Differential effects of tyrosine kinase inhibitors in haemostasis, thrombosis and inflammation

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

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DECLARATION

I certify that, except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work that has been carried out since the official commencement date of the approved research programme; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

14/12/2018

Abdullah Hamadi
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Special thanks to my friends in the lab. They are my brothers Gasim (special lovely thanks), Fahd and Fehaid. I will miss you all so much. Special thanks to my brother Ali Mahzari for his support during my study. I appreciate that.

Here, strongly and warmly, I love to thank my heart, my mum, my amazing mother for her full support to me during my study, her pray which give me the great power to achieve my and her dream. My father, may Allah (God) has mercy on you, I’ve achieved your dream and send it to your pure soul. I love you mum and dad.

Gratefully I love to thank my great wife for her full support for me during my study, and for her patience and standing beside me.

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PUBLICATIONS


ACCEPTED CONFERENCE ABSTRACTS

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  Annual Scientific Meetings of the HAA (Haematology Society of Australia and New Zealand, the Australian & New Zealand Society of Blood Transfusion and the Australasian Society of Thrombosis and Haemostasis)-ASTH stream. Adelaide, Australia 2016.
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABL</td>
<td>Abelson gene</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoid leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
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<td>AP</td>
<td>Accelerated Phase</td>
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<td>Adenosine triphosphate</td>
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<td>BP</td>
<td>Blastic Phase</td>
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</tr>
<tr>
<td>BSS</td>
<td>Bernard–Soulier syndrome</td>
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<td>CaCl₂</td>
<td>Calcium chloride</td>
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<tr>
<td>CCB</td>
<td>Calcium channel blocker</td>
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<td>Complete cytogenetic response</td>
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<td>Extracellular matrix</td>
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<td>Epidermal growth factor</td>
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<td>eNOS</td>
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<td>HSCs</td>
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<td>HUVEC</td>
<td>Human vascular endothelial cell</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular cell adhesion molecule</td>
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IFN-γ  Interferon gamma
IL-6  Interleukin-6
IM  Imatinib
INN  International Nonproprietary
IP  Intraperitoneal
KCl  Potassium chloride
LDL  Low density lipoprotein
LOX  Lipoxygenases
MgCl$_2$-6H$_2$O  Magnesium chloride hexahydrate
MKs  Megakaryocytes
MMR  Major molecular response
MQ H$_2$O  Milli-Q water
Na$_2$HPO$_4$  Disodium hydrogen phosphate
NaCl  Sodium chloride
NaHCO$_3$  Sodium bicarbonate
NaOH  Sodium Hydroxide
NIL  Nilotinib

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<tr>
<td>NRTKs</td>
<td>Non-receptor protein tyrosine kinase families</td>
</tr>
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<td>NSAID’s</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
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<td>OCS</td>
<td>Open canalicular system</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAC-1</td>
<td>activated integrin αIIbβ3</td>
</tr>
<tr>
<td>PAOD</td>
<td>Progressive arterial occlusive disease</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>Ph+</td>
<td>Philadelphia chromosome positive</td>
</tr>
<tr>
<td>PON</td>
<td>Ponatinib</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein Tyrosine kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatse</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PVD</td>
<td>Prinzmetal Variant angina</td>
</tr>
<tr>
<td>QD</td>
<td>Once daily</td>
</tr>
<tr>
<td>RCD</td>
<td>Ringer citrate dextrose</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinases</td>
</tr>
<tr>
<td>sP-sel</td>
<td>Soluble P-selectin</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Angiopoietin receptor</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor-activating peptide</td>
</tr>
<tr>
<td>TP</td>
<td>Thromboxane receptor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
ABSTRACT

The detection of the Bcr-Abl gene in chronic myeloid leukaemia (CML) has resulted in the development of novel tyrosine kinase inhibitors (TKIs), such as imatinib that particularly interfere with the ATP binding pocket of the Abl kinase domain of the Bcr-Abl protein. Imatinib is currently used as a frontline therapy, with impressive outcomes at producing long-term clinical remission among many CML patients; however, resistance to imatinib has been reported in patients due to the development of Bcr-Abl mutations and/or intolerance to the drug. Potent second generation TKIs, namely nilotinib and dasatinib, have since been developed and approved to address this therapeutic challenge. With their prompt and noticeable deep molecular and haematological remission properties, dasatinib and nilotinib have provided a significant effect and efficiency against leukaemic cells. However, due to the side effects and challenges linked to dasatinib, imatinib, and nilotinib, ponatinib which is a new third generation TKI inhibitor multi-kinase drug has been developed to be effective against leukemic cells containing the Bcr-Abl resistance mutation T315I. Nevertheless, ponatinib can inhibit T315I Bcr-Abl mutant enzymes selectively especially the ones which are not sensitive to new Bcr-Abl inhibitors obtainable at clinics.

The management of malignancy in a significant proportion of patients and their survival is improved using these later generation drugs, therefore, recent studies have concentrated on their toxicity. For instance, in the case of ponatinib and nilotinib, clinical studies have revealed excessive and unnecessarily severe vascular problems, such as peripheral arterial occlusive disease (PAOD) and arterial occlusive events compared with other TKIs. Consequently, this had led to an increased desire to limit the pathophysiology of cardiovascular risk factors.
Cardiovascular disease is a major clinical issue and a leading cause of illness, disability and mortality in the world, specifically in industrialised countries. Platelets are essential in normal haemostasis as they prevent blood loss following trauma by blood clot formation and they sustain vascular integrity. They also play a crucial role in the pathophysiological processes of atherosclerotic plaque and arterial thrombotic disease by contributing to vaso-occlusive thrombotic mechanisms that generate ischaemic traumatic damage in cardiovascular and peripheral blood vessel disease. Platelets’ attachment under some extreme conditions of excessive flow shear forces in stenotic blood arteries enhances the reaction of platelets leading to increased in vivo arterial coagulation.

In the context of vasculature, the pro-atherothrombotic role of platelets is presently well defined and involves pro-incendiary viscid particles in the interface between endothelium and platelets at sites of ruptured atherosclerosis plaques. This promotes the recruitment and adhesion of leukocytes, thereby leading to enhancement of atherosclerotic plaque formation and development of arterial thrombosis. The anti-leukaemic therapies, such as TKIs, are commonly associated with modifications of platelets and endothelial function that lead to the production of pathological thrombi.

The primary purpose of this study was to determine the effect of ponatinib, nilotinib, dasatinib and imatinib on the function of platelets and thrombus growth in mouse and human arteries by in vitro, ex vivo and in vivo methods. The overarching goal was to provide a comprehensive analysis that would improve the understanding of potential mechanisms by which the TKI, ponatinib, potentiates a prothrombotic state.
In vitro experiments demonstrated that dasatinib and imatinib, but not ponatinib or nilotinib, inhibited ADP-, CRP-, and collagen-induced platelet aggregation. Ponatinib also potentiated PAR-1-mediated alpha granule release, whilst imatinib and dasatinib showed inhibitory effects on platelet alpha granule exocytosis following agonist stimulation.

In this study, we demonstrated using intravital microscopy of vascular injury of mesenteric arterioles and carotid arteries induced by ferric chloride (FeCl₃) that there was a significant increase in in vivo thrombus formation over time in mice treated with ponatinib (3 mg/kg), nilotinib (25 mg/kg), but not with imatinib (25 mg/kg) or dasatinib (5 mg/kg). In addition, the effect of ponatinib was independent of platelet glycoprotein surface expression. Ponatinib potentiated alpha granule exocytosis in humans and mice.

The data presented in this thesis confirmed the prothrombotic effects of ponatinib and nilotinib in humans where it was shown that in whole blood from drug-treated CML patients there was increased ex vivo platelet adhesion under flow conditions and increased plasma levels of soluble P-selectin (sP-selectin) and pro-inflammatory cytokines.

The results from this study also demonstrated that ponatinib and nilotinib TKIs have a prothrombotic effect that can be reversed by pretreatment with the L-type calcium channel blocker, diltiazem and the COX-2 inhibitor, diclofenac, and to a lesser extent by the eNOS synthase inhibitor, L-NNA. However, the study suggested that nilotinib or ponatinib-treated CML patients who develop vascular complications of coronary artery vasospasm or cerebrovascular accident (CVA) could potentially be treated with L-type calcium
channel blockers to reduce arterial occlusive events. Further clinical trials are indicated to examine the efficacy and safety of using calcium channel blockers in the clinical setting of ponatinib and nilotinib-treated CML patients.
Chapter 1: LITERATURE REVIEW
1.1 INTRODUCTION

Blood has four main components in the body. These components are red blood cells, white blood cells, plasma and platelets [1]. Haematopoiesis is the process of blood cell production and formation from haematopoietic stem cells (HSCs) which occurs during embryogenesis and throughout life. It occurs mainly in bone marrow within adults but also occurs in liver, spleen and thymus [2]. The haematopoietic stem cell (HSC) is a multipotent stem cell that resides in the bone marrow and produces all of the cells of the blood and immune system [3]. In addition, HSC is a rare population of cells that gives rise to all of the cells comprising the two main differentiation pathways of the haematopoietic lineage: the myeloid arm and the lymphoid arm (Figure 1). However, defects can occur in the haematopoietic system leading to serious haematologic diseases such as anaemia and leukemia [4]. Leukemia is divided into two main types which are acute and chronic based upon onset of the disease, and various types of leukaemia including acute myeloid leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myeloid leukaemia (CML), and chronic lymphocytic leukaemia (CLL) occur in adults [5]. However, CML is rare accounting for only 0.3 % of all adult cancers in Australia [6].
Figure 1. Haematopoietic stem cell system. The HSC differentiate into two different kinds of progenitors common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), which further differentiate to various blood cells including platelets, granulocytes, lymphocytes and monocytes [7].

Chronic Myeloid Leukaemia (CML) is a malignancy of haematopoietic stem cells caused by the formation of the Philadelphia (Ph) chromosome that carries a reciprocal translocation of chromosomal 22 and 9 [8]. The translocation leads to a Bcr-Abl (breakpoint cluster region gene-Abelson gene) fusion gene which results from a portion of the Abl gene from chromosome 9 fusing with the remaining portion of the Bcr gene on chromosome 22 [9] (Figure 2). The classic fusion in CML is b3a2 or b2a2 fusion of exon 13 (b2) or exon 14 for b3 of Bcr to Abl’s exon 2 (a2) [10]. However, the reciprocal translocation of the chromosome results in a constitutively active tyrosine kinase (TK) protein producing myeloid hyper-proliferation associated with pathogenesis of CML. The reciprocal translocation can be identified through
routine karyotyping. TK activates several downstream signaling pathways resulting in uncontrolled proliferation and survival of myeloid cells [11].

CML progresses in multiple stages that include a long initial chronic phase that is able to last for more than ten years (CP-CML) [11]. The next stage (AP-CML) is an accelerated phase which is then followed by the blastic phase (BP). The survival rate of patients in the chronic phase is approximately 68% a year [10]. A number of patients are asymptomatic during the first stage where the diagnosis occurs by incidental discovery of elevated white cell counts [12]. In a number of cases, the dysfunction of the myeloid cells mostly results in a more severe, prolonged infection. The condition can be diagnosed in any person at any age; however, it is more common in older people aged above fifty years [13]. It is also reported that the susceptibility of CML is increased by family leukemic history and Down syndrome which increase the chances for DNA mutations and of development of precursor leukaemia cells [10].
Figure 2. Formation of the philadelphia chromosome in chronic myeloid leukaemia. A part of chromosome 9 (9q) and a part of chromosome 22 (22p) breaks off and site places. The bcr-abl gene is formed on chromosome 22 where the piece of chromosome 9 attaches. The changed chromosome 22 is called the Philadelphia chromosome which is a hallmark of adult CML [14].

Ubiquitous intracellular TK pathways regulate basic cellular pathways such as proliferation, progression of cell cycle, survival, apoptosis and differentiation through phosphorylated signal transduction molecules. Dysregulation of tyrosine kinase expression by mutations in promoters governing their expression, or, as in Bcr-Abl, by mutations creating novel protein tyrosine kinases (PTKs), often contribute to dysregulated cell division, survival and metastatic properties as seen in cancer cells [15]. Some cancer therapies involve small-molecule inhibitors that target TKs, although there are others that are directed against serine and threonine kinases [16]. Under normal conditions the level of TK phosphorylation is closely controlled through antagonistic actions of SH2 domain containing protein-tyrosine phosphatases. Genetically
mutated or over-expressed TK results in unwarranted tyrosine phosphorylation and gene transcription changes that leads to tumorigenic processes [17].

TKs are classified into two receptors: receptor TK and non-receptor TK (Figure 3). Receptor tyrosine kinases (RTK) are made up of transmembrane domain, extracellular ligand-binding and intracellular TK catalytic domains. Most RTKs have a single polypeptide chain and are monomeric due to a lack of ligands (Figure 3) [18]. On the other hand, non-receptor TK relay intracellular signals given that they are found within the cell. An example of non-receptor TK is Abl [19]. A constitutive TK activity occurs through CML Bcr-Abl fusion gene. The activity is characterised by predominant proliferation of mature granulocytes within the bone-marrow and myeloid cells’ unregulated growth [20].

