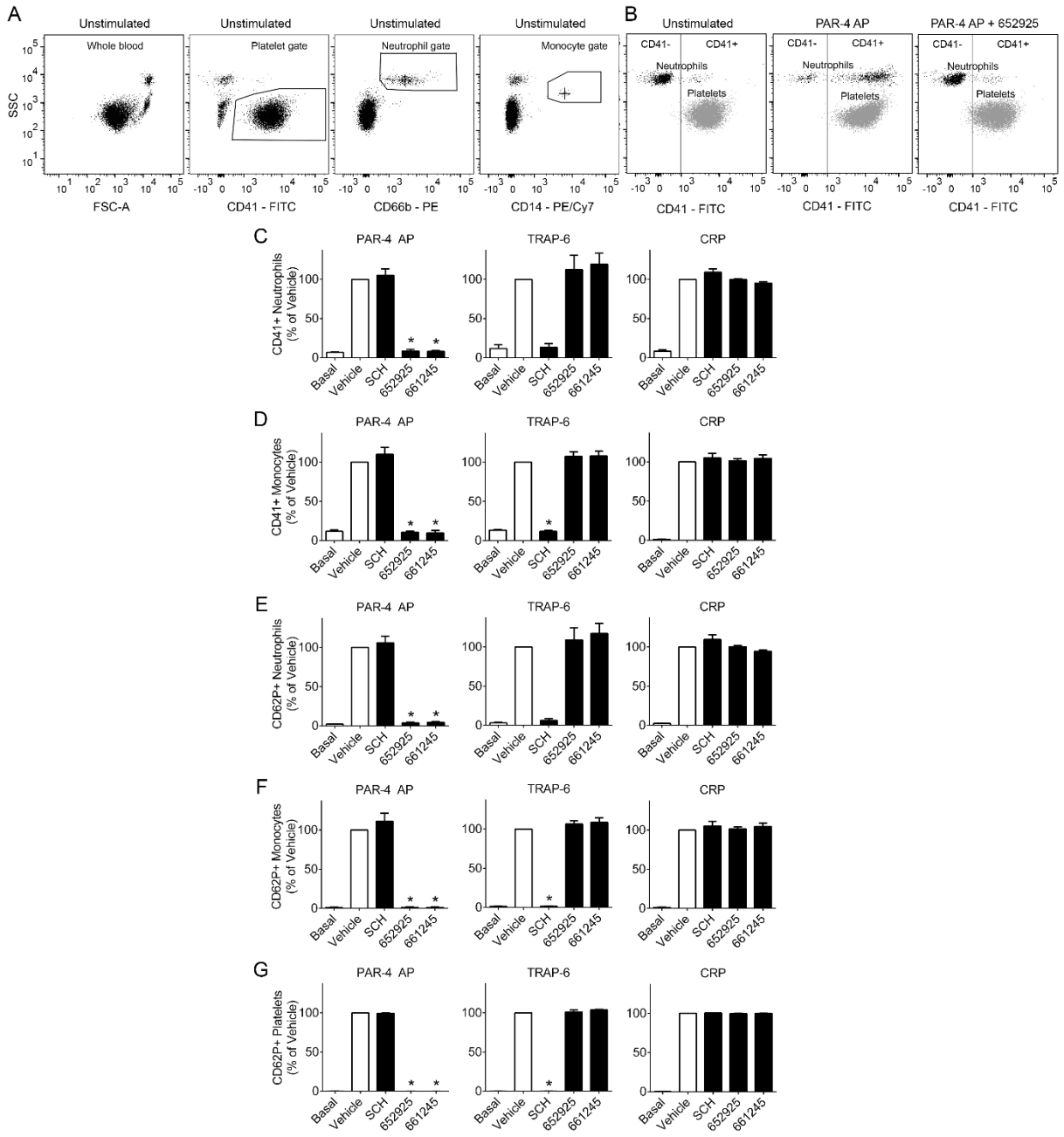


Figure 5.6. PAR4 inhibitors diminish platelet-leukocyte interactions and platelet alpha granule release in whole human blood. Whole human blood was pretreated with the PAR1 inhibitor SCH 79797 (3 μM), PAR4 inhibitors VU0652925 (10 μM) and VU0661245 (10 μM), or vehicle (0.2% DMSO), stimulated with the agonists PAR4 AP (activating peptide, 200 μM), TRAP-6 (20 μM), or CRP (10 μg/ml), and stained with markers for granulocytes (CD66b), platelets (CD41), activated platelets (CD62P), and monocytes (CD14). (A) Samples were gated for platelets (CD41+), granulocytes (CD66b+), and monocytes (CD14+). (B) Granulocytes (CD66b+) or monocytes (CD14+, not shown) were plotted by CD41 and a line drawn for CD41+; platelets (CD41+) shown for comparison. Bar graphs show (C) percent of CD41+ granulocytes, (D) percent of CD41+ monocytes, and (E) percent of activated (CD62P+) platelets. Basal = no agonist; $n = 3$ independent experiments; * = $p < 0.05$ vs. vehicle.

5.5.4 *PAR4 activity promotes platelet-leukocyte interactions in whole baboon blood*

Human and nonhuman primate platelets express PAR1 and PAR4, while murine platelets express PAR-3 and PAR4 and demonstrate other differences in PAR function, making non-human primates a preferred model for *in vivo* studies of PAR4 function for translational studies evaluating safety versus efficacy.[209, 210] In order to validate our whole blood flow cytometry approach in nonhuman primates before further studies, we performed flow cytometry analysis of whole baboon blood *ex vivo* (Figure 5.7). Platelet-granulocyte interactions were measured as expression of the platelet marker CD41 in the granulocyte gate (Figure 5.7C). In alignment with our results in human blood, PAR4 inhibitors blocked interactions between baboon platelets and granulocytes stimulated by PAR4 AP (AYPGKF-NH_2), while SCH 79797 had no effect. Platelet-granulocyte interactions stimulated by TRAP-6 were blocked by SCH 79797 but not PAR4 inhibitors. Neither inhibitor blocked platelet-granulocyte interactions stimulated by CRP. These results were recapitulated for the activation state of platelets bound to granulocytes, measured as platelet P-selectin (CD62P) exposure in the granulocyte gate (Figure 5.7D). Finally, platelet activation was measured as P-selectin (CD62P) exposure in the platelet gate (Figure 5.7E). For platelet P-selectin exposure, PAR4 AP stimulation was blocked by PAR4 inhibitors but not SCH 79797. TRAP-6-stimulated P-selectin exposure was blocked by SCH 79797 but not PAR4 inhibitors. Neither PAR4 inhibitor nor the PAR1 inhibitor blocked P-selectin exposure stimulated by CRP. These results demonstrate efficacy of these inhibitors and feasibility of future studies in a nonhuman primate model.