There is a likelihood that CML starts through mutation of a pluripotential stem cell. If true, this implies that the treatment of clonal haematological malignancy may occur through a single pathway. Consequently, the generation of a discerning tyrosine kinase inhibitor (TKI) is a
representation of a promising therapeutic strategy in the treatment of CML [21]. While targeting the Abl kinase domain using TKIs is generally effective, the genetic instability in primitive CML stem cells leads to abnormalities in DNA repair mechanisms. As a result, additional mutations in these primitive stem cells develop leading to resistance with some TKI therapies.

Other TKs such as epidermal growth factor receptor (EGFR) are involved in tumours like prostate, colon and breast cancer. Application of specific tyrosine kinase inhibitors block tyrosine phosphorylation mediated by protein dimerization (Figure 3.1) [22]. The resultant effect is the blocking of the initiation of signaling cascade and stops the cell for proliferating and dividing. Compound cancer growth inhibitors can block one or more type of RTK.

**Figure 3.1. Epidermal growth factor receptor (EGFR) pathway.** Ligands, such as epidermal growth factor (EGF), transforming growth factor (TGF)-α, or others, bind to the homo- and heterodimer kinase domain (TK), resulting in activation and receptor transphosphorylation. This creates docking sites for the adaptor proteins, Grb2 and Sos, which recruit Ras and phosphatidylinositol 3-kinase (PI3K), leading to the formation of two major signalling pathway branches, Ras/MAPK and PI3K/Akt. These networks result in, amongst others, proliferation, evasion of apoptosis and angiogenesis. MAPK: mitogen-activated kinase-like protein [23].
1.1.1 Protein tyrosine kinase involvement

Protein tyrosine phosphorylation (PTP) has been implicated as one of the essential cell-signaling pathways. The transfer of phosphate groups by adenosine triphosphate (ATP) on tyrosine residues within proteins encoded by multicellular organisms gives rise to intracellular connections through biochemical signaling [24]. PTP is mediated and regulated by specific enzymatic molecules called protein-tyrosine kinases (PTKs), which catalyse the transfer of the terminal phosphate of ATP to tyrosine residues on protein substrates [25, 26]. PTKs belong to a heterogeneous superfamily of proteins with specific importance in numerous physiological events, including the mediation and regulation of numerous responses, such as cell growth, proliferation, differentiation, metabolism, migration and apoptosis [24]. PTKs can be subdivided into two broad families based on their supposed structures [27]. Receptor tyrosine kinases (RTKs), are characterised by having both an intracellular and an extracellular domain. This family includes platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and the c-Kit receptor [28]. The second family, non-receptor protein tyrosine kinases (NRTKs), is characterised by a lack of extracellular sequences. NRTKs contain modular domains, which play an important role for subcellular objectives and the regulation of catalytic actions (Figure 4). NRTKs include distinct families such as the Src family kinases (SFK) (Figure 4.1), Abl and spleen tyrosine kinase (Syk) play a critical role as a cytosolic enzymes that catalyse the transfer of phosphate group from ATP to tyrosine residues in proteins [29].
Figure 4. Non-receptor protein tyrosine kinase families (NRTKs). This figure shows domain structures of the main subfamilies of NRTKs. The carboxyl group is on the right side and the amino group is on the left. Adapted from [26, 30].
Figure 4.1. Diagram of Src kinase activation. Diagram represents the inactive (left) and active (right) conformations for comparison. The two intramolecular interactions that regulate Src activation are indicated by arrows. Upon phosphorylation of the autoinhibitor site (Y529 for mouse Src, see text for more details) the C-terminus of Src binds to the SH2 domain and the linker region binds to the SH3 domain, resulting in a closed molecular structure. This closed (inactive) conformation reduces the access of Src substrates to the kinase domain. On the other hand, dephosphorylation of Y529 results in an open (active) molecular structure, giving Src substrates access to the kinase domain. Full Src activation requires the autophosphorylation of Y418 (in mouse; see text for more details). Binding to other molecules such as CAS (Crk-associated substrate) or FAK (focal adhesion kinase) displaces the intramolecular inhibitory interactions and results in activation of Src (not shown in the figure). The gray line indicates myristoylation sites important for membrane localization.

1.1.2 The Efficacy, Safety, and Complication of Tyrosine Kinase Inhibitor Therapy

In the past, treatment of chronic myeloid leukemia was ineffective for the majority of patients [31]. Historically, there are different types of treatment for CML including chemotherapy with hydroxyurea, cytarabine, cyclophosphamide and stem cell transplantation which has improved survival of leukemia patients [32-34]. The treatment of CML utilises tyrosine kinase inhibitors which has increased the 10 years survival rate of chronic phase CML patients to more than 80 percent [35]. Imatinib (Gleevec) was introduced in 2000 as the first TKI for treatment of CML.
Imatinib has been clinically approved in all clinical phases of CML and has provided an efficient and advantageous safety profile [36]. It revolutionised therapy for chronic phase CML significantly improving quality of life and survival outcomes. Some of the TKIs developed for the treatment include second generation compounds such as nilotinib and dasatinib. Despite the high 10 year survival rate of patients who are treated with TKIs such as imatinib, there are emerging issues on the treatment of CML using these TKIs [37]. Some of the emerging issues include increase in the development of resistance to dasatinib, nilotinib and imatinib. There are factors that have made the outcomes of between 20 and 30 percent of CML patients insufficient leading to “multi-TKI-insensitivity” due to mutations of the Abl tyrosine kinase ATP binding pocket [38].

Imatinib, a 2-phenylaminopyrimidine is the first CML-targeted TKI. Imatinib works to inhibit ATP binding to Bcr-Abl kinase domain resulting in the stabilisation of an inactive, closed conformation (Figure 5). Most CML patients treated with imatinib are capable of achieving complete cytogenetic response (CCyR) and complete haematological response (CHR). However, there are number of forms of resistance to imatinib messylate (IM) based therapies described in CML patients [39].

One of the related resistances to the therapy comes from leukaemic stem cells develop a residual cancer cell population that is capable of surviving the therapy [40]. This is often referred to as minimal residual disease. When therapy is stopped, the residual cells rebuild the population of the leukaemic cells leading to relapse of the disease. Imatinib fails to effectively target dormant CML stem cells. There is a high likelihood that quiescent cells can retain proliferation potential together with being reservoir for the relapse of disease. These quiescent cells can as well lead to progression of the condition to an advanced stage. This also shows a
possibility of the recurrence of the disease caused by the amplification of Bcr-Abl gene. On the other hand, second (nilotinib & dasatinib) and third (ponatinib) generations were developed as a solution to imatinib-resistant CML and has a higher linking affinity than imatinib producing a $>30$-fold greater effectiveness [41]. Recent reports suggest that chronic phase CML patients treated with nilotinib remain in remission after withdrawal of drug but not imatinib [42]. However, the two drugs demonstrate different side-effects and target selectivity.

Figure 5. Mechanism of action of the Bcr-Abl TKI. (A) The Bcr-Abl tyrosine kinase (Bcr-Abl TK) is a constitutively active kinase that binds ATP. Transfer a phosphate from ATP to tyrosine residues on different substrates lead to an alteration in cellular activation which leading to myeloid hyper-proliferation CML development. (B) The imatinib TKI blocks the binding of ATP to the Bcr-Abl and inhibits the TK phosphorylation of Bcr-Abl-mediated signaling pathways. (C) In imatinib resistant CML, ATP binds Bcr-Abl TK that leads to hyperactivity of tyrosine kinases resulting in relapse of CML. The second & third generation of TKI, nilotinib, dasatinib and ponatinib effectively overcomes imatinib resistance via potent inhibition on Bcr-Abl TK phosphorylation and ponatinib will be effective for all Bcr-Abl mutations including T315I.
Nilotinib is a structural analogue of imatinib that interacts with both active and inactive conformation of Bcr-Abl [43]. Nilotinib inhibits cells that express kinase-activating mutants in a variety of tyrosine kinases such as c-Kit. Moreover, nilotinib is well tolerated in patients with CML-CP after imatinib failure or intolerance [12]. However, nilotinib-treated CML patients have been observed with side effects of impaired glucose metabolism, hypercholesterolaemia and cardiotoxicity [44]. The rapid development of progressive peripheral arterial occlusive disease (PAOD) with patients who were treated by nilotinib 300 or 400 mg twice daily within 8 months after moving from imatinib to nilotinib is of major concern [45]. The common tyrosine kinase inhibitor drugs details are shown in Table 1.
**Table 1.** Common tyrosine kinase inhibitor drugs; branded name, market authorisation holder, target tyrosine kinases, indications. INN: International Nonproprietary [46].

<table>
<thead>
<tr>
<th>TKI (INN)</th>
<th>Branded</th>
<th>Market authorisation</th>
<th>Target tyrosine kinases</th>
<th>Indication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>Glivec</td>
<td>Novartis</td>
<td>BCR-ABL, KIT, PDGFR-A, PDGFR-B</td>
<td>CML, GIST, BCR-ABL-positive ALL, dermatofibrosarcoma protuberans, myeloproliferative neoplasms, hypereosinophilic syndromes</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>Tasigna</td>
<td>Novartis</td>
<td>BCR-ABL, KIT, PDGFR-A, PDGFR-B</td>
<td>CML</td>
</tr>
<tr>
<td>Ponatinib</td>
<td>Iclusig</td>
<td>Ariad</td>
<td>BCR-ABL</td>
<td>Patients with CML for which Imatinib, Nilotinib and Dasatinib are not appropriate (or patients carrying a T315I single-point mutations)</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Sprycel</td>
<td>Bristol-Myers Squibb</td>
<td>BCR-ABL</td>
<td>CML</td>
</tr>
</tbody>
</table>
The initial design of dasatinib was as a Src family kinase (SFK) inhibitor which targets mutated Bcr-Abl by a distinct binding to inactive and active Abl-tyrosine kinase [47]. However, studies have shown that the drug affects activation of platelets induced by thrombin, collagen and ADP [48]. The platelet activation is possibly a contributory factor towards inhibition of SFK including Fyn and Lyn induced by vWF/GP1b-IX-V complex [49]. In addition, increased gastrointestinal bleeding has been linked to dasatinib treatment [50]. As a result of these challenges and the side effects associated with imatinib, dasatinib and nilotinib, a new multikinase inhibitor drug named ponatinib has been developed for T315I-based resistance (Figure 6). T315I Abl-Bcr mutant is a common mutation occurring in 15% CML patients. However, ponatinib has the ability to selectively inhibit cells carrying the T315I Bcr-Abl mutant that are insensitive to other clinically available Bcr-Abl inhibitors [51]. Ponatinib, a third generation TKI, uses a different interaction mode as compared to other TKI [52]. It binds with higher affinity and stronger than other TKIs.
Figure 6. Molecular structure of nilotinib, imatinib, dasatinib and ponatinib. Nilotinib is an analogue of imatinib. However, nilotinib is characterised by incorporating alternative linking groups to the N-methylpiperazine moiety and conserving an amide pharmacophore to maintain four H-bond associations with Glu286 and Asp381. Dasatinib and ponatinib are structurally distinct from imatinib and nilotinib. Both nilotinib and ponatinib contains trifluoro groups in their chemical structure designed to increase biological activity. Adapted from [53].
1.2 Ponatinib

1.2.1 Mechanism of action and contribution to the development of vascular complications.

This drug was designed through a structure-based and computational approach to generate a pan Bcr-Abl primary target enzymatic activity that includes T315I which is the most common resistance mutation detected in CML [54] (Figure 7). T315I forms hydrogen bonds between Abl and imatinib at an allosteric site resulting in a conformational change at the active site. The structure permits the drug to link to isoleucine T315I in the mutated side chain through van der Waals bonding [51, 55]. Recently, a T315L mutation of Abl kinase was described that is associated with ponatinib resistance and poor prognosis [56]. Ponatinib also has an inhibitory activity against platelet derived growth factor receptor alpha (PDGFR) families, fibroblast growth factor receptor (FGFR) members and vascular endothelial growth factor receptor (VEGFR) [57]. A major factor reducing the utility of ponatinib is that, 22% of patients receiving ponatinib experience arterial thrombosis that may induce a stroke or heart attack in patients receiving it [58].

The increased risk of the thrombotic complications can lead to a patient’s death unless a concurrent use of anti-platelet agents, such as 100 mg of aspirin per day, is applied [12]. It may be argued that the thrombotic events are as a result of a number of cardiovascular risk factors such as diabetes mellitus, smoking, advanced age, or arterial hypertension [59]. However, the thrombotic events have been observed in both the absence and presence of the risk factors [45]. This is an indication of a direct drug effect of ponatinib to produce prothrombotic effects. Following the observed increase of 19 % in arterial thrombotic events, ponatinib was withdrawn and suspended from the market by the FDA and Ariad Pharmaceuticals in October 2013 [60]. Following introduction of safety measures, ponatinib was reintroduced into the market in January 2014 with recommendations for drug dose reduction strategies, patient
selection, the need to evaluate cardiovascular and thrombotic risk before use and dosage was reduced from 30 mg to 15 mg QD.

Figure 7: Ponatinib with Triple bond (yellow) unique structural feature evades the T315I gatekeeper mutation (blue) [61].

1.2.2 The Management of Side Effects

The side effects of Bcr-Abl inhibitors can be debilitating to some CML patients. Even side effects defined as low-grade may affect a patient after a prolonged period of time [62]. For this reason, it is important to have a long-term plan for the management of side effects to maintain the patient’s quality of life. In all the Bcr-Abl inhibitors, the most common side effects seen in
CML patients are low blood counts such as anaemia 22% and thrombocytopenia 4% while different drugs have a different spectrum of other side effects (Table 2) [63].

**Table 2**: Safety profiles of TKI. CNS: central nervous system; TKI: tyrosine kinase inhibitors. INN: International Nonproprietary Name x: rare, xx: uncommon [46].

<table>
<thead>
<tr>
<th>TKI</th>
<th>CNS</th>
<th>Heart disorders</th>
<th>Vascular disorders</th>
<th>Renal disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Nilotinib</td>
<td>X</td>
<td>X X</td>
<td>X X</td>
<td>X</td>
</tr>
<tr>
<td>Ponatinib</td>
<td></td>
<td>X X</td>
<td>X X</td>
<td>X X</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>X</td>
<td>XX</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Gefitinib</td>
<td></td>
<td></td>
<td>X</td>
<td>X X</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X X</td>
</tr>
</tbody>
</table>
Oncologists and other clinicians are, therefore, required to assist their patients to understand the benefits of reporting the side effects such that they can be managed prior to becoming serious. This would allow the treatment of CML to continue uninterrupted [62]. Most of the side effects are generally managed through reduction of the drug dosage or the interruption of the treatment, but in some cases, the discontinuation or change to another TKI of the treatment is needed [64]. Oncologists and other clinicians should provide guidance to their patients to manage side effects resulting from additional medications (30% of elderly patients) e.g to follow their instruction to avoid any complications. They are required to reassure the CML patients with regard to the effects of the medication on Bcr-Abl inhibitor efficacy [62]. However, CML patients with cardiovascular risk factors are not given nilotinib or ponatinib as frontline treatment to avoid thrombotic complications [65]. Data obtained in October 2013 from the ongoing PACE trial with ponatinib-treated CML patients showed that after a median of 24 months of follow-up, ponatinib was associated with an increased frequency of 19% serious arterial occlusive events, compared with the frequency reported after 11 months of follow-up. Recommendations for dose reduction were implemented in October 2013 to mitigate the risk of additional serious adverse events [66].

1.3 PLATELET OVERVIEW

Platelets are well identified that play crucial role in the pathogenesis of CVD, particularly involving arterial thrombosis. Platelets are tiny disc shaped cell fragments that are derived from precursor megakaryocytes. Platelets were discovered in 1882 by Giulio Bizzozero, who described these unique cells as granular, irregular and anuclear discs [67]. Platelets in mammalian blood systems are required for the formation of blood clots, healing of wounds and for maintaining haemostasis [68]. Platelets are also involved in angiogenesis in both adults and embryos. Insufficient platelets in the blood stream (thrombocytopenia) can result in excessive
bleeding. Platelets are also involved in certain diseases such as atherosclerosis, and thrombophlebitis [69]. Moreover, the angiogenic properties of platelets promote metastatic diseases like tumour spreading. Anti-platelet therapies are geared toward the prevention of myocardial infarction and excessive clotting [70]. Platelets are a main source of growth factors and their proteins used in the healing of wounds. These factors include transforming growth factor beta (TGFB), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) [71]. During the clot formation process, the shedding of platelets occurs releasing the growth factors together with chemotactic signals [70].

Megakaryocytes are the precursor of platelets which originate from haematopoietic stem cells. These are the rarest (0.01 percent) as well as the largest cells (50-100 micrometer) found in the bone marrow. It is through endomitosis that platelets are released from the megakaryocytes [72]. This process involves several replications of the DNA without cell division. In addition, the other process that follows the replication of DNA includes the synthesis of protein and formation of granules [72].

Platelets are formed from proplatelets which are long cytoplasmic extensions of the megakaryocyte plasma membrane. In the cytoplasmic expansion phase, immediately after endomitosis, megakaryocytes synthesise specific organelles [73]. The resultant process is the assembly of microtubules at the centrosome (Figure 8). They run parallel to the cell membrane as they spread out towards the cell’s periphery. Prior to the initiation of the proplatelet, microtubules are assembled under the cell surface [74]. They assist in forming pseudopods that enlarge into becoming processes of proplatelets. Organelles are then moved within tracks of the microtubule toward the proplatelets ends in which the assembling of platelets occur [75]. Proplatelets are then constricted, resulting in a beaded appearance. The process results in the
conversion of megakaryocytes into proplatelets which divide and fragment to produce individual platelets [76]. It is estimated that about $10^{11}$ platelets are produced daily in an average human at circulating concentration of about 150-400 x $10^9$ per litre of blood [77] (Figure 7). The platelet development and formation process are influenced by growth factors such as thrombopoietin (TPO) that regulate the formation of megakaryocytopoiesis [78]. It is believed that the differentiation processes and maturation of MK are promoted by various interleukins (IL-11, IL-6, and IL-3) that work in association with TPO and cytokines [79].

Endoreduplication, polyploidisation, polyploidy and addition of the cytoplasmic mass distinguish the maturation process, proliferation, and terminal differentiation of MK [80]. Immature MKs are characterised by rough endoplasmic reticulum and high levels of ribosomes as well as ribonucleic acid (RNA) content [81]. As the MKs mature, their cytoplasm continually develops, which includes platelet organelle amplification, intricate membrane system formation, and the production of specific platelet proteins such as fibrinogen and von Willebrand Factor (vWF) [82] (Figure 8). The amplified platelet organelles include alpha granules, dense granules, lysosomes, and T granules. After the maturation process, the centrosomes are disassembled and form microtubules which move to the cortex of the cell. Thick pseudopods are then produced from the tough cytoplasmic mass of the MK [83].

During the expansion of organelles and microtubules into the proplatelet ends, the assembly point of the elongated proplatelets begin to develop. Mature platelets are later released after the conversion of MK cytoplasmic fragments into proplatelets [78]. Platelets are estimated to circulate for an average of between 7 to 10 days in the human body before macrophages situated in the spleen and liver remove them from the blood stream.
Figure 8. (A-E) Several stages promote megakaryocyte transition from immature cells to produce platelets. (A-B) The first stage, the immature megakaryocyte undergoes nuclear endomitosis, organelle synthesis and cytoplasmic maturation and expansion. (C-D) The next stage involves dissociation of the centrosomes and formation of microtubules, which move to the cell cortex and the proplatelet generation that also occurred with the development of thick pseudopods. Slipping of overlapping microtubules leads to proplatelet elongation as organelles are moved independently at proplatelet ends, where emerging platelets assemble. (E) The complete megakaryocyte cytoplasm is altered into a mass of proplatelets and new platelets are produced from proplatelet ends. Adapted from [84].

1.3.1 PLATELET ULTRASTRUCTURE

Platelets are the smallest of all blood cells with average diameters ranging from 2.0 to 5.0 micrometers [85]. Although they are characterised by the absence of genomic DNA, they often contain the translation apparatus essential for the synthesis of proteins as well as megakaryocyte-derived messenger RNA (mRNA) [86]. Platelets do not have a fixed shape
While in a resting physiological state, platelets have a discoid shape as they flow freely in the bloodstream. When platelets are stimulated, their cytoskeleton is modified to result in the platelet becoming a small sphere with numerous long extensions from its discoid shape to enhance adhesion through activation and then aggregation of platelets [88]. The ultra-structure of platelets can be divided into four zones: the structural zone, the peripheral zone, membrane systems, and the organelle zone [89] (Figure 9).

**Figure 9. Platelet ultrastructure:** The three main structural zones are peripheral, membranous, organelle and T-granules. Function of peripheral zone is adhesion and aggregation. In addition, the membranous zone play as a main role in structure and support and finally, the organelle zone is for the secretion and storage of platelets [90].
1.3.2 The Peripheral Zone Structure

The peripheral zone comprises a coagulation layer, a unit membrane, a phospholipid membrane, and glycocalyx [91]. This zone has a key role in the transport of substances from the surface of the platelet into the cell and in the release of alpha granule products secreted throughout platelet activation. The platelet membrane has two main components, phosphatidylserine, and phospholipids which facilitate and act as the place for configuration of the prothrombinase complex of coagulation, and the membrane also contains tissue factor that are exposed during the activation process with the production of microparticles [92]. At the same time, the membrane contains tissue factor that is exposed during the activation of platelets [93]. The process also results in the production of microparticles. Underneath the peripheral zone sits the glycocalyx, a dynamic surface that facilitates the adhesion and aggregation of platelets [94]. It is covered by integrin αIibβ3 molecules and glycoprotein GPIb-V-IX complex receptors. The complex cytoskeleton located underneath the membrane of the platelet contains microfilaments and microtubules that facilitate the dynamic morphological changes of the platelet during the activation process. The primary inner structure of the platelet contains the platelet cytoskeleton that comprises a rigid network of actin cross filaments and central microtubules that strengthen the spectrin mesh [95]. Thus, it is evident that the cytoskeleton is pivotal in maintaining the platelet’s discoid morphology as well as safeguarding the integrity of the platelets from extensive shear stress when they are forced by the flow of the blood against the endothelium [96]. Additionally, the cytoskeleton takes part in the process of platelet activation by facilitating continual shape variations and the interactions that occur when platelets spread over an injured endothelial surface [97].

1.3.3 The Organelle Zone

Platelets have secretory organelles such as lysosomes, dense granules, alpha granules and T granules (Table 3). Platelets contain high granule concentrations which are essential for platelet adhesion, activation and for thrombus formation. In response to the secretion of calcium during
the activation of platelets, dense bodies and alpha granules release their contents [98]. To promote platelet aggregation, all the secretory organelles are specialised to release their contents during the activation process [98].

**Table 3.** The contents of organelles of platelets.

<table>
<thead>
<tr>
<th>Granules</th>
<th>Contents of Granule</th>
</tr>
</thead>
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| **1. Dense granules** | Secretion of ions  
Guanine: GTP, GDP.  
Nucleotides: Adenine: ATP, GTP, ADP, GDP  
Serotonin (Transmitters), Histamine, Bivalent cations, Amines. |
| **2. Alpha-granules** | **Adhesion molecules:** P-selectin (CD62P), Glycoprotein IIb/IIIa (GPIIb/IIIa, integrin αIIbβ3, CD41/CD61), Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), vWF, Thrombospondin-1 (TSP1), Vitronectin, Fibronectin.  
**Protease inhibitors:** Plasminogen activator inhibitor-1 (PAI-1), Tissue factor pathway inhibitor (TFPI), C1 inhibitor.  
**Coagulation factors:** Fibrinogen, Kininogen, Factors V, VII, XI, XIII. |
Acid hydrolases, cathepsins D, LAMP-1, LAMP-2 and WAMP-1. |
1.3.4 The Structural Zone

The sol-gel zone is the matrix of the cytoplasm of the platelets. Situated at the platelet’s center, it comprises various fibre systems in numerous polymerisation configurations and maintains the appearance of the platelet [89]. Its central function is the supply of a contractile system involved in the alteration of the morphology of the platelets, granule release, platelet activation, and the expansion of pseudopodia during the commencement of spreading [99].

1.3.5 Membrane Systems

The plasma membrane of the platelet contains a phospholipid bilayer, with several tiny indentations. Each platelet contains a dense tubular system (DTS) and an open canalicular system (OCS) [100]. The OCS comprises numerous tortuous invaginations on the surface of the platelet that are evenly interposed along its structure [101]. It helps in improving the direct association between the interior surface of the platelet to create a path for the cells to access plasma substances and ingredients. At the same time, it provides a passage that helps in the transportation of particulate and chemical substances useful in the discharge reaction [102]. The platelet membrane is the primary site for the storage of calcium and cyclooxygenase, an enzyme responsible for converting arachidonic acid to thromboxanes and prostaglandins endoperoxide precursors [103]. Previous studies suggested that there is a close connection between the platelet membrane and the two platelet channels and highlighted that the platelet membrane might be essential in the activation of cells, which is achieved via direct communication with signals from the plasma. The transfer occurs through the OCS to DTS elements containing calcium ions and enzymes responsible for the synthesis of thromboxanes and prostaglandins [104]. Additionally, the membrane contains glycoprotein receptors such as fibronectin, collagen, and vWF that aid in the enhancement of platelet adhesion and activation pathways as well as platelet shape change [100]. The DTS helps in regulating the activation of platelets by controlling the release of calcium. It exists as thin elongated membranes in resting
platelets. Ten seconds after the addition of thrombin, there is a distinct ultrastructural change in the platelet’s DTS; from the thin elongated form to a rounded vesicular form [105].