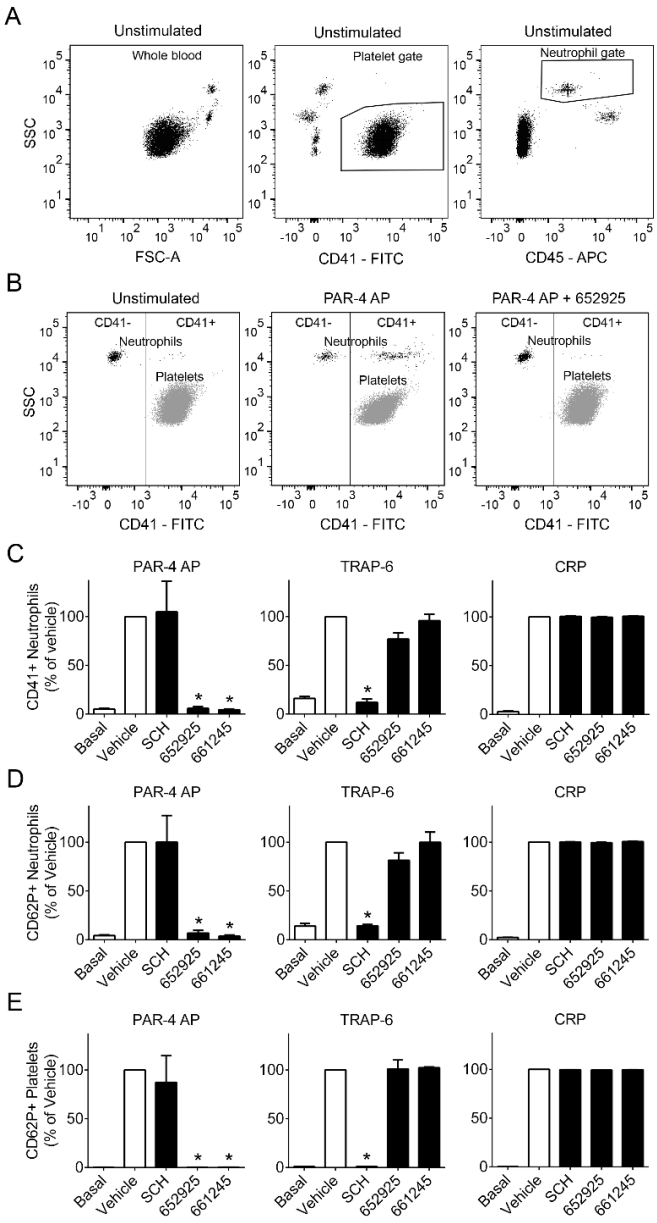


Figure 5.7. PAR4 inhibitors diminish platelet-leukocyte interactions and platelet alpha granule release in whole baboon (Papio anubis) blood. Whole baboon blood was pretreated with the PAR1 inhibitor SCH 79797 (3 μM), PAR4 inhibitors VU0652925 (10 μM) and VU0661245 (10 μM), or vehicle (0.2% DMSO), then stimulated with the agonists PAR4 AP (activating peptide, 200 μM), TRAP-6 (1 mM), or CRP (10 μg/ml) and stained with markers for leukocytes (CD45), platelets (CD41), and activated platelets (CD62P). (A) All samples were gated for platelets by CD41+ and granulocytes by CD45+ and high side scatter (SSC, granularity). (B) Granulocytes (CD45+, high SSC) were plotted against CD41, and a line was drawn to designate the CD41+ region. Platelets (CD41+) are shown for comparison. Bar graphs designate (C) percent of CD41+ granulocytes and (D) percent of activated (CD62P+) platelets for each treatment. Basal = no agonist; n = 3-4 independent experiments; * = $p < 0.05$ vs. vehicle.

5.6 Discussion

Release of dense granule contents from activated platelets facilitates recruitment of leukocytes to the site of injury; this process is often dysregulated in inflammatory disease.[193, 194] Given the described role of platelet PAR4 in supporting the phosphorylation and activation of a number of PKC substrates requisite for platelet dense granule release,[126] we examined whether PAR4 plays a disproportionate role relative to PAR1 in dense granule release. Intriguingly, PAR4 inhibitors diminished dense granule release in response to α-thrombin, while the PAR1 inhibitor SCH 79797 on its own had no effect. Furthermore, upon stimulation with the inflammatory mediators cathepsin G or plasmin, PAR4 inhibitors fully blocked dense granule release, while SCH had no effect either alone or in combination with a PAR4 inhibitor. Given that cathepsin G and plasmin are proteases known to specifically cleave PAR4[197, 198] and are released during inflammation mediated by leukocytes or during fibrinolysis,[211, 212] this suggests a unique and important role for PAR4 in facilitating platelet dense granule release under inflammatory conditions.

Our results also suggest that platelet PAR4 may perform a unique role in the steps of thrombus formation. Other studies have shown that inhibition of PAR1 with a vorapaxar analog was fully rescued with 10 nM but not 2 nM thrombin, suggesting PAR4 can act without PAR1 only at thrombin levels above 2 nM,[202] which we also observed in our assay of dense granule release and our flow cytometry assay of platelet P-selectin using 5 nM thrombin. Higher concentrations of thrombin are likely present in the inner core of a thrombus,[213] supporting the physiological relevance of PAR4 in promoting thrombus formation. Furthermore, PAR4 may play a more prominent role than PAR1 in the release of platelet microparticles and preactivated factor V from platelet α-granules, both events representing a procoagulant phenotype.[126] Platelet stimulation with PAR4 AP also supports thrombin generation in plasma up to 5 minutes faster than stimulation

with TRAP-6.[126] These prior observations combined with our findings that PAR4 mediated dense granule release by 5 nM thrombin suggest that PAR4 plays a distinct role apart from PAR1 to promote thrombus formation.

Activated platelets express surface P-selectin, which binds to the PSGL-1 receptor on circulating leukocytes such as neutrophils and monocytes; importantly, these interactions are upregulated in cardiovascular disease, inflammation, and sepsis, suggesting platelet-leukocyte interactions may be a potential disease biomarker.[214-219] Given our observation that platelet PAR4 plays a specific role in dense granule release via neutrophil cathepsin G, we hypothesized that PAR4 activity also plays a role in platelet-leukocyte interactions. We first investigated the role of PAR4 in facilitating platelet-granulocyte interactions in a flow cytometry assay of isolated platelets and granulocytes, which are largely composed of neutrophils. Our results demonstrate that the PAR4 inhibitor VU0652925 impaired platelet α-granule release (P-selectin exposure) following stimulation with α-thrombin, cathepsin G, or plasmin, while the PAR1 inhibitor SCH 79797 had no effect. Furthermore, platelet-granulocyte interactions stimulated by plasmin were completely blocked by the PAR4 inhibitor VU0652925, suggesting a PAR4 specific effect. Surprisingly, addition of cathepsin G did not stimulate platelet-granulocyte interactions, as the basal (no agonist) condition was similar to the vehicle (with agonist) condition. This could be due to a saturation effect in part from cathepsin G released by neutrophils in the platelet-granulocyte mixture. Meanwhile, unlike the strong inhibition seen with VU0652925, the second PAR4 inhibitor VU0661245 was unable to block platelet-granulocyte interactions by plasmin and platelet activation by thrombin or cathepsin G. Our data show that the IC50 values for activation by 100 nM γ-thrombin are very different for the two PAR4 inhibitors: 229 pM for VU0652925 and 8.42 nM for VU0661245 (Figure 5.2). Thus, we believe that VU0661245 has a lower affinity for PAR4, possibly due to its smaller size (Figure 5.1), and therefore

it is more easily outcompeted by the tethered ligand than VU0652925, despite being effective against PAR4 AP.