1.3.6 Alpha Granules

Alpha granules are the majority of the organelles found in platelets with 50 to 80 granules in every platelet. They have an elliptical shape with a diameter ranging between 200 to 500 nm [106]. The electron density of granules varies with granules in the outer zone have less electron density than the granules situated in the central zone. Produced within MK cells, alpha granules have high functional protein concentration as they comprise anti-coagulants, coagulation proteins, adhesion molecules (such as P-selectin, CD40L), growth factors, angiogenic factors, and chemokines [107]. When stimulated, proteins such as CD40L and P-selectin are translocated and expressed on the platelet’s surface membrane. Furthermore, proteolytic enzymes can cleave these proteins into soluble forms resulting in their secretion into the plasma [108]. It is worth noting that the expression of CD40L and P-selectin is essential to various atherosclerotic and inflammatory pathways that are involved in different pathophysiological processes. Alpha granules also contain vWF, a large multimeric glycoprotein that is synthesised specifically in the MKs and endothelial cells [109]. It is situated in the subendothelial matrix and is responsible for the regulation of platelet activation and aggregated at vascular injury sites. Primary and secondary haemostatic processes are regulated by the release of the contents of alpha granules [108]. During primary haemostasis, fibrinogen acts as the αIIbβ3’s ligand functioning as an adhesive protein. Fibrinogen is also cross-linked in some of the stimulated platelets to aid in stabilising the growth of the thrombus. The release of coagulation factors V, VII, XI, and XIII is useful in mediating fibrin thrombi formation for the initiation of coagulation and stabilisation of a clot [110].
1.3.7 Dense Granules

Dense granules are also formed in the MK. However, their number is about ten-fold lower than that of alpha granules [111]. Each platelet is estimated to have 3 to 8 dense granules. Some dense granules are elongated in shape while others are spherical [112]. Additionally, dense granules contain specific non-protein components, unlike alpha granules. A key distinguishing trait of these granules is their high electron density that is attributed to the high concentration of substances such as phosphate, serotonin, calcium, adenosine triphosphate (ATP), nucleotides, magnesium, and adenosine triphosphate (ADP) [113]. These molecules are essential in platelet activation and aggregation as well as thrombus formation. Furthermore, dense granules influence the phosphorylation of protein kinase C and other tyrosine kinases useful in the aggregation of platelets by the release of calcium and ATP granules [114].

1.3.8 Lysosomes

In addition, primary platelet lysosomes are formed in the MK. They are found in small numbers, about 1-3 lysosomes in each platelet [115]. They contain a variety of different enzymes; acid hydrolases such as proteases, glycosidases, and cathepsins [116]. They function as a reservoir of different digestive enzymes. In addition, they play a role in digesting phagocytosed particles following their fusion with phagosomes and formation of phagolysosomes. Platelet receptors that localise to lysosomes contain a lysosomal targeting sequence in their cytoplasmic domain, Tyr XX hydrophobic residue such as the lamp proteins and CD63 tetraspanin superfamily member [117].

1.3.9 T-Granules

Toll-like receptors (TLRs) were first described in 1994 by Nomura and colleagues [118]. TLRs are proteins that play a main role to stimulate the innate immunity system. TLRs are pattern recognition receptors that recognise pathogen associated molecular patterns (PAMPs) expressed on pathogens and induce an inflammatory response on binding these PAMPs [119]. These responses normally, involve sentinel cells such as dendritic cells and macrophages [120].
TLRs are expressed by platelets resulting in a link between innate and adaptive immune response. In addition, they help the immune system to recognise molecules that are found by pathogens which lead to activated immune cells [121].

1.3.10 Platelet Function

The basic role of platelets is to offer vascular system integrity and maintain normal haemostasis. These functions rely on the ability of the platelets to provide particular receptors to bind type 1 collagen and vWF exposed form extracellular matrix in order to activate signaling pathways [122]. It has been shown that vWF, fibronectin, and collagen are capable of stimulating platelet adhesion and aggregation. Platelets are generated and activated by locally-produced agonists to induce a change in their shape from discs to tiny spheres with reorganised cytoskeleton and filopodia as discussed in 1.3.1 [123] (Figure 9). These activated platelets release their alpha granule contents including P-selectin that translocates to the surface. It basically mediates the rolling and tethering of leucocytes that are needed for strong extravasation and adhesion. Upon the release of platelet contents, other granules such as lysosomes cause the aggregation of platelets eventually forming platelet plugs at injury sites that assist to stem bleeding [124].

1.3.11 Platelet Activation

Following injury of endothelial cells, when the blood vessel is damaged or diseased, collagen and vWF becomes exposed and serve as substrate for adherence of platelets at the injury site. Platelets are then recruited, activated and aggregated [125]. The activation of platelets can occur via thrombin stimulation, a process assisted by subendothelium tissue factor [126]. Activated platelets change in shape to develop pseudopods. They then elongate to cover the injured or diseased endothelium. The contents of dense and alpha granules are released into the plasma [70]. Dense bodies secrete calcium, serotonin and ADP which then stimulate aggregation. In the same way, serotonin weakly accelerates the aggregation of platelets and also enables glycoprotein IIb/IIIa receptor binding to a number of agents [127]. On the other
hand, alpha granules secrete fibronectin, vWF, fibrinogen, and thrombospondin that serve as agents of connection during aggregation. In addition, GPVI, GPIa and vWF bind to GPIb-V-IX which firmly attaches the platelets to the injured or diseased endothelium (Figure 10) [128]. Eventually, aggregated platelets develop a plug at the site of the wound to stop the blood flow [129].
Figure 10. Stages of platelet activation and thrombus formation. Platelets adhere to a von Willebrand factor (vWF)/collagen matrix, get activated, secrete granular contents, aggregate via integrins, produce thrombin after developing a procoagulant surface, and form a contracted thrombus with fibrin. Heat map with color codes from green (low Ca$^{2+}$ signal) to red (high Ca$^{2+}$ signal). Interactions of platelets with coagulation factor are indicated, as described. Note that procoagulant platelets provide a phosphatidylserine (PS)-exposing surface for the tenase complex (activated FVIII and FIX) and the prothrombinase complex (activated FV and FX). Formed thrombin provides positive-feedback reactions to activate platelets via GPCR and secretion of ADP and TXA2, which bind to their ligands, the P2Y$_{12}$ and TP receptors, respectively to activate coagulation factors, and to convert fibrinogen into fibrin [130].
1.4 PLATELET RECEPTORS AND INTEGRINS

Platelets contain numerous receptors which are important for normal function of platelets including platelet activation, adhesion, spreading and platelet aggregation [131]. These receptors include direct collagen-binding proteins, G-Protein coupled, thromboxane receptor (TP), integrins, immunoglobulin superfamily receptors, ADP purinergic receptors, leucine-rich superfamily member receptors and tyrosine kinase receptors (Figure 11) [132].

Figure 11. Diagram of platelet agonists and their receptors. Platelet agonists involved in platelet activation pathways on the site of vascular injury [133].
1.4.1 Glycoprotein Ib-IX-V complex (GPIb-IX-V).

Glycoprotein Ib-IX-V complex comprises the subunits: GPIbα, GPIbβ, GPV and GPIX. Each of these subunits contain a variable number of leucine-rich repeats. GPIbα and GPIbβ are linked by disulfide bridges, while the GPV and GPIX associate non-covalently with the complex with a ratio of 1:2:1 [134] (Figure 12). The binding between GPIbα and vWF mediates the capture of platelets to the injured vascular wall. In addition, GPIbα subunit bears the binding site for von Willebrand factor (vWF), α-thrombin, leukocyte integrin αMβ2 and P-selectin [135]. GPIb-IX-V complex plays an important role in the initiation of tethering and adhesion of platelets at high shear stress [136].

A deficiency or abnormal glycoprotein Ib-IX-V complex synthesis created by structural mutations in functionally important sites of platelet GPIb-IX-V complex leads to Bernard Soulier Syndrome (BSS) [137]. This is a congenital bleeding disorder that is characterised by macrothrombocytopenia and an inability of these platelets to aggregate in response to ristocetin, an antibiotic that normally causes platelets to aggregate in vitro by binding and activating vWF [138]. In addition, this complex plays a main role in thrombosis, metastasis, the life cycle of platelets, and is implicated in a number of thrombotic pathological processes such as ischaemic stroke and myocardial infarction [139].
**Figure 12. GPIb-IX-V structure.** The GPIb-IX-V complex composed of GPIbα disulfide-linked to two GPIbβ subunits, and associated with GPIX and GPV. Disulfide bonds within domains either side of leucine-rich repeat domains are depicted as solid black bars. The position of sulphated tyrosine residues (Sulfo-Tyr at 276, 278 and 279 of GPIbα), phosphorylated serine residues (Phospho-Ser) and palmitylated Cys residues of GPIbβ and GPIX are indicated. C, C-terminus; N, N-terminus; TM, transmembrane domain [140].

### 1.4.2 Glycoprotein VI (GPVI)

Glycoprotein VI (GPVI) is one of the main important members of the immunoglobulin superfamily expressed on the surface of platelets. There are approximately 2,000-4,500 copies of GPVI per resting platelet and this protein is exclusively expressed on platelets. [141]. GPVI is a major receptor on the platelet membrane surface as a direct collagen-binding protein, which plays role in platelet activation and aggregation upon vascular injury [142]. GPVI forms functional bonds with the GPIb-IX-V complex through a direct association between the
extracellular sequences of GPVI and GPIbα. As a result, the binding of GPVI/GPIb-IX-V complex with extracellular matrix ligands- vWF and collagen quickly induces platelet integrin activation, such as αIIbβ3 that binds vWF or fibrinogen to mediate platelet aggregation and integrin α2β1 that attaches to collagen [141]. GPVI has two extracellular immunoglobulin domains, a mucin-like domain, a transmembrane domain and a short cytoplasmic tail [143].

![Diagram of GPVI structure](image)

**Figure 13. Structure of GPVI.** It has two extracellular immunoglobulin domains, a mucin-like domain, a transmembrane domain, and a short cytoplasmic tail. The proline rich region is linked to the Src homology domains (Fyn and Lyn). Basic region within C-terminal region is connected to calmodulin and associated with FcRγ-chain. Modified from [144].

A salt bridge between an arginine residue in the transmembrane sequences of the GPVI and an aspartic acid residue in the transmembrane sequences of the FcRγ chain leads to linkage of GPVI with FcRγ subunit that contains an immunoreceptor tyrosine-based activation motif (ITAM) [145]. The FCR gamma chain provides the ITAM motif that is required for GPVI signaling pathway. The Fc receptor pathway of GPVI activation involves FcRγ chain (GPVI
transmembrane domain associates with FcR γ chain), Src kinase Fyn / Lyn, and LAT adaptor protein, all participating in phospholipase C activation [146] (Figure 14). When collagen binds to GPVI, platelets are activated initially by tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif of FcR-γ- chain [147]. Cross-linking FcRγ subunit with GPVI leads to the activation of Syk, PI3K, PKC and PLCγ. The phosphorylated FcRγ-chain-ITAM is subsequently associated with the Syk via SH2 domains, and Syk is phosphorylated by Src family kinases (SFKs) which promotes its major activation and initiates interactions with other signaling pathways [148] (Figure 13). However, deficiency in GPVI on the human platelet membrane results in impaired collagen adhesion, thrombus formation and is associated with prolonged bleeding [149]. On the other hand, increased GPVI expression is associated with thrombotic events [150]. GPVI plays a main role for the initiation of early inside-out signaling through platelet activation involving integrins αIIbβ3 and α2β1 that modulates platelet adhesion and aggregation [151].
Figure 14. Immunoreceptor tyrosine-based activation motif (ITAM) receptors in platelets. Glycoprotein VI (GPVI) is a type I transmembrane protein that is constitutively associated with the Fc gamma chain receptor \( \gamma \) (FcR-\( \gamma \)) chain, which bears an ITAM. It consists of two extracellular immunoglobulin domains, a mucin-like domain, a transmembrane domain, and a short cytoplasmic tail. The proline rich region is linked to the Src homology domains (Fyn and Lyn). Basic region within C-terminal region is connected to calmodulin and associated with FcR\( \gamma \)-chain [152].

1.4.3 Integrin \( \alpha 2\beta 1 \)

The integrin \( \alpha 2\beta 1 \) works as a direct receptor for collagen type I and IV [153]. It consists of two subunits (\( \alpha 2 \) and \( \beta 1 \)) with approximately 2000-4000 copies occurring per resting platelet. Integrin \( \alpha 2\beta 1 \) is expressed mainly on epithelial cells and platelets. The \( \alpha 2 \) subunit includes a domain homologous to von Willebrand factor domain attaching to collagen [154]. However, the \( \beta 1 \) subunit has four cysteine-rich regions and a structure similar to other \( \beta \)-integrins. The interaction of integrin \( \alpha 2\beta 1 \) with collagen is essential for form platelet adhesion (Figure 15).
The surface expression of this complex shows high variability, particularly in relation to the polymorphism of GPIa subunit gene [156].

Figure 15. Structure, activation and inhibition of integrin \( \alpha_2\beta_1 \). The upper Left image is inactive conformation of integrin \( \alpha_2\beta_1 \) which bound to collagen with the C-terminal \( \alpha_7 \) helix of the I-domain (red) displaced downward to engage the metal ion (purple) in the I-like domain. The upper Right Inactive conformation of integrin \( \alpha_2\beta_1 \) with stabilising salt bridges formed by the pair E318 and R288 as well as R310 and E338 (Lower) Inhibitor (orange) attached into integrin \( \alpha_2\beta_1 \) I-like domain locking the compound into the inactive conformation [157].
GPVI-collagen binding plays a role in the increasing the affinity of integrin α2β1 for collagen from a low-affinity state to intermediate or high-affinity states. However, platelet integrin α2β1, αIIbβ3 or others promote firm adhesion and further support GPVI-mediated signaling pathways leading to pro-coagulant activity [158].

**Figure 16.** Diagram of platelet adhesion to collagen by interaction of GPVI and integrin α2β1. GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to high-affinity state and the release of second agonists, most importantly ADP and TxA₂. Adapted from [159].
1.4.4 Integrin αIIbβ3

This integrin attaches to fibrinogen and thus plays an important role in platelet adhesion and aggregation to damaged endothelial surfaces. Activation of this complex prescribes the platelet aggregation and the formation of the primary platelet plug, a fibrin clot [160]. The GPIIb/IIIa complex otherwise known as Integrin αIIbβ3 is a major platelet membrane component. There are around 80000 copies of αIIbβ3 found on the surface of unstimulated platelets [161]. Integrin αIIbβ3 is composed of a heterodimeric complex consisting of an αIIb and β3 subunit linked by disulphide bridges [162]. These subunits form Ca²⁺-dependent complexes on the surface of platelet membrane in a 1:1 ratio [162].

Integrin αIIbβ3 plays an important role in haemostasis and thrombosis in clot retraction, cytoskeletal reorganisation (platelet spreading) and stabilisation of platelet aggregates [163] (Figure 17). In the resting platelet state, the integrin is thought to be expressed in a low-affinity state and that the conformation of αIIbβ3 switches to a high-affinity state in response to specific agonist-induced platelet activation, such as ADP and thrombin, through ‘inside-out signaling’ events [164]. Activated αIIbβ3 is able to bind its ligand fibrinogen, which essentially induces important processes involving ‘outside-in’ signaling events [163]. In addition, integrin αIIbβ3 and the immunoglobulin family receptor GPVI signal through similar but distinct tyrosine kinase-based signaling cascades causing activation and aggregation development [165].

Dysfunction or lack of integrin αIIbβ3 due to structural mutations result in Glanzmann thrombasthaenia which is a bleeding syndrome characterised by impaired adhesion and failure of platelet aggregation [166]. In vivo experiments using β3-knockout mice or αIIb-knockout mice elucidated that β3-deficient mice have significantly prolonged tail bleeding times and show defective thrombus formation [156].

Intravital microscopy investigations also verified that β3 deficiency causes defective binding of the integrin to fibrinogen which leads to abnormal platelet aggregation and spontaneous
haemorrhage [167]. Integrin αIIbβ3 clearly has an important role in haemostasis and thrombosis through platelet adhesion, cytoskeletal reorganisation and aggregation events [168].

**Figure 17. Structure & Inside out signaling to induce activation of the integrin αIIbβ3.**
The three major conformational states of integrin αIIbβ3. It contains αIIb domains and β3 subunits. The conversion of integrin αIIbβ3 from low-affinity (a bent state) into high-affinity conformation and stabilisation of high-affinity conformation (an extended closed headpiece state)[169].
1.4.5 G-protein Coupled Receptors

G-protein Coupled Receptors components comprise seven transmembrane receptors that bear different features for use in controlling the actions of thrombin. Specifically, G-protein coupled receptors (GPCRs) have both extracellular and intracellular loops that function as domains that link to G-protein signaling pathways [170]. One vital characteristic common to all GPCRs is the existence of a large cytoplasmic tail that may interact with protein kinase A, or C, or GPCR kinase. When a blood vessel is damaged due to injury, blood usually flows out. Thrombin-mediated platelet activation engages GPCR to initiate the GP signaling pathway. Released secondary mediators of platelets, like TXA$_2$ and ADP, amplify the process that leads to enhanced platelet aggregation of the blood tissue [171]. Specific forms of platelet activators can be used to engage respective ADP receptors, thrombin protease-activated Receptor (PAR) for PAR-1, PAR-2, and PAR-4, TXA$_2$ receptor, and P2Y$_{12}$. These elements respond differently to particular functions of the blood clot control process [172].

1.4.6 Thrombin Receptors

GPCR comprises seven members that includes thrombin. Unlike other components of GPCR and other receptors that may be activated multiple times, thrombin receptors can be activated only once due to the fact that their activation involved a protease cleavage step [173]. Thrombin catalytic activity targets the N terminal of GPCR receptor leading to its cleavage and activation of the receptor [174] (Figure 18). However, studies indicate that PAR receptors are not just restricted to platelets but also in the vasculature in smooth muscle cells and endothelial cells [175]. PAR receptor was found to have a significant role in the control of the spread of tumour growth. Specifically, PAR is composed of four components that include PAR-1 to PAR-4 that varies in expression in different parts of the body. For instance, PAR-1 appears on the plasma membrane of the platelets, which is very sensitive in humans but less efficient in mice [176].
In addition, PAR-1 is the predominant thrombin receptor mediating platelet activation especially in humans with expression of approximately 1,000-2,000 copies per platelet. Mice express PAR-4 and PAR-3, while humans express PAR-1 and PAR-4 that work as signaling systems. However, PAR-4 only becomes more sensitive when PAR-3 is absent which works as a co-factor in the activation of platelets [177].
1.4.7 ADP Receptors

Some of the very first platelet agonists discovered were adenosine diphosphate (ADP) receptors. ADP is stored within platelet dense granules as discussed above and are secreted when platelets are activated [179]. ADP is largely kept at high concentrations in dense granules. It is released in reaction to P2Y_1 and P2Y_{12} receptors mediated by platelet activation. Among the seven members of the transmembrane ADP purinergic receptors are P2Y_1 and P2Y_{12} [180]. Platelet procoagulant activity is activated by the two receptors [25]. On the other hand, P2Y_1
is solely responsible for shape change control [181]. It has been shown that the absence of P2Y12 results in an impaired ADP-induced aggregation which eventually leads to an unstable thrombi and excessive bleeding in-vivo [182]. One striking feature of ADP is its aggregating properties that allow it to change shape of platelets [183]. This observation implies that one can apply P2Y1 receptor to act as moderator. An assessment of the scenario revealed that when ADP participates in the activation of P2Y1, the ultimate effect is further activation of phospholipase under control of a Gq protein [184]. Uniquely, platelets in Gp knockout mice fail to undergo shape changes in the presence of ADP [185]. Thus, expression of ADP purinergic receptors is restricted to certain parts of the body including neuronal cells, platelets and smooth muscle cells [180]. ADP is recognised by nucleotide receptors (P2 purinergic receptors) consisting of the P2X ion-channel family and the P2Y GPCR family [186].

Briefly, ADP mediates platelet activation through binding to two P2Y receptors, P2Y1 and P2Y12 which link to Gαq and Gαi, respectively. In brief, P2Y1 is responsible for Ca2+ mobilisation, thus mediating platelet shape change and transient aggregation in response to ADP [187]. In contrast, P2Y12 plays an important role in inhibition of adenylyl cyclase which reduces the level of cAMP synthesis and promotes the activation of platelets. This occurs by dephosphorylation of vasodilator-stimulated phosphoprotein which carries out activation of integrin αIIbβ3 and increases the cyclic Ca2+ flux and platelet granule release. The signal transductions pathway downstream of P2Y1 and P2Y12 receptors have revealed an involvement of Src tyrosine kinase (Figure 19) [188]. A lack of platelet shape change, impaired platelet aggregation was demonstrated in P2Y1 knockout mice in response to ADP [118]. In contrast, P2Y12 -/- mice demonstrated normal platelet shape change but impaired ADP-induced aggregation. The P2Y12 -/- mice experienced significantly increased bleeding times and unstable thrombus formation [189].
Figure 19. Different subtypes of ADP receptors. P2Y<sub>1</sub> and P2Y<sub>12</sub> are GPCRs. There are two main ADP receptors. The P2Y<sub>12</sub> receptor leads to inhibition of adenylyl cyclase (AC) and a decrease in cyclic AMP (cAMP), resulting in platelet secretion of thrombotic mediators and in platelet aggregation. The P2Y<sub>1</sub> receptor leads to improved calcium influx (Ca<sup>2+</sup>), resulting in platelet shape change. MRS-2179 and Cangrelor (formerly AR-C9931MX) are selective P2Y<sub>1</sub> or P2Y<sub>12</sub> antagonists, respectively [190].

1.4.8 Thromboxane A<sub>2</sub> receptors

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is a platelet agonist. It is a member of the prostanoid family of arachidonic acid metabolites which is generated by the sequential action of three enzymes – phospholipase A<sub>2</sub>, COX-1 or COX-2 and TXA<sub>2</sub> synthase (TXAS) [191]. It is produced during platelet activation through a series of actions of cyclo-oxygenase-1 (COX-1) stimulation in platelets and is synthesised following arachidonic acid generation [192]. TXA<sub>2</sub> amplifies platelet activation signals causing shape change, protein phosphorylation, secretion, and aggregation. TXA<sub>2</sub> is lipid soluble, therefore it diffuses across the platelet membrane to recruit more platelets for promoting platelet plug formation via binding to its receptor [193]. The blockade of TXA<sub>2</sub> secretion that occurs via inhibition of COX-1 by aspirin results from the
conversion of arachidonic acid into prostaglandin H₂ which serves to prevent thrombotic diseases such as atherosclerosis, myocardial infarction and pulmonary embolism. The TXA₂ receptor (TP) presents two splice variants (TPα and TPβ), which differ only at their C-terminal domains and both are generated from a single gene. In human platelets, TPα expression has been identified at the protein level and has been suggested to possibly stimulate adenylyl cyclase. In contrast, TPβ has also been identified at the protein level but it inhibits adenylyl cyclase (Figure 20) [194]. When the TP receptor is activated by prostaglandin endoperoxides PGG₂ and prostaglandin H₂ predominantly couples to Gq and G12/13, and TP receptor has a minimal involvement in tyrosine kinase signal transduction [195]. TP-deficient mice are unresponsive to TXA₂ and show an impaired response to collagen. TP⁻/⁻ mice show delayed bleeding times and are unable to generate stable thrombi. These data suggest that the decreased activation of platelets in TP⁻/⁻ mice might contribute to a reduction in cardiovascular risk [196].
Figure 20. Arachidonic acid metabolism. Arachidonic acid (AA) is oxidised by lipoxygenases (LOX), cyclooxygenases (COX), ETE- Eicosanoid, LTA and LTB-leukotriene A and B, LXA-lipoxin A and cytochrome P450 (CYP 450) to produce either pro-inflammatory (red border) or anti-inflammatory and pro-resolving (green border) metabolites. Adapted from [197].

1.5 Role of coagulation cascade in Haemostasis

The coagulation cascade is triggered to produce a stable clot through crosslinking of fibrin clotting factor complex by factor XIIIa. According to the in vivo cell-based theory, the process takes place in four overlapping stages [198]. The stages include initiation and amplification as the first and second stages and propagation and stabilisation of the formation of thrombus as the third and fourth stages [26]. The coagulation is essentially initiated by the injury of
endothelium resulting in the release of tissue factor [199]. The tissue factor activates the extrinsic pathway resulting in fibrinogen being converted to fibrin. The amplification stages involve aggregation of platelets, activation of protein C, coagulation factors including Xla, Vα and VIIIa and endothelial cell effects [70]. Thrombin then cleaves fibrinogen to develop fibrin clots with platelets [70]. There are a number of different receptors for vWF for the mediation of platelet adhesion and involvement in leukocyte binding [199] (Figure 21). In conditions associated with hypertension with high shear stress, platelet can become activated, predisposing to thrombosis. In addition, hypercoagulability results in elevation of coagulation factors; factor VII, vWF and prothrombin [200]. Clotting of blood occurs either in veins or arteries. Arterial thrombosis always occurs from deposition of platelets under high shear flow rates and the presence of the collagen reach atherosclerotic plaque. Venous thrombosis is mainly linked to reduce blood flow stasis and hypercoagulability with large amounts of fibrin deposited at the vascular injury site [199, 201].
Figure 21. The coagulation cascade. Proposed cell-based model of coagulation in three steps: initiation, amplification and propagation occurring at the surface of TF-bearing cells and platelets. Adapted from [202].
**Gap In knowledge:**

The mechanisms by which ponatinib TKI contributes to vascular occlusive events remains unclear. Whether ponatinib directly induces arterial thrombotic episodes or accelerated atherosclerosis or pathological induction of vasospasm is unclear. By defining the mechanism will help to define ways of ameliorating the prothrombotic effects of ponatinib. These gaps in knowledge provide a basis for the hypothesis and study aims of the project.

**Hypothesis:**

Ponatinib has a prothrombotic effect that leads to thrombotic complications.