Next, we expanded our investigation to a whole blood flow cytometry assay in order to validate the potency and specificity of the PAR4 inhibitors in whole blood. In this assay, we examined platelet P-selectin along with platelet-granulocyte and platelet-monocyte interactions, the two most prevalent platelet-leukocyte interactions and potential markers of inflammation. Both PAR4 inhibitors impaired platelet P-selectin, platelet-granulocyte interactions, and platelet-monocyte interactions upon stimulation with PAR4 AP but not TRAP-6 or CRP, demonstrating validation of these PAR4 inhibitors in whole blood. This inhibition of platelet P-selectin and platelet-granulocyte interactions was recapitulated in a flow cytometry assay with whole baboon blood *ex vivo,* demonstrating feasibility for future *in vivo* studies with PAR4 inhibitors. Given the differences between VU0652925 and VU0661245 seen in our assays with purified cells, future work will focus on exploring the differential effects of these PAR4 inhibitors *in vivo*. Overall, these results demonstrate that in platelet-granulocyte preparations or in whole blood, inhibition of PAR4 impairs platelet α-granule release and platelet-leukocyte interactions. This suggests that PAR4 may be a new druggable target against platelet-leukocyte interactions to reduce inflammation, including thrombo-inflammation.

This novel finding that PAR4 activity promotes platelet-leukocyte interactions extends the role of platelet PAR4 beyond thrombosis to interactions between platelets and immune cells that are activated in inflammatory disease states. The pathological role of platelet-leukocyte interactions was highlighted years ago when clinical trials of the platelet integrin $\alpha_{IIb}\beta_3$ inhibitor eptifibatide was halted due to increased cardiac events; studies determined that despite inhibiting platelet aggregation, the drug also triggered platelet-neutrophil and platelet-monocyte interactions.[220, 221] More

recently, race-specific PAR4 polymorphisms have been identified that confer a pro-atherothrombotic phenotype, pointing to potential use of PAR4 inhibitors for targeting inflammation in specific populations. While donor demographics were not taken into account as a variable in the current study, the effect of race-related PAR4 variants on the pharmacodynamics of PAR4 inhibitors is an important area of future investigation.[222] Meanwhile, the literature supports roles for platelet PAR4 in platelet-leukocyte interactions and inflammation, though many studies have been limited by the use of murine models. Some studies have shown roles for PAR4 activity in leukocyte recruitment in animal models of inflammation and pain but do not define the role of platelet PAR4 in these conditions.[201] Others have shown that PAR4 activity by cathepsin G plays a role in the inflammatory bowel disease ulcerative colitis, and either a PAR4 inhibitor or a cathepsin G inhibitor were beneficial in a mouse model of the disease.[223, 224] In addition to cathepsin G, high levels of plasmin can also activate platelets through PAR4,[197] and elevated plasmin may be partly responsible for the cytokine storm in the deadly macrophage activation syndrome,[225] raising questions about the role of PAR4 in inflammation that involves plasmin activity. Our results show for the first time that inhibition of PAR4 but not PAR1 with small molecule antagonists impairs cathepsin G- and plasmin-induced human platelet granule release and plasmin-induced platelet-granulocyte interactions. This study provides a new rationale for the use of PAR4 activity inhibitors as therapeutic agents in disease conditions that are driven by platelet-leukocyte interactions.

5.7 Conclusions

This study points to an expanded role for the platelet receptor PAR4 in facilitating platelet granule release and platelet-leukocyte interactions. We demonstrate that PAR4 activity via PAR4 AP, thrombin, cathepsin G and plasmin can be targeted with PAR4 inhibitors to block platelet granule release and platelet-leukocyte interactions. These results suggest broader roles for platelet PAR4 than

previously presumed and open up new possibilities for PAR4 as a therapeutic target for inhibition in diseases where platelet-leukocyte interactions play a pathogenic role.

Chapter 6. Conclusions and Future Directions

6.1 Conclusions

The studies outlined in this thesis have investigated pathways of platelet activation and inhibition with the goal of improving therapeutic approaches to treat thrombotic and inflammatory diseases. Initially, clinical reports of bleeding side effects with the Btk inhibitor ibrutinib prompted our study of Btk inhibition in platelet function. We determined that this class of inhibitors targets multiple tyrosine kinases downstream of GPVI, which are required for hemostasis, providing an explanation for antihemostatic side effects and a rationale for improved Btk inhibitor development. Our second study also investigated the importance of GPVI signaling in platelet function, this time discovering a novel role for the molecular chaperone Hsp70 in LAT signolosome assembly, a potential concern for future development of Hsp70 inhibitors. Our final study examined an emerging target of antiplatelet drugs, the platelet PAR4 receptor. We found that, in addition to facilitating activation by thrombin, PAR4 activity also promotes platelet-leukocyte interactions observed in inflammatory states. This points to potential uses for PAR4 inhibitors beyond thrombotic disease, contributing to a growing field investigating the role of platelets in innate immunity. In this chapter, we summarize the results of these studies and describe future work to characterize interactions between platelets, endothelial cells, and leukocytes as an elaboration of existing studies of platelet function.

6.2 Summary

6.2.1 *Btk inhibition impairs platelet function*

Our initial study sought to investigate the effect of Btk inhibitors on platelet function, in light of mild bleeding effects seen in patients taking the Btk inhibitor ibrutinib. Functional assays found that Btk

inhibitors analogous to ibrutinib cause impairment of platelet granule release, spreading, and aggregation under shear flow. A short-term oral dosing study of Btk inhibitors *in vivo* also demonstrated impairment of aggregation but no serious bleeding effects, in line with the mild bleeding seen in patients. Biochemical assays determined that the Btk inhibitors target not only Btk but also Lat and other downstream targets, suggesting that the side effects seen in ibrutinib patients may result from off-target effects on Src, Syk, or Tec kinases. Recently, other groups have shown that ibrutinib has off-target effects on EGFR, ITK, and Tec family kinases, confirming and extending the results of our study.[226] This study provided a rationale for the development of more specific second-generation Btk inhibitors such as acalabrutinib, which is now in clinical trials.[102]

6.2.2 *Hsp70 coordinates assembly of the LAT signolosome*

While Btk kinase is a target in B cell malignancies, another potential drug target is the molecular chaperone heat shock protein 70 (Hsp70), which is known to be upregulated in certain cancers and inflammatory diseases.[227] While other molecular chaperones such as PDI and ERp57 are known to promote platelet integrin activation, it is unknown whether Hsp70 also promotes specific aspects of platelet activation. Therefore, our next study sought to investigate the role of Hsp70 in platelet function. Initial imaging studies identified Hsp70 was abundantly expressed throughout platelets though not colocalized with PDI, suggesting different functions for the two chaperones. Functional studies found that Hsp70 inhibition impaired platelet aggregation by CRP, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation on collagen under shear, suggesting Hsp70 is acting downstream of GPVI activation. Biochemical studies confirmed that Hsp70 inhibition diminishes phosphorylation of members of the LAT signalosome such as PLCγ2. Furthermore, Hsp70 associated with LAT members Vav and PLCγ2 in pull-down assays, and Hsp70 inhibition abolished this association. This suggests that Hsp70 performs chaperone activities in the assembly and coordination of the LAT signalosome

required for platelet activation via GPVI. These results define a novel mechanism for Hsp70 in facilitating platelet activation and suggest potential antihemostatic effects of Hsp70 inhibitors, warranting further studies.

6.2.3 *PAR4 activity facilitates platelet-leukocyte interactions*

While tyrosine kinases such as Btk are targets in B cell malignancies, with resulting off-target effects on platelet function, an alternative approach is to target platelet receptors such as the protease-activated receptor 4 (PAR4). Animal models have shown PAR4 antagonists prevent thrombosis without causing bleeding, while a clinical trial of a PAR4 antagonist for recurrent stroke has been recently completed.[128] However, given the potential for PAR4 activation by proteases other than thrombin such as neutrophil cathepsin G, we investigated the role of PAR4 in promoting platelet-leukocyte interactions and dense granule release by several proteases. Assays of platelet dense granule release showed that PAR4 activity promotes dense granule release through thrombin, cathepsin G, or plasmin. Furthermore, PAR4 antagonists blocked platelet-leukocyte interactions potentiated by plasmin, demonstrating a novel functional role for PAR4 activity. These results suggest PAR4 antagonists may have utility in treating diseases of inflammation as well as thrombosis.

6.3 Future directions

6.3.1 *BCR-ABL tyrosine kinase inhibitors and thrombosis*

In addition to ibrutinib, a number of other platelet tyrosine kinase inhibitors have been developed for the treatment of B cell malignancies, with unintended hematological side effects. One class of inhibitors targets the BCR-ABL protein, a product of the BCR-ABL oncogene formed by a reciprocal

translocation between the long arms of chromosomes 9 and 22 in chronic myelogenous leukemia (CML) and, less commonly, in acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML).[228] The BCR-ABL protein is a tyrosine kinase with elevated activity that causes increased myeloid cell proliferation and decreased apoptosis of hematopoietic stem cells or progenitor cells, defects in adherence of myeloid progenitors to marrow stroma leading to the release of immature progenitors into circulation, and disease progression resulting from genetic instability. As a result, BCR-ABL tyrosine kinase inhibitors were developed as a treatment for CML.

Imatinib, which competitively inhibits the ATP binding site of BCR-ABL tyrosine kinase, was the first BCR-ABL inhibitor developed for the treatment of patients with CML.[228] While imatinib significantly improves patient outcomes, follow-on studies found that nearly 20% of patients on imatinib do not exhibit a complete cytogenetic response and develop resistance.[228] Nilotinib, an aminopyrimidine derivative, was rationally designed to be more selective against the BCR-ABL tyrosine kinase than imatinib, and has been shown to effectively inhibit the growth of 32 of the 33 cells lines containing imatinib-resistant mutations. However, some mutants, particularly the T315I mutation, are also resistant to these second-generation compounds.[229-231] As a result, ponatinib was developed as a third-generation pan-BCR-ABL inhibitor. Ponatinib has been shown to exhibit potent activity against native, T315I, and most other clinically relevant BCR-ABL mutants.[232] Ponatinib has also been shown to inhibit a subset of the class III/IV family of receptor tyrosine kinases.[233]

Tyrosine kinase inhibitors, including BCR-ABL inhibitors such as ponatinib, have been associated with cardiovascular complications, including bleeding diathesis and thrombosis.[234] Specifically, hemorrhaging is a common adverse event reported in patients receiving imatinib, and imatinib has been associated with abnormalities in platelet aggregation.[235, 236] Meanwhile, the second-generation inhibitor nilotinib has been associated with occlusive vascular diseases in up to 36% of patients.[237, 238] Ponatinib treatment is also associated with thrombotic complications such as peripheral arterial occlusive disease, myocardial infarction, and venous thromboembolism, such that the FDA temporary suspended the drug in late 2013.[239, 240] However, the pathogenic mechanisms underlying the increased risk of thrombosis associated with nilotinib and ponatinib remain ill-defined. Recent studies have suggested BCR-ABL inhibitors cause endothelial inflammation in addition to platelet dysfunction.[237, 241] Overall, the prevention and treatment of prothrombotic complications requires an understanding of the effect of BCR-ABL inhibitors not only on platelet function but on interactions between platelets and endothelial cells and between platelets and leukocytes. In preliminary studies, we have defined the effects of ponatinib on key steps in platelet signaling, activation, and aggregation. In future work, we will investigate platelet-endothelial cell and platelet-leukocyte interactions to better define the prothrombotic phenotype induced by BCR-ABL inhibitor treatment.

6.3.2 *Effect of BCR-ABL inhibitors on tyrosine phosphorylation in platelets*

We initially hypothesized that BCR-ABL inhibitors such as ponatinib may interfere with platelet function through an inhibition of tyrosine kinase activation proximal to the engagement of platelet activating receptors, including GPVI and integrin $\alpha_{IIb}\beta_3$. Accordingly, we examined the effects of ponatinib, nilotinib, and imatinib on the intracellular signaling cascades mediating platelet activation following exposure to fibrillar collagen or fibrinogen. As seen in Figure 6.1A, treatment of platelets

with ponatinib partially inhibited the tyrosine kinase activation profile of platelets on surfaces of fibrinogen or fibrillar collagen as determined by Western blot analysis of platelet lysates with 4G10 antisera; imatinib and nilotinib had only minor effects on the activation of platelet tyrosine kinases. In addition to Abl, ponatinib has been shown to inhibit a number of tyrosine kinases *in vitro*, including Src family kinases (SFKs).[242] To specify a role for the inhibition of Src by ponatinib in mediating platelet inhibition, we next examined the phosphorylation state of the Src kinase activation loop (Tyr416) which serves as a marker of Src kinase activation.[243] As seen in Figure 6.1B, exposure of platelets to surfaces of fibrinogen or fibrillar collagen upregulated Src Tyr416 phosphorylation in platelets. Like PP2, ponatinib inhibited Src Tyr416 phosphorylation in response to fibrinogen or collagen (Figure 6.1B). In contrast, platelet Src Tyr416 phosphorylation was not effected by nilotinib or imatinib treatments (Figure 6.1B).