**Aims:**

- To determine the effect of ponatinib on platelet function and haemostasis *in vitro* (Use nilotinib, imatinib and dasatinib as comparison).
- To assess the acute effect of TKI treatment (ponatinib, nilotinib, imatinib and dasatinib) on platelet and endothelial activation and thrombus formation *ex vivo* and *in vivo* using a C57BL/6 mouse model.
- To determine strategies to ameliorate the prothrombotic effects of ponatinib *ex vivo* and *in vivo* thrombus formation in mouse models (calcium channel blocker, COX-2 inhibitor and L-NNA inhibitor).
- To examine the *ex vivo* thrombus formation and pro-inflammatory markers of ponatinib-treated CML patients versus healthy human controls and other TKI treated CML patients.
Chapter 2: MATERIALS AND METHODS
2.1 MATERIALS & METHODS

2.2 Materials

2.2.1 Chemicals and reagents

Sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate (NaHCO₃), glucose, tri-sodium citrate, magnesium chloride hexahydrate (MgCl₂-6H₂O), disodium hydrogen phosphate (Na₂HPO₄) were purchased from Merck, Kilsyth (Victoria, Australia). Potassium, Iron (III) Chloride (FeCl₃), quinacrine, dimethyl sulfoxide (DMSO) and Rhodamine G6 dye (Rh6G) were purchased from Sigma Aldrich (St Louis, MO, USA). Type I collagen fibrils were purchased from Nycomed (Linz, Austria). Ketamine was purchased from Pfizer, (Auckland, New Zealand) and xylazine was purchased from Ilium, Troy Laboratories Pty Ltd (Smithfield, NSW, Australia) TRAP peptide SFLLRN, PAR-4 agonist peptide, AYPGKF-NH₂ was purchased from GL-Biochem, Shanghai, China. Thrombin was purchased from Sigma Aldrich (St Louis, MO, USA). ADP was purchased from DKSH (Hullam, Victoria, Australia).

2.2.2 Antibodies

FITC-conjugated anti-mouse CD62P antibodies, PE-labeled mouse IgG isotype control, anti-mouse integrin β3-FITC conjugated (CD61) - FITC conjugated and anti-mouse CD3e were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ). PE-conjugated JON/A monoclonal antibodies, anti-mouse GPIbα- FITC conjugated anti-mouse GPVI (JAQ₁), anti-mouse integrin α2-FITC conjugated and anti-mouse CD9- FITC conjugated antibodies were purchased from Emfret Analtics (Würzburg, Germany).
2.2.3 Preparations of stocks of tyrosine kinase inhibitors

Ponatinib was provided from ARIAD/Takeda Pharmaceuticals Company (Boston, USA) as a pure powder. Both nilotinib and imatinib were purchased from Novartis Pharmaceuticals as a pure powder. Dasatinib was purchased from Bristol Myers Squibb (BMS) (Mulgrave, VIC) as a pure powder form. They were dissolved in DMSO to give a 10 mM stock solution in phosphate buffer solution (PBS) pH 7.4 and stored at -20°C. Fresh dilutions of TKIs were made in phosphate buffered saline (PBS) pH 7.4 that was prepared by dissolving (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) in 1000 mL total volume of Milli-Q water (Millipore, Australia). The maximum final concentration of DMSO in the TKI mixture was less than 0.2% (v/v).

2.2.4 Preparation of tyrosine kinase inhibitors for in vivo work

The following procedure was used to prepare 1 mL of dosing formulation at a 2.5 mg/mL concentration of compounds. 2.5 of each mg of compound was added to 500 μL of 25 mM citrate buffer (pH 2.75) and warmed for one minute in hot water (60-70 deg C). Then extra 500 μL of 25 mM citrate buffer; pH 2.75 was added and the solution stirred for another 5 min until a clear dose solution was obtained. Final concentration of the solution was 2.5 mg/mL of compound. All daily aliquots intended for dosing were administered within 2 hours of preparation. To prepare 1 mL of a 0.5 mg/mL dosing solution, we took 200 μL of the 2.5 mg/mL dosing solution and mixed thoroughly with 800 μL of 25 mM citrate buffer; pH 2.75. The volume of dosing solution to be administered was based on the weight of each individual mouse. For example, 250 μL of a 2.5 mg/mL dosing formulation administered to a 0.025kg (25g) mouse, will yield a dose of 25 mg/kg; 300 μL of a 2.5 mg/mL dosing formulation administered to a 30g mouse, will yield a dose of 25 mg/kg; 250 μL of a 0.5 mg/mL dosing formulation administered to a 0.025kg (25g) mouse, will yield a dose of 5 mg/kg. The dosing
of mice TKI drugs was based on achieving in vivo plasma drug Cmax concentrations within the four time frame (provided by Ariad/Takada Pharmaceuticals).

### 2.2.5 Healthy human donors

The collection of healthy human blood for this research project was approved by RMIT University Human Research Ethics Committee (HREC/14/Austin/404). Each blood donor voluntarily gave informed consent prior to blood collection. Eligible donors were healthy adults of both sexes between 18-58 years of age without known history of disease and donors must not have taken any anti-platelet medications such as aspirin and clopidogrel within the last 7 days. Venous blood was obtained from healthy human volunteers using a 21-gauge needle into a syringe containing 3.2% (w/v) trisodium citrate (9 parts blood to 1 part anti-coagulant).

### 2.2.6 Animal Model

Male and female (age- and sex matched) C57BL/6 mice were obtained from Animal Resource Centre (Perth, Western Australia). These mice were accommodated and monitored at RMIT University animal house facility in accordance with the National Health and Medical Research Council animal guidelines. Four to eight-week-old WT mice were used in the experiments. The experiments procedures conducted on these mice were approved by the RMIT University animal ethics committee (#AEC 1333) or (#AEC 1704). For ex vivo and in vivo model studies, cohorts of C57BL/6 mice were orally administrated by oral gavage once as a single dose with certain concentrations of TKIs according to mouse body weight. Mice were treated with 3, 10 and 30 mg/kg of ponatinib, 25 mg/kg of imatinib, 25 mg/kg of nilotinib and 5 mg/kg of dasatinib. Vehicle control mice were orally given PBS pH 7.4 or 25 mM citrate buffer pH 2.75 alone using the same method of TKIs-treated mice with similar corresponding doses of drugs vehicle- and TKI- treated mice were left for 4 hours (C max peak drug concentration) and had free access to fresh water and food supplement.
2.3 METHODS

2.3.1 Preparation of human platelets

Whole blood samples obtained from either healthy volunteers or CML patients were immediately collected into tubes containing 3.2 % (w/v) tri-sodium citrate. Platelet rich plasma (PRP) was generated by centrifuging the citrate blood at 190 ×g for 10 minutes without brake at room temperature using an Allegra X-15R centrifuge (Beckman Coulter, Brea, USA). Washed platelets were generated from PRP by centrifugation at 1000 ×g for 15 minutes without brake in the presence of 50 ng/mL PGE\textsubscript{1}. The platelet pellet then was washed twice in Ringer-citrate-dextrose (RCD) buffer (108 mM NaCl, 38 mM KCl, 1.7 mM NaHCO\textsubscript{3}, 21.2 mM sodium citrate, 27.8 mM glucose and 1.1 mM MgCl\textsubscript{2}-H\textsubscript{2}O, pH 6.5) in the presence of 50 ng/mL of PGE\textsubscript{1} gently resuspended in RCD buffer. Finally, the platelet pellet was then resuspended in RCD buffer, pH 7.4, to give a final concentration of 100×10\textsuperscript{9}/L of platelets [203]. The remaining citrate blood was further centrifuged at 1000 ×g for 10 minutes without brake at room temperature to obtain platelet poor plasma (PPP) from the top layer of the centrifuged blood sample to be used as an optical baseline standard for platelet aggregation studies.

2.3.2 Preparation of mouse platelets

Blood was collected from C57BL/6 mice under universal anaesthesia using inhalation of 2% isoflurane from an Isotec3 vaporiser linked with two litres/minute oxygen flow to initiate a deep plane of anaesthesia. Whole blood was collected from mice by cardiac puncture using a 26-gauge needle attached to a 1 mL syringe and immediately placed into 100 μL 3.2% (w/v)
trisodium citrate in a ratio of 9 parts blood to 1 part anti-coagulant. Approximately 600 to 900 μL blood was collected from each mouse. PRP was obtained through centrifugation of the whole blood at 115 ×g for 8 minutes at room temperature without brake in the presence of 50 ng/mL of PGE₁. Washed platelets were obtained via re-centrifuged PRP at 640 ×g for 10 minutes without brake at room temperature. The platelet pellet was then resuspended gently in RCD buffer, pH 7.4, at a concentration of 100×10⁹ platelets/liter. Finally, mice were killed by cervical dislocation immediately following cardiac puncture. Mouse plasma samples were generated from whole blood for measurement of soluble levels of P-selectin and other cytokines. Plasma was isolated from whole blood by centrifugation at 1000 ×g for 15 minutes at room temperature, and this process was repeated twice. Finally, plasma was separated by aliquoting into microcentrifuge tubes and immediately frozen at -80°C until analysis.

2.3.3 Platelet aggregation studies

As described in section 2.2.1 for the separation of PRP from human whole blood. Platelet aggregation responses were recorded by measuring the changes in light transmission using a four channel lumi-aggregometer (Chrono-Log Co, Havertown, PA) [403]. PRP was normalised to 100×10⁹/L in RCD buffer, pH 7.4 using a Cell-Dyn Emerald haematology analyser (Abbott Diagnostics, Abbott Park, Illinois). PRP aggregation was performed in the presence of 1 mM CaCl₂ with continuous stirring (1000 rpm) at 37°C. Human PPP was set as baseline diluted 1:2 in ringer citrate dextrose (RCD) buffer, pH 7.4. PRP was pre-incubated with different concentrations (0.05, 0.1, 1, 2.5, 5 and 10 μM) of ponatinib, imatinib, nilotinib or dasatinib for 10 minutes at 37°C. Subsequently, platelet aggregation was initiated by adding certain agonist, including 1 μg/mL collagen, 0.5 μg/mL CRP or 5 μM ADP. The platelet aggregation response was measured as the maximal increase of light transmittance platelet aggregation over 10 minutes was recorded.
2.3.4 Measurement of alpha granule exocytosis

Washed platelets (100×10^9/L; 50 µL) were pre-incubated with or without different concentrations of ponatinib, nilotinib, imatinib or dasatinib at 37°C for 10 min. Platelets were then fixed in 1% (w/v) paraformaldehyde with RCD buffer pH 6.5 for a 10 min at room temperature followed by termination of the reaction with 1 mL of RCD buffer pH 6.5 containing 0.2% (w/v) BSA and the samples were centrifuged at 640 xg for 10 minutes without brake. Fixed platelets were pre-labelled with 10 μg/mL P-selectin antibody (FITC-conjugated - anti-mouse-CD62P antibody) for 30 min at room temperature in the dark. Following, samples were washed in RCD buffer pH 6.5 with 0.2% (w/v) BSA and centrifuged at 640 xg for 10 minutes without brake. Lastly, platelets were then diluted with 300 µL of RCD buffer pH 7.4. Platelet alpha granule secretion (P-selectin expression) was recognised using platelet population characteristic side and forward light scatter channels. A total of 10,000 platelet events were analysed for each individual sample using FACS Canto II flow cytometer and Weasel software Version 3.0.2 (Walter Eliza Hall Institute, Victoria).

2.3.5 Measurement of platelet dense granule exocytosis

Washed human platelets (100×10^9/L; 50 µL) were pre-labelled with 100 µM quinacrine dye for 30 minutes in the dark at 37°C, allowing the platelet dense granules to take up the dye. The reaction was terminated by adding 1 mL RCD, pH 6.5 containing 0.2% (w/v) BSA and centrifuged the samples at 640 xg for 10 minutes at room temperature without brake. Platelets were then incubated with or without various dose responses of ponatinib, nilotinib, imatinib or dasatinib at 37°C for 10 minutes. A range of platelet agonists such as 0.25 U/mL thrombin, 1.25 μM TRAP peptide SFLLRN and 1.25 μg/mL CRP were added to samples and incubated at 37°C for 10 minutes to stimulate platelet activation and trigger dense granule exocytosis. The stimulation was stopped by adding 300 µL of RCD buffer, pH 7.4. Immediately, platelet dense granule release was quantitated as the percentage of decrease in quinacrine fluorescent intensity.
compared to resting platelets using flow cytometry. The flow cytometer settings were recognised for the acquisition of platelets via logarithmic signal intensification. The dot-plot template was configured specifically for platelet-FITC/PE protocol based on characteristic side and forward light scatter channels and fluorescence parameters in log scale. A total of 10,000 platelet events were analysed for each individual sample using FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) and Weasel software Version 3.0

2.3.6 Analysis of PAC-1 mAb binding

The ability of PAC-1 monoclonal antibody binding to the active conformation of integrin αIIbβ3 (GPIIb/IIIa) on human platelets was tested as previously described [204]. In brief, 50 μL of washed platelets (100×10⁹/L) derived from normal human donor blood were incubated with PE-conjugated PAC-1 mAb (1:50 dilution) for 1 hour at room temperature in the dark. Platelets were then terminated with 0.2% (w/v) BSA-RCD buffer pH 6.5, and then centrifuged at 640 × g for 10 minutes without brake. Labelled-platelets were then stimulated with certain agonists such as thrombin (0.25 U/mL) for 60 minutes at room temperature. Platelets were then washed in 0.2% (w/v) BSA-RCD buffer pH 6.5, and then centrifuged at 640 ×g for 10 minutes without brake. The activation of platelets was terminated by the addition of 1% (w/v) paraformaldehyde for 10 min at room temperature. Finally, platelets were diluted in 300 μL of RCD buffer, and a total of 10,000 events were quantified using FACS Canto II flow cytometer and Weasel software Version 3.0.2.

2.3.7 Analysis of JON/A-PE mAb binding

The ability of JON/A monoclonal antibody binding to the active conformation of integrin αIIbβ3 (GPIIb/IIIa) on murine platelets was tested as previously described [205]. In brief, 50 μL of washed platelets (100×10⁹/L) derived from mice untreated or treated TKIs were
incubated with PE-conjugated JON/A mAb (1:50 dilution) for 1 hour at room temperature in the dark. Platelets were then terminated with 0.2% (w/v) BSA-RCD buffer pH 6.5, and then centrifuged at 640 × g for 10 minutes without brake. Labelled-platelets were then stimulated with certain agonists such as thrombin and CRP for 60 minutes at room temperature. Platelets were then washed in 0.2% (w/v) BSA-RCD buffer pH 6.5, and then centrifuged at 640 ×g for 10 minutes without brake. The activation of platelets was terminated by the addition of 1% (w/v) paraformaldehyde for 10 min at room temperature. Finally, platelets were diluted in 300 μL of RCD buffer, and a total of 10,000 events were quantified using FACS Canto II flow cytometer and Weasel software Version 3.0.2.

2.3.8 In vitro and ex vivo flow thrombus formation onto immobilised type I collagen

Briefly, glass μ-slide VI 0.1 with specific dimensions (0.1×1.0×45 mm, HxW×L) (Ibidi Company, Martinsried, Germany) was coated with 500 μg/mL type I equine ligament collagen (Nycomed, Linz, Austria) for two hours at 37°C followed by rinsing with PBS pH 7.4 to remove non adherent collagen.

For in vitro arterial thrombus growth studies, anticoagulant whole blood from human or mice were normalised with RCD pH 6.5 to 200×10⁹/L and 300×10⁹/L respectively. Blood was then pre-incubated with different concentration of drugs including 0.1 μM ponatinib, 5 μM imatinib, 5 μM nilotinib, or 0.1 μM dasatinib or vehicle control for 10 minutes at 37°C.

For ex vivo thrombus formation studies, mice were treated with ponatinib (3, 10 and 30 mg/kg), imatinib (25 mg/kg), nilotinib (25 mg/kg) or dasatinib (5 mg/kg) or vehicle for 4 hours before blood collection that is a Cmax peak drug concentration [206]. Whole blood from both human and mice in vitro or ex vivo thrombus formation studies were fluorescently labelled with 0.05
% (w/v) rhodamine 6G dye at 37°C for 30 minutes to allow visualisation of platelets. Labelled platelets in whole blood were then perfused over a matrix of type I collagen-coated μ-slide VI 0.1 microcapillaries for 6 minutes at shear stress rate of 1800 seconds⁻¹ using a infuse/withdraw PHD 22/2200 syringe pump (Model 999; Harvard Apparatus, Holliston, MA). The flow system was rinsed with PBS pH 7.4 subsequently each perfusion at the same shear rate for 6 minutes. The intact thrombus formation derived from initial platelet adhesion with type I fibrillar collagen and accumulation of platelet aggregation on coated surface was directly visualised in real time using a Zeiss Axiovert 135 M1 microscope (Carl Zeiss, Gottingen, Germany) (objective LD 20×/0.4 NA) equipped with Halogen 100-W bright light and HBO 100-W fluorescent light lamp source. Image series of the time-lapse recording and examination of the extent of platelet thrombi production on the surface were carried out off-line from digital photos of the microchannel surface using Axiovision Rel version 4.6 software (Carl Zeiss Imaging Solution GmbH, Munich, Germany). The fluorescent profile of platelets attached in each image was determined and expressed as an arbitrary ‘pixel unit’ which is the total surface area of the thrombi on the surface in a 3D deconvolved thrombus area. The determination of fluorescent platelet thrombus generation on a recognised surface coverage area is basically utilised for comparing thrombus formation between different TKI phenotypes on human and mouse samples. Z-stack slices of fluorescent images of aggregated platelets were captured and recorded by an AxioCam MRm camera (Carl Zeiss, Gottingen, Germany). Deconvolution process was carried out from the three dimensional (3D) reconstructed rendered Z-stack images using the AxioVision Rel4.6 software (Carl Zeiss). Following deconvolution, the threshold was adjusted to be lower intensity for distinguishing platelets from the background and the same threshold characteristic was applied for all Z-stack images to keep analysis consistent. The thrombus volume (μm³) was determined from the surface coverage area (μm²) multiplied by the height (μm) of the platelet thrombi.
2.3.9 In vivo thrombosis model experiments

The ferric chloride (FeCl₃) induced vascular injury is well-defined and extensively performed to examine arterial thrombosis and thrombogenesis in experimental mouse models in vivo [207]. The FeCl₃ mechanically induces arterial thrombosis through oxidative injury to vasculature without complete damage to the inner layers of vessel wall, leading to endothelial cell denudation and exposure of the basement of membrane elements including collagen and tissue factor, resulting in platelet and leukocyte adhesion as well as development of occlusive thrombus formation. Characteristically, FeCl₃ contains pro-oxidative properties (redox-active iron) which trigger endothelial denudation. The moving of ferric ion (Fe³⁺) to the lumen initiates lipid peroxidation of erythrocyte membrane leading to direct red blood cell (RBC) haemolysis [208]. The haemolytic cells secrete haemoglobin that is oxidised by iron generating reactive oxygen species. RBC haemolysis and oxidative haemoglobin generation are required for endothelial cell denudation exposing their molecules e.g. type I collagen [209].

2.3.10 Ferric chloride-induced vascular injury model of mesenteric arterioles

Male and female C57BL/6 mice (4-6 weeks old) were orally administrated with the TKIs, 3, 10 and 30 mg/kg ponatinib, 25 mg/kg imatinib, 25 mg/kg nilotinib, 5 mg/kg dasatinib or vehicle control PBS pH 7.4 (vehicle control mice) or 25 mM citrate buffer pH 2.75 using an oral gavage procedure. Treated mice were left for 4 hours (peak drug concentration) before surgical procedures were performed. Mice were anaesthetised with a ketamine/xylazine (100:10 mg/kg) mixture intraperitoneally (IP). Mice were tested to ensure for receiving sufficient anaesthesia by using the pedal reflex test by pinching the front/hind paw of the mice with a pair of forceps to confirm the mouse is suitably anaesthetised. Once the mice were sufficiently anaesthetised, FeCl₃ induced injury and intravital microscopy (IVM) were performed. A midline incision was made in the abdomen exposing the intestines. Mesenteric arterioles of the ideal diameter (80-100 μm) were then selected and visualised with a Zeiss Axiovert 135 microscope (Carl Zeiss,
Gottingen, Germany) and Z-stack slices of the arterioles were captured with AxioCam MRm camera (Carl Zeiss, Gottingen, Germany) and projected onto the computer screen. The selected vessels were visualised under a long distance lens 20 ×/0.4 NA. A strip of Whatman filter paper (4 mm x 1 mm) was soaked in freshly prepared (7.5% w/v) FeCl₃ solution for around 3 seconds, applied to a 2-to-5-mm length of mesenteric arterioles for 4 minutes and then removed. A volume of 75 μL of 0.05 % w/v rhodamine 6G dye was injected through the jugular vein catheter for platelet labelling. The monitoring of blood flow and thrombus development within vessels was recorded in real time over 10 minutes. Z-stack images of the artery were progressively captured over 10 minutes in 2 minute cycles (defined as the artery vessel starts to occlude).

The rendered Z-stack images were deconvolved with Zeiss Axiovert Rel 4.6 software to generate 3D reconstructions (Carl Zeiss Imaging Solution GmbH, Munich, Germany). Analysis of thrombus growth parameters including thrombus area (μm²), thrombus height (μm) and thrombus volume (μm³) were carried out, excluding any thrombi less than 50 μm². The threshold was adjusted to be lower consistently for distinguishing platelets from the background and the same threshold typical was applied for all Z-stack images to analyse in similar conditions. The calculations of thrombus volume and the percentage of vessel occlusion and stability scores were performed using Zeiss Axiovert Rel 4.6 software. Thrombus area was determined by measuring the selected surface average of the thrombus by scaling outline in μm². Thrombus volume (μm³) was calculated as thrombus area (μm²) multiplied by thrombus height (μm) of the platelet thrombi. The vessel volume was calculated based on this formula (The vessel volume= π × (vessel diameter ÷ 2)² × vessel length).

2.3.11 Ferric chloride-induced vascular injury model of carotid arteries

C57BL/6 mice (sex and age-matched 8 weeks; weight 20±2 g) were orally administrated with TKIs, 3 mg/kg ponatinib, 25 mg/kg imatinib, 25 mg/kg nilotinib, 5 mg/kg dasatinib or PBS pH
7.4 (vehicle control) using an oral gavage. Treated mice were left for 4 hours (peak drug concentration) prior to the surgical procedure. The animals were anaesthetised by IP injection of ketamine and xylazine (100:10 mg/kg). The right common carotid artery was completely exposed utilising surgical scalpel and blunt dissection. A Doppler flow probe was placed proximal to surface of the carotid artery allowing to Doppler monitoring and baseline blood flow was recorded on a laser Doppler perfusion monitor using Moor Instruments Ltd (Millwey, Axminster, Devon, UK). Following surgical incision and the blood flow baseline adjusted, a piece of filter paper (0.5 × 1.0 mm) was saturated in 20 % (w/v) FeCl₃ for 3 seconds and placed on the adventitial surface of carotid artery for 4 minutes (to initiate sufficient injury mediated thrombus formation). The filter paper was carefully removed after 4 minutes and blood flow of carotid artery was recorded. The blood flow reading persisted till flow recording dropped to 50 AU (corresponding to 95 % vessel occlusion). The initial time of artery injury to 95% vessel thrombotic occlusion was calculated [210].

2.3.12 Enzyme linked immunosorbent assay

Levels of soluble P-selectin (sP-selectin) were measured by solid phase sandwich enzyme linked immunosorbent (ELISA) assay according to manufacturer’s instructions. The purpose of ELISA tests of multiple cytokines (IL-6, sP-selectin, TNF-α and IFN-γ) to examine the effect of TKIs on the inflammatory biomarkers of platelets and endothelium for ex vivo studies. Plasma was isolated from whole blood of TKI treated human patient or mice treated with PBS 7.4, either 3 mg/kg ponatinib, 25 mg/kg imatinib, 25 mg/kg nilotinib or 5 mg/kg dasatinib. The samples were separated by double-centrifugation at 1000 ×g for 10 minutes to obtain platelet poor plasma. Plasma was collected from upper half volume of sample and aliquoted in portions of 20 microliters and the PPP samples are quickly frozen at -80°C. The frozen plasma samples were thawed at room temperature prior to processing.
2.3.13 Soluble P-selectin measurement

Mouse sP-selectin/CD40 Quantikine ELISA kit and human sP-Selectin ELISA kit were purchased from (R&D Systems, Minneapolis, MN, USA) and performed according to the manufacturer’s instructions. Briefly, for measuring sP-selectin in mouse plasma, wells of 96 well polystyrene microplates have been pre-coated with monoclonal anti-mouse P-selectin antibodies. Plasma samples were diluted in 1:50 in ‘Calibrator Diluent’ and 100 μL of diluent was added to wells in duplicates. A standard curve ranging from 0 to 10 ng/mL (mouse sP-selectin standard) was generated and a lyophilised control was used. The plate was incubated at room temperature for 2 hours. After five washes with 400 μL ‘Wash Buffer’ containing PBS, 100 μL of a polyclonal antibody against mouse P-selectin (conjugated) was then added and incubated at room temperature for 2 hours. The plate was then washed five times and colour was developed by adding 100 μL of substrate solution (tetramethyl-benzidine). After 30 minutes, 100 μL of stop solution (NaOH) was applied for terminating the reaction. For measuring sP-Selectin levels in patient plasma, ELISA plate was pre-incubated with anti-human sP-selectin monoclonal antibody. Plasma diluted in 1:20 in ‘sample diluent’ human sP-selectin standard and a lyophilised control was added. Subsequently, the ‘sP-selectin conjugate’ containing a polyclonal anti-human sP-Selectin antibody cross-linking with human P-selectin was then applied to each well and incubated for 1 hour at room temperature. The plate was washed three times with 400 μL ‘wash buffer’ and incubated with 100 μL of substrate in the dark. After 15 minutes, the reaction was stopped by adding 100 μL of stop solution (NaOH). Optical density (OD) 450 nm was determined with wavelength correction set to 650 nm using A PerkinElmer’s Victor™ X3 Multilabel Plate Reader (Waltham, Boston, USA).