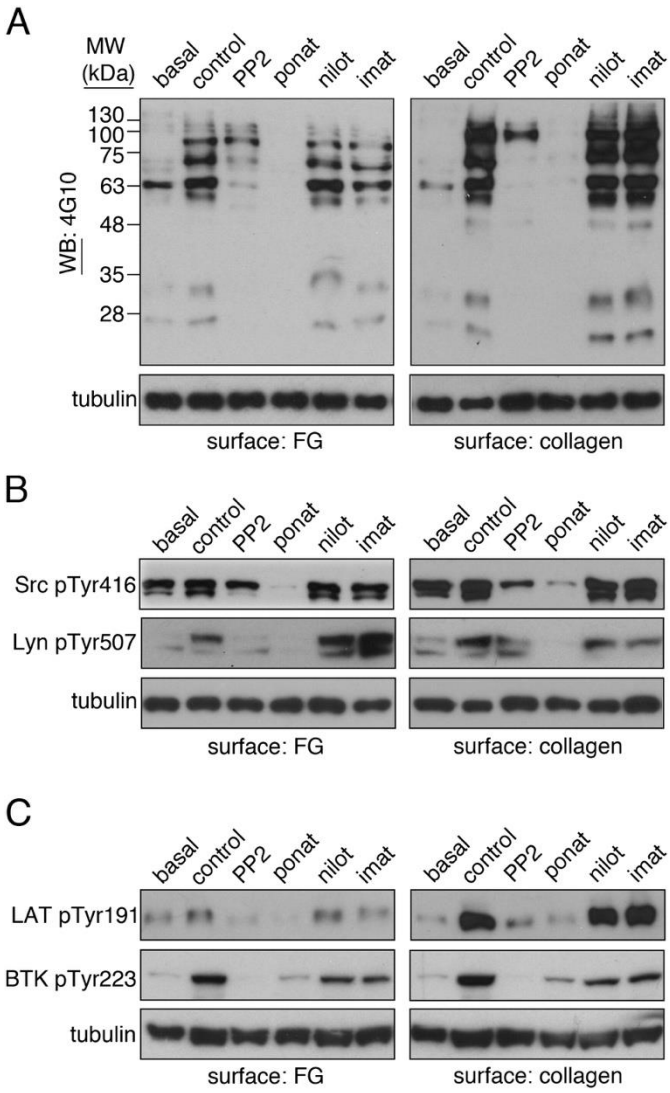


Figure 6.1. Ponatinib inhibits platelet tyrosine kinase activation. Replicate samples of washed human platelets (5×10^8/ml) treated for 10 minutes with vehicle (DMSO, 0.1%), ponatinib (1 μM), nilotinib (1 μM), or imatinib (1 μM) were spread on fibrinogen (50 μg/ml) or fibrillar collagen (100 μg/ml) at 37 °C. After 45 min, non-adherent platelets were removed and adherent platelets were washed three times with PBS before lysis into sample buffer, separation by SDS-PAGE and Western blotting (WB) for (A) phosphotyrosine moieties with 4G10 antisera as well as (B) phospho-Src Tyr416, phospho-Lyn Tyr507 and (C) phospho-BTK Tyr223 and phospho-LAT Tyr191. Tubulin serves as a loading control. Western blots are representative of four experiments.

Previous work has shown that BCR-ABL inhibitors, including ponatinib, inhibit the phosphorylation of the SFK member Lyn and have inhibitory effects on the activation of Bruton's tyrosine kinase (Btk).[242] As both of these kinases have roles in platelet activation, we examined the effect of

ponatinib, nilotinib, and imatinib on the activation state of Lyn as well as Btk in response to platelet spreading on fibrillar collagen and fibrinogen.[135, 136] Similar to the Src family kinase inhibitor, PP2, treatment of platelets with ponatinib markedly reduced the levels of Lyn Tyr507 phosphorylation in response to spreading on fibrinogen or fibrillar collagen (Figure 6.1B). Phosphorylation of Btk Tyr223, an autophosphorylation site that serves as a marker of Btk activation, was also reduced by ponatinib or PP2 treatment (Figure 6.1C).[244] Imatinib and nilotinib had only marginal inhibitory effects on Lyn and Btk phosphorylation (Figure 6.1). Similar to Btk phosphorylation, platelet SFKs also support the phosphorylation of the linker for activation of T cells protein, LAT, which serves roles in platelet PLCγ activation, calcium signaling and the later stages of platelet activation.[21] As shown in Figure 6.1C, both ponatinib and PP2 inhibited the LAT Tyr191 phosphorylation in platelets bound to surfaces of fibrinogen or fibrillar collagen. LAT phosphorylation was minimally affected by nilotinib or imatinib treatments (Figure 6.1C). Together, these studies support a role for ponatinib as an inhibitor of platelet Src family tyrosine kinase activation and signaling.

6.3.3 *Effect of BCR-ABL inhibitors on platelet P-selectin and phosphatidylserine exposure*

In vivo, platelet activation and aggregation is associated with the secretion of P-selectin from platelet alpha granules, which is associated with thrombus stability, as well as the externalization of phosphatidylserine (PS), which regulates blood coagulation by providing a platform for the coagulation factor assembly required for thrombin generation.[89, 165] Given the reports of thrombotic complications in ponatinib-treated patients and the ability of ponatinib to upregulate PS-exposure on apoptotic cells in culture, we next assayed the effect of ponatinib, nilotinib, and imatinib on platelet P-selectin and PS surface levels during platelet activation.[245] Replicate samples of washed human platelets were treated with BCR-ABL inhibitors prior to stimulation with CRP and/or

thrombin and staining with anti-P-selectin (CD62P) antibodies or FITC-conjugated Annexin-V prior to flow cytometry analysis. As seen in Figure 6.2A, ponatinib (1 μM) significantly decreased platelet P-selectin exposure in response to CRP. The addition of thrombin reversed the inhibitory effect of ponatinib, indicating that the effects of ponatinib on P-selectin exposure can be bypassed through GPCR-mediated signaling. Ponatinib also significantly decreased PS exposure, as measured by Annexin V binding, in response to CRP alone as well as in response to CRP in combination with thrombin (Figure 6.2B). Treatment of platelets with nilotinib or imatinib (1 μM) did not significantly affect P-selectin or PS exposure in response to CRP or a combination of CRP and thrombin (Figure 6.2A, B). This demonstrates that ponatinib impairs both platelet alpha granule secretion and platelet procoagulant activity.

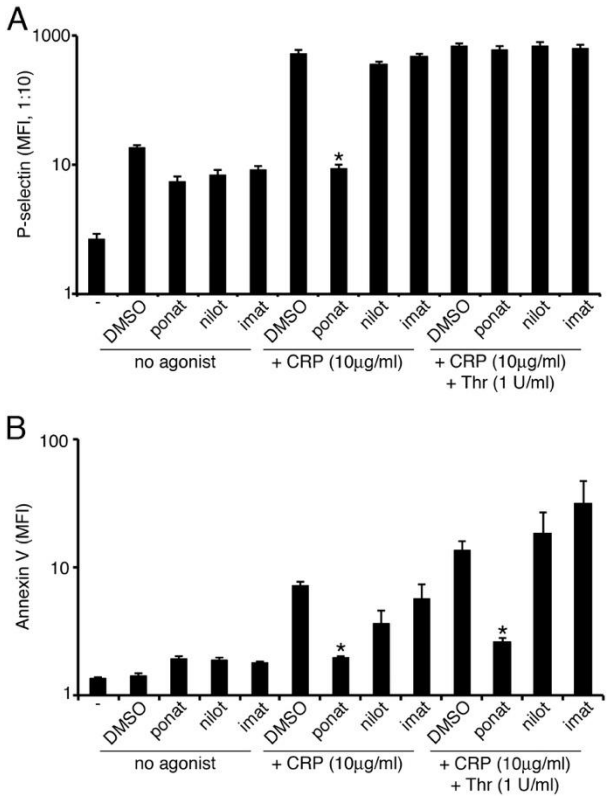


Figure 6.2. Ponatinib blocks P-selectin and phosphatidylserine exposure. (A) Platelet surface P-selectin levels analyzed by flow cytometry following vehicle (DMSO), ponatinib (1 μM), nilotinib (1 μM), or imatinib (1 μM) treatment and incubation with vehicle, CRP (10 μg/ml), or CRP (10 μg/ml) and thrombin (1 U/ml). (B) Phosphatidylserine levels analyzed by flow cytometry following vehicle (DMSO), ponatinib (1 μM), nilotinib (1 μM), or imatinib (1 μM) treatment and incubation with vehicle, CRP (10 μg/ml), or CRP (10 μg/ml) and thrombin (1 U/ml). Results shown as mean fluorescence intensity (MFI). * signifies $p < 0.05$ with respect to DMSO control; $n = 3$, error bars represent S.E.M.

6.3.4 *Effect of BCR-ABL inhibitors on aggregate formation under flow*

We also investigated the effect of the BCR-ABL inhibitors on aggregate formation in a flow chamber model of platelet aggregate formation. Whole human blood was pretreated with BCR-ABL inhibitors prior to flow over a surface of fibrillar collagen at an initial shear rate of 2200 sec^{-1} for 5 min, fixed, and visualized. As seen in Figure 6.3, ponatinib reduced aggregate formation under shear, as evidenced by a 52.4 ± 11.2% reduction in platelet aggregate surface area coverage in the presence of ponatinib as compared to vehicle alone (0.1% DMSO). Interestingly, imatinib also significantly

decreased platelet aggregate formation on collagen under shear. These results show that ponatinib, and to a lesser extent, imatinib, impair the ability of platelets to form aggregates under shear.

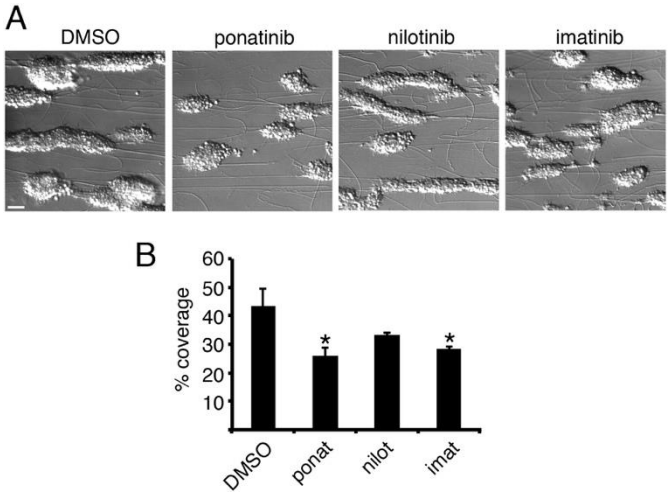


Figure 6.3. Ponatinib inhibits platelet aggregate formation under shear. (A) Whole blood was treated with vehicle (DMSO, 0.1%), ponatinib (1 μM), nilotinib (1 μM), or imatinib (1 μM) and perfused at 2200 s^{-1} at 37 °C through capillary tubes coated with collagen (100 μg/ml) to form platelet aggregates. (B) The percent surface area covered by aggregates was computed by outlining and quantifying platelet aggregates. * signifies $p < 0.05$ with respect to DMSO control; error bars represent S.E.M.; $n = 3$; scale bar = 10 μm.

6.3.5 *Effect of BCR-ABL inhibitors on platelet-endothelial interactions*

Given our preliminary studies suggesting BCR-ABL inhibitors impair several aspects of platelet function, the prothrombotic effects observed in patients remain contradictory and require further investigation. Several studies have suggested inflammatory marker expression by endothelial cells may play a role in the prothrombotic phenotype induced by nilotinib and ponatinib treatment. Two proposed mediators are the endothelial VEGF receptor 2 (VEGFR2), which binds platelet integrin $\alpha_v\beta_3$, and lectin-like ox-LDL receptor-1 (LOX-1), which binds platelet CD36.[241] First, to determine if BCR-ABL inhibitors activate endothelial inflammation independent of platelets, cultured HUVECs will be pre-treated with imatinib, nilotinib, ponatinib, or vehicle and stained for E-

selectin, P-selectin, ICAM-1, VCAM-1, and vWF release before being analyzed for fluorescence in a plate reader. Next, to determine whether BCR-ABL inhibitors induce platelet-endothelial cell binding, a platelet binding assay will be used. Washed human platelets will be stained with CD41, pre-treated with imatinib, nilotinib, ponatinib, or vehicle, and incubated on cultured HUVECs. Wells will be washed and analyzed for platelet adherence as fluorescence in a plate reader. Blocking antibodies will be used against endothelial cell receptors PSGL-1, E-selectin, P-selectin, ICAM-1, VCAM-1, integrin $\alpha_v\beta_3$, or GPIbα, as well as VEGFR2, LOX-1, and CD40 in order to determine which receptor interactions mediate platelet-endothelial cell adhesion following BCR-ABL inhibitor treatment.

Platelet interactions with the endothelium typically occur under shear flow, with important effects on tethering and adhesion compared to static conditions. To probe the effect of BCR-ABL inhibitors on platelet-endothelium interactions under flow, we will utilize an endothelialized, perfusable microvessel system that has been developed in our group.[246] Custom-molded PDMS channels will be seeded with extracellular matrix proteins and the resulting channels cultured with HUVECs under constant media flow for 24 hours to develop confluent microvessels. Whole human blood with be pretreated with imatinib, nilotinib, ponatinib, or vehicle and stained for markers of endothelial cells (VE-cadherin), platelets (CD41), leukocytes (CD45), and markers of inflammation including ICAM-1, VCAM-1, and vWF. Blood will be perfused through the microvessels at a venous shear rate for 10 minutes. Channels will be washed and visualized with fluorescence microscopy to detect platelet adhesion, vascular permeability as leakage of cells into the ECM, and endothelial expression of inflammatory markers. These studies will determine whether BCR-ABL inhibitor treatment induces inflammation from endothelial cells alone, platelet-endothelial cell interactions, or both and whether this is a flow-dependent phenomenon.

6.3.6 *Effect of BCR-ABL inhibitors on platelet-leukocyte interactions*

Finally, the effect of BCR-ABL inhibitor treatment on platelet-leukocyte interactions will be assayed to determine if the prothrobotic effects of the inhibitors are mediated by leukocytes interacting with platelets. Whole blood anticoagulated with sodium citrate and PPACK and diluted 1:1 in buffer will be incubated with imatinib, nilotinib, ponatinib, or vehicle for 15 minutes at room temperature. Treated blood will be added to FACS tubes containing and antibody and agonist mixture and incubated 20 minutes at room temperature. Fluorophore-conjugated antibodies will be used against CD41 (platelets), CD66b (granulocytes), and CD14 (monocytes). Agonists will include collagen-related peptide, PAR1 activating peptide, PAR4 activating peptide, thrombin, or vehicle. Samples will be analyzed by flow cytometry for platelet-leukocyte interactions. Additional blocking antibodies against leukocyte receptors PSGL-1, Mac-1, and CD40 will be used to probe which receptors mediate changes in platelet-leukocyte interactions following BCR-ABL inhibitor treatment.