2.3.14 Mouse sTNF-α measurement

This mouse TNF-α ELISA test is designed to enable the quantitative measurement of natural and/or recombinant TNF-α in serum, plasma and cell culture media. It contains one strip-well
plate pre-coated with mouse TNF-α capture antibody along with sufficient key reagents for plate development. Firstly, solutions were prepared as required by following the manufacturer’s instructions. Then, 50 μL of assay diluent 1B were added to each well. Then, we added 50μL of standards, samples and zero standard controls to each well and seal the plate with the adhesive cover provided and incubate for 2 hours at room temperature. Plates were then washed four times with wash buffer (250 μL). Then, 100μL of biotin labelled detection antibody was added to each well and incubated for 2 hours at room temperature. The aspiration/wash steps were repeated as previous. Then, 100μL of freshly diluted streptavidin-HRP conjugate was added to each well and seal the plate with the adhesive cover provided and incubated for 45 minutes at room temperature. The washing step were repeated as previous. Finally, 100μL of TMB substrate were added to each well and incubated for approximately 20 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped with 50 μL of stop solution. The wells colour changed from blue to yellow in colour. The optical density (OD) of the plate were determined using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.15 Mouse sIL-6 measurement

This mouse sIL-6 ELISA test is designed to enable the quantitative measurement of natural and/or recombinant IL-6 in serum and cell culture media. It contains one strip-well plate pre-coated with mouse IL-6 capture antibody along with sufficient key reagents for plate development. Solutions were prepared according to manufacturer’s instructions. 100 μL of standards (2000 to 31.25 pg/ml), samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed with wash buffer (250 μL). 100 μL of biotin labelled detection antibody to each well and incubated for 2 hours at room temperature. The washing step was repeated followed by adding 100 μL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes
at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally, we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.16 Mouse IFN-γ measurement

This mouse IFN-γ ELISA test is designed to enable the quantitative measurement of natural and/or recombinant IFN-γ in serum and cell culture media. It contains one strip-well plate pre-coated with mouse IFN-γ capture antibody along with sufficient key reagents for plate development. Solutions were prepared according to manufacturer’s instructions. 100 µL of standards (2000 to 31.25 pg/ml), samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed with wash buffer (250 µL). 100 µL of biotin labelled detection antibody to each well and incubated for one hour at room temperature. Washing step was repeated followed by adding 100 µL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally, we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.
2.3.17 Human sTNF-α measurement

Human TNF-α ELISA test is designed to enable the quantitative measurement of natural and/or recombinant TNF-α in serum, plasma and cell culture media. It contains one strip-well plate pre-coated with human TNF-α capture antibody along with sufficient key reagents for plate development. Firstly, solutions were prepared as required by following the manufacturer’s instructions. Then, 50 μL of assay diluent 1B were added to each well. Then, we added 50 μL of standards, samples and zero standard controls to each well and seal the plate with the adhesive cover provided and incubate for 2 hours at room temperature. Plates were then washed four times with wash buffer (250 μL). Then, 100μL of biotin labelled detection antibody was added to each well and incubated for 2 hours at room temperature. The aspiration/wash steps were repeated as previous. Then, 100 μL of freshly diluted streptavidin-HRP conjugate was added to each well and seal the plate with the adhesive cover provided and incubated for 45 minutes at room temperature. The washing step were repeated as previous. Finally, 100 μL of TMB substrate were added to each well and incubated for approximately 20 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped with 50 μL of stop solution. The wells colour changed from blue to yellow in colour. The optical density (OD) of the plate were determined using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.18 Human sIL-6 measurement

This human sIL-6 ELISA test is designed to enable the quantitative measurement of natural and/or recombinant IL-6 in serum and cell culture media. It contains one strip-well plate pre-coated with human IL-6 capture antibody along with sufficient key reagents for plate development. Solutions were prepared according to manufacturer’s instructions. 100 μL of standards (2000 to 31.25 pg/mL), samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed
with wash buffer (250 µL). 100 µL of biotin labelled detection antibody to each well and incubated for 2 hours at room temperature. The washing step was repeated followed by adding 100 µL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally, we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.19 Human IFN-γ measurement

This human IFN-γ ELISA test is designed to enable the quantitative measurement of natural and/or recombinant IFN-γ in serum and cell culture media. It contains one strip-well plate pre-coated with human IFN-γ capture antibody along with sufficient key reagents for plate development. Solutions were prepared according to manufacturer’s instructions. 100 µL of standards (2000 to 31.25 pg/mL), samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed with wash buffer (250 µL). 100µL of biotin labelled detection antibody to each well and incubated for one hour at room temperature. Washing step was repeated followed by adding 100 µL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally,
we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.20 Human sICAM-1 measurement

Human sICAM-1 ELISA test is designed to enable the quantitative measurement of natural and/or recombinant sICAM-1 in serum and cell culture media. It contains one strip-well plate pre-coated with human sICAM-1 capture antibody along with sufficient key reagents for plate development. Solutions were prepared according to manufacturer’s instructions. 100 µL of standards, samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed with wash buffer (250 µL). 100µL of biotin labelled detection antibody to each well and incubated for one hour at room temperature. Washing step was repeated followed by adding 100 µL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally, we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.21 Human sVCAM-1 measurement

This human ELISA test is designed to enable the quantitative measurement of natural and/or recombinant sVCAM-1 in serum and cell culture media. It contains one strip-well plate pre-coated with human sVCAM-1 capture antibody along with sufficient key reagents for plate
development. Solutions were prepared according to manufacturer’s instructions. 100 µL of standards, samples and zero standard controls were added to the plate and incubated for 2 hours at room temperature. Then, the plate wells were aspirated and washed with wash buffer (250 µL). 100µL of biotin labelled detection antibody to each well and incubated for one hour at room temperature. Washing step was repeated followed by adding 100 µL of freshly diluted streptavidin-HRP conjugate to each well and incubated for 45 minutes at room temperature. Again, aspirating each well and washing of the plate five times with wash buffer (250 µL). 100 µL of TMB substrate was added to each well and incubated for approximately 15 minutes at room temperature. The development process (blue colour) was monitored every 5 minutes to prevent over development of the plate. The reaction was stopped by adding 50 µL of stop solution resulting in change the colour from blue to yellow. Finally, we determined the optical density (OD) of the plate using a micro-plate reader set at 450 nm and wavelength correction, set at 570 nm.

2.3.22 Statistical analysis

Statistical analysis was conducted using GraphPad Prism software program version 7.02 (GraphPad, San Diego, CA). All results are expressed as a mean ± standard error of mean (SEM). The statistical significance of differences was determined using unpaired students t-test for two groups for multiple comparisons. A statistically significant difference was indicated by P values less than 0.05.
Chapter 3: THE IN VITRO EFFECTS OF DIFFERENT TYROSINE KINASE INHIBITORS IN REGULATING HUMAN AND MOUSE PLATELET FUNCTION
3.1 INTRODUCTION

The results of the Ponatinib Ph\textsuperscript{+} ALL and CML Evaluation (PACE) trial showed 22% arterial thrombotic events, 12 cardiovascular events, 8% cerebrovascular events and 8% peripheral vascular events with ponatinib TKI therapy [211]. The complications of ponatinib treatment have led to clinical sequence of heart attacks, hypertension, PAOD, and significant stenosis [212]. These finding suggest, TKIs exert several biological effects on the functioning of vascular endothelium and platelets [213]. Various clinical studies have shown that ponatinib has a potent significant impact on the vascular endothelium and platelets by development of arterial thrombotic events [65]. Similarly, nilotinib TKI can selectively potentiate in vitro thrombus formation on type I collagen for both human and mouse [214]. Conversely, imatinib and dasatinib inhibited platelet aggregation and reduced in vitro thrombus formation on type I collagen. However, other previous studies showed that essentially, dasatinib inhibited in vitro creation of thrombus on type I collagen under arterial shear conditions using either murine and human blood [215]. The same sources also revealed that CML patients who were subjected to the imatinib treatment demonstrated a reduced thrombus formation under arterial flow conditions.

The process of unregulated platelet activation and aggregation has been found to contribute to the development of arterial thrombotic occlusion that could result in cerebral ischaemic stroke or myocardial infarction. Among patients with leukemia, thrombosis is linked to cancerous lesions and is the second highest cause of mortality [216]. This makes platelets a crucial component of blood to target during treatment [217]. Once a vascular injury has occurred, platelets, by binding vWF to GPIb-IX-V and collagen to GPVI, stick to exposed thrombogenic molecules of the subendothelial matrix. This consequently results in platelet adhesion, activation, and aggregation. A number of soluble agonists such as ADP and thrombin are secreted during platelet activation. These agonists can induce pathways for signaling, as well
as further activation of platelets [218]. After their activation, platelets undergo a series of biological alterations that prompt the initiation of intracellular signaling events that activate inside-out signaling pathways through the conversion of integrin αIIbβ3 from its low-affinity inactive condition to an active state. This conversion to a high-affinity state then enables the integrin αIIbβ3 to bind with fibrinogen that consequently triggers “outside-in” signaling pathways, hence resulting in an irreversible, stable platelet adhesion, cytoskeletal reorganisation, aggregation of platelets and thrombus development [219].

Type I fibrillar collagen is the principal bonding moderator in the vascular subendothelium, and acts as a soluble agonist that can promote platelet adhesion, signaling events and aggregation. Thus, in most cases, in vitro and ex vivo research has employed the use of vWF-coated or type I collagen-covered flow microcapillary chambers in studying the shear-induced formation of platelet thrombus with or without drugs in identifying characteristics of thrombus growth and stability, in real-time development of arterial thrombi [220]. Evidence from a particular study demonstrated that dasatinib therapy inhibited the activation and aggregation of platelets as well as impairing thrombus growth [221]. Other emerging clinical trials suggest that nilotinib and ponatinib potentiate arterial events [222]. However, it is notable that no single study has been able to demonstrate how TKIs affect platelet activation, secretion and aggregation of platelets.

The objective of this chapter is to determine the ability of TKIs, in particular, ponatinib compared with other TKIs to regulate the formation of thrombus using either human or C57BL/6 mouse blood under in vitro physiological flow conditions and the effect of TKIs on platelet activation and aggregation.
3.2 RESULTS

3.2.1 Effect of TKIs on platelet alpha granule exocytosis

In order to investigate the effect of TKI’s on alpha granule release, P-selectin expression was monitored by flow cytometry to determine whether ponatinib, imatinib, nilotinib or dasatinib can modulate agonist induced platelet alpha granule release.

Flow cytometry detection using an FITC-labelled monoclonal antibody (mAb) against P-selectin is one of most frequently used examinations for assessing the platelet alpha granule release following agonist stimulation as a marker of platelet activation.

The ability of washed human platelets to release alpha (P-selectin) granules in response to soluble agonists with and without TKIs was determined by flow cytometric detection of CD62P-FITC mean fluorescence intensity. Interestingly, P-selectin exposure induced by 1.25 µM TRAP peptide was significantly potentiated by ponatinib and nilotinib when compared to the vehicle control at 5 and 10 µM concentrations. Treatment with 0.25 U/ml thrombin or 1.25 µM TRAP peptide and 1.25 µg/mL CRP significantly inhibited alpha granule secretion induced by agonists following treatment with dasatinib (Figure 22-24). In addition, inhibition of alpha granule release induced by 0.25 U/ml thrombin or 1.25 µM TRAP peptide was also observed with imatinib-treated platelets (Figures 22-24). Therefore, different TKIs exhibit opposing actions in modulating platelet alpha granule secretion.

In these experiments, imatinib inhibited thrombin and TRAP peptide-mediated alpha granule release (P-selectin exposure) whilst ponatinib and nilotinib enhanced TRAP mediated alpha granule release at 5 and 10 µM concentrations (Figure 22 and 23). Our data also are consistent with the view that dasatinib had a potent inhibitory effect on agonist-mediated alpha granule exocytosis.
Figure 22. The effect of TKIs on thrombin-mediated platelet alpha granule exposure.
Washed platelets incubated with dose responses of imatinib, nilotinib or dasatinib for 10 minutes at 37°C following agonist activation of thrombin (0.25 U/mL) or unstimulated (resting). The platelets were then labelled with FITC-P-selectin monoclonal antibody. Flow cytometric analysis was used for determining platelet P-selectin expression. Results are represented as MFI±SEM from four independent experiments (**P<0.01 and ***P<0.001; n=4) using unpaired student’s t test.
Figure 23. The effect of TKIs on TRAP peptide-mediated platelet alpha granule exocytosis. P-selectin surface expression for washed human platelets was measured by flow cytometry. Platelets were either untreated or treated with various concentrations of ponatinib, nilotinib, imatinib or dasatinib and then stimulated by 1.25 µM TRAP peptide for 15 minutes at 37°C. Platelets were then stained with FITC-P-selectin CD62P monoclonal antibody. Data are expressed as MFI±SEM from four independent experiments (*P<0.05; **P<0.01 and ***P<0.001; n=4) using unpaired student’s t test.
Figure 24. The effect of TKIs on CRP-mediated alpha granule exocytosis was assessed by flow cytometry. Washed human platelets were either untreated or treated with a dose-dependent concentration of ponatinib, nilotinib, imatinib or dasatinib (0.05-10 μM) for 10 minutes at 37°C and then stimulated with selective agonist CRP (1.25 μg/mL) for 15 minutes at 37°C. Platelets were stained with FITC-P-selectin CD62P monoclonal antibody for 30 minutes at room temperature. Platelet P-selectin CD62P expression was determined by flow cytometry. Results are cumulative data from four independent experiments and presented as mean±SEM (*P<0.05; **P<0.01 and ***P<0.001; n=4) using unpaired student’s t test.
3.2.2 Impact of TKIs on platelet dense granule exocytosis

As TKI-treated platelets showed different agonist-mediated alpha granule release responses, the effect of ponatinib, imatinib, nilotinib or dasatinib over a dose-dependent range 0.05 to 10 µM on agonist-induced dense granule exocytosis (quinacrine) in human platelets was examined. Quinacrine staining was performed that labels only dense granules and then flow cytometry analysis was used to evaluate agonist-induced dense granule release with and without TKI treatment.

Analysis of dense granule secretion revealed that ponatinib, imatinib, nilotinib or dasatinib had no effect