Overall, these studies of BCR-ABL inhibitors on platelet-endothelial interactions and platelet-leukocyte interactions will define the interactions that generate thrombotic complications following BCR-ABL inhibition. The results of these studies will inform the design of more potent and specific BCR-ABL tyrosine kinase inhibitors in the future. Furthermore, these future investigations will extend our previous studies of tyrosine kinase inhibition in platelet function to the platelet-vascular interface, providing clinically relevant insights for improving the treatment of thrombosis and inflammation.

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Biographical Sketch

Rachel Anne Rigg was born on January 2, 1981 in Austin, Texas to David and Sally Rigg. The family moved to Sugar Land (near Houston) in 1990. Rachel attended Dulles High School and was active in the debate team, orchestra, and academic decathlon.

Rachel attended Rice University and obtained a B.A. in Religious Studies in 2004, while also developing interests in human evolution and the brain. She attended the University of Chicago in 2006 to study human evolution and psychology, receiving an M.A. in the Social Sciences.

In 2008, Rachel moved to Portland, Oregon to pursue an Americorps position in environmental restoration and volunteer coordination. Afterwards, she pursued postbaccaulareate studies at Portland State University in biology and chemistry. During this time, she volunteered in the lab of Owen McCarty as a microscopist and also worked as an aide in the lab of Rosalie Sears.

After graduating with postbaccaulareate B.S. in Chemistry, Rachel joined the lab of Owen McCarty as a graduate student in the Department of Biomedical Engineering. Her work is focused on platelet signaling and functional studies of platelet biology. In 2015-2016, she was awarded a Whitaker International Fellowship to study in the lab of Dr. Thomas Renné at the Karolinska Institutet in Stockholm, Sweden. She has published her research in peer-reviewed journals and presented at conferences throughout the U.S. and Europe. Rachel's current publications, presentations, and mentoring experiences are listed below.

Publications/Creative Work:

Peer-reviewed

1. Phillips, K.G., Kolatkar, A., Rees, K.J., **Rigg, R.**, Marrinucci, D., Luttgen, M., Bethel, K., Kuhn, P., McCarty, O.J. Quantification of cellular volume and sub-cellular density fluctuations: comparison of normal peripheral blood cells and circulating tumor cells identified in a breast cancer patient. *Frontiers in Oncology*. 2012; 2(96).
2. Loren, C.P., Aslan, J.E., **Rigg, R.A.**, Nowak, M.S., Healy, L.D., Gruber, A., Druker, B.J., McCarty, O.J. The BCR-ABL inhibitor ponatinib inhibits platelet immunoreceptor tyrosine-based activation motif (ITAM) signaling, platelet activation and aggregate formation under shear. *Thrombosis Research*. 2015; 135(1):155-160.
3. Phillips, K.G., Lee, A.M., Tormoen, G.W., **Rigg, R.A.**, Kolatkar, A., Luttgen, M., Bethel, K., Bazhenova, L., Kuhn, P., Newton, P., McCarty, O.J. The thrombotic potential of circulating tumor microemboli: computational modeling of circulating tumor cell-induced coagulation. *American Journal of Physiology: Cell Physiology*. 2015; 308(3):C229-36.
4. Phillips, K.G., Lee, T., de Guillebon, A.M., Chandrasekaran, S., McGuire, M.J., Carr, R.T., Baker-Groberg, S.M., **Rigg, R.A.**, Kolatkar, A., Luttgen, M., Bethel, K., Kuhn, P., Decuzzi, P., McCarty, O.J., King, M.R. A physical sciences network characterization of circulating tumor cell aggregate transport. *American Journal of Physiology: Cell Physiology*. 2015; 308(10):C792-802.
5. Aslan, J.E., **Rigg, R.A.**, Nowak, M.S., Loren, C.P., Baker-Groberg, S.M., Pang, J., David, L.L., McCarty, O.J. Lysine acetyltransfer supports platelet function. *Journal of Thrombosis and Hemostasis*. 2015; 13(10):1908-17.
6. **Rigg, R.A.**, Aslan, J.E., Healy, L.D., Wallisch, M., Loren, C.P., Pang, J., Hinds, M.T., Gruber, A., McCarty, O.J. Oral administration of Bruton's Tyrosine Kinase inhibitors impairs GPVI-mediated platelet function. *American Journal of Physiology: Cell Physiology*. 2016; 310(5):C373-80.
7. **Rigg, R.A.**, Healy, L.D., Nowak, M.S., Mallet, J., Thierheimer, M.L., Pang, J., McCarty, O.J., Aslan, J.E. Heat shock protein 70 regulates platelet integrin activation, granule secretion and aggregation. *American Journal of Physiology: Cell Physiology*. 2016; 310(7):C568-75.
8. Labberton, L., Kenne, E., Long, A.T., Nickel, K.F., Di Gennaro, A., **Rigg, R.A.**, Hernandez, J.S., Butler, L., Maas, C., Stavrou, E.X., Renné, T. Neutralizing blood-borne polyphosphate in vivo provides safe thromboprotection. *Nature Communications*. 7:12616.
9. Ngo, A.T., Thierheimer, M.L., Babur, Ö., Rocheleau, A.D., Huang, T., Pang, J., **Rigg, R.A.**, Mitrugno, A., Theodorescu, D., Burchard, J., Nan, X., Demir, E., McCarty, O.J., Aslan, J.E. Assessment of roles for the Rho-specific guanine nucleotide dissociation inhibitor (RhoGDI) Ly-GDI in platelet function: a spatial systems approach. *American Journal of Physiology: Cell Physiology*. 2017; 312(4):C527-C536.
10. Mitrugno, A., **Rigg, R.A.**, Laschober, N.B., Ngo, A.T.P., Pang, J., Williams, C.E., Aslan, J.E., McCarty, O.J.T. Potentiation of TRAP-6-induced platelet dense granule release by blockade of P2Y12 signaling with MRS2395. *Platelets*. 2017; In press.

11. **Rigg, R.A.**, Healy, L.D., Chu, T.T., Ngo, A.T.P., Mitrugno, A., Zilberman-Rudenko, J., Aslan, J.E., Hinds, M.T., Dirling Vecchiarelli, L., Morgan, T.K., Gruber, A., Temple, K.J., Lindsley, C.W., Duvernay, M.T., Hamm, H.E., and McCarty, O.J.T. Protease-activated receptor 4 (PAR4) activity promotes platelet granule release and platelet-leukocyte interactions. *Platelets*. 2017; In press.

Reviews, Editorials, and Book Chapters

1. Puy, C., **Rigg, R.A.**, McCarty, O.J. The hemostatic role of factor XI. *Thrombosis Research*. 141 Suppl 2:S8-S11.
2. **Rigg, R.A.**, McCarty, O.J., Aslan, J.E. Heat shock protein 70 (Hsp70) in the regulation of platelet function. *Heat Shock Protein 70 in Biology and Medicine*. 2017; In press.
3. Mitrugno, A., Sylman, J.L., **Rigg, R.A.**, Yunga, S.T., Shatzel, J.J., Williams, C.D., McCarty, O.J.T. *Carpe* low-dose aspirin: the new anti-cancer face of an old anti-platelet drug. *Platelets*. 2017; In press.

Abstracts and Posters

1. **Rigg, R.A.**, Baker-Groberg, S.M., Phillips, K.G., Kolatkar, A., Luttgen, M., Bethel, K., Decuzzi, P., King, M.R., Kuhn, P., McCarty, O.J.T., "Linking CTC cluster measurements to *in vitro* flow experiments and *in silico* models of CTC transport", National Cancer Institute Physical Sciences-Oncology Center (PS-OC) Site Visit, Scripps Institute, La Jolla, CA (Oct 2013).
2. Phillips, K.G., **Rigg, R.**, Kolatkar, A., Luttgen, M., Newton, P., Bazhenova, L., Kuhn, P., McCarty, O.J., "Spatial-temporal characterization of circulating tumor cell biophysical metrics before, during, and after pulmonary lobectomy", 5th Annual National Cancer Institute Physical Sciences-Oncology (PS-OC) Center Conference, Bethesda, MD (Apr 2014).
3. Aslan, J.E., **Rigg, R.A.**, Loren, C.P., Itakura, A., Baker, S.M., Pang, J., McCarty, O.J., "p300-based acetylation signaling regulates cytoskeletal reorganization and procoagulant phenotype of platelets", Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions, Toronto, Canada (May 2014).
4. Loren, C.P., **Rigg, R.A.**, Aslan, J.E., Healy, L.D., Gruber, A., McCarty, O.J., "The prothrombotic profile of BCR-ABL inhibitors ponatinib, nolotinib and imatinib", Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions, Toronto, Canada (May 2014).
5. Loren, C.P., **Rigg, R.A.**, Aslan, J.E., Healy, L.D., Gruber, A., McCarty, O.J., "The BCR-ABL inhibitor ponatinib inhibits platelet signaling, platelet function, and thrombus formation," 23rd Biennial International Congress on Thrombosis, Valencia, Spain (May 2014).
6. **Rigg, R.A.**, Phillips, K.G., McCarty, O.J.T., "Elucidating the role of circulating tumor cells in metastasis and thrombosis via biophysical characterization and transport modeling," Cell, Developmental & Cancer Biology and OHSU Center for Spatial Systems Biomedicine First Annual Joint Retreat, Stevenson, WA (Jul 2014).
7. Aslan, J.E., **Rigg, R.**, Baker, S., Pang, J., David, L., McCarty, O., "Lysine acetyltransfer supports platelet function," XXV Congress of the International Society on Thrombosis and Haemostasis (ISTH), Toronto, Canada (Jun 2015).

8. **Rigg, R.A.**, Aslan, J., Pang, J., Nowak, M., McCarty, O., "Platelet Hsp70 regulates integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation," XXV Congress of the International Society on Thrombosis and Haemostasis (ISTH), Toronto, Canada (Jun 2015).
9. **Rigg, R.A.**, Healy, L.D., Nowak, M.S., Mallet, J., Thierheimer, M.L.D., Pang, J., McCarty, O.J.T., Aslan, J.E. "Heat shock protein 70 (Hsp70) regulates platelet integrin activation, granule secretion and aggregation," 62nd Annual SSC Meeting of the ISTH, Montpellier, France (May 2016).
10. Aslan J.E., **Rigg R.A.**, Ngo A., Nelson J.W., Zilberman-Rudenko J., Pang J., Burchard J., David L.L., McCarty O.J. "Deciphering roles for reversible protein lysine acetylation in the regulation of platelet function," Gordon Research Conference: Hemostasis, Stowe, VT (Jul 2016).
11. Aslan, J.E., **Rigg R.A.**, Ngo A.T., Nelson J.W., Pang J., Burchard J., Shinde U., David L.L., McCarty O.J.T. "Determining structural and systems-level roles for reversible protein lysine acetylation in the regulation of platelet function," Earl W. Davie Symposium, Vancouver BC, Canada (Dec 2016).
12. Aslan J.E., **Rigg R.A.**, Ngo A.T., Nelson J.W., Burchard J., David L.L., McCarty O.J.T. "Determining roles for reversible lysine acetylation in the regulation actin cytoskeletal dynamics," Annual Meeting of The American Society for Cell Biology (ASCB), San Francisco, CA (Dec 2016).
13. Ngo A.T., Thierheimer M.L., Pang J., Babur Ö., Rocheleau A.D., **Rigg R.A.**, Mitrugno A., Huang T., Nan X., Burchard J., Demir E., McCarty O.J., Aslan J.E. "Identification of roles for the Rho-specific guanine nucleotide dissociation inhibitor (RhoGDI) Ly-GDI in platelet function," Arteriosclerosis, Thrombosis, and Vascular Biology, Minnesota, MN (May 2017).
14. **Rigg, R.A.**, Labberton, L., Nickel, K.F., Long, A.T., Butler, L.M., McCarty, O.J.T., Renné, T. "Characterization of Polyphosphate in Endothelial Cells Under Cellular Stress," 2017 Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany (Jul 2017).
15. **Rigg, R.A.**, Mitrugno, A., Healy, L.D., Ngo, A.T.P., Duvernay, M.T., Lindsley, C.W., Hamm, H.E., Gruber, A., McCarty, O.J.T. "Role of Protease-Activated Receptor 4 in Regulating Platelet-Leukocyte Interactions in Whole Blood," 2017 Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany (Jul 2017).
16. Chu, T.T., **Rigg, R.A.**, Healy, L.D., Ngo, A.T.P., Mitrugno, A., Aslan, J.E., Gruber, A., Lindsley, C.W., Duvernay, M.T., Hamm, H.E., and McCarty, O.J.T. "Role of Protease-Activated Receptor 4 in Regulating Platelet Dense Granule Release," Annual Meeting of the Biomedical Engineering Society (BMES), Phoenix, AZ (Oct 2017).
17. McCarty O.J.T., Aslan J.E., Babur O., Buchanan A., Ngo A.T., Pang J., **Rigg R.A.**, Mitrugno A., David L., Demir E. "Pathway analysis reveals a p38 MAP kinase-MAPKAPK2/Nogo axis regulating intracellular calcium dynamics in procoagulant platelets," Annual Meeting of the Biomedical Engineering Society (BMES), Phoenix, AZ (Oct 2017).

Invited Lectures and Conference Presentations

1. **Rigg, R.A.**, Healy, L.D., Nowak, M.S., Mallet, J., Thierheimer, M.L.D., Pang, J., McCarty, O.J.T., Aslan, J.E., "Heat shock protein 70 (Hsp70) regulates platelet integrin activation, granule secretion, and aggregation," 24th Biennial International Congress on Thrombosis, European and Mediterranean League Against Thrombosis (EMLTD), Istanbul, Turkey (May 2016).

Research Mentor:

1. Liam Wong (2013), undergraduate, Oregon State University Chemical Engineering
2. Marie Nowak (2014), Master's student, Lille University of Science and Technology, France; current position: Radiation Therapy, Lausanne University Hospital, Switzerland
3. Ishan Patel (2014), medical student, OHSU; currently Resident, Internal Medicine, OHSU
4. Cassandra Loren (2014), undergraduate, Oregon State University Chemical Engineering; currently medical student, Vanderbilt University
5. Marisa Lian Dahl Thierheimer (2015), undergraduate, Oregon State University Chemical Engineering
6. Tiffany Chu (2017), undergraduate, Johns Hopkins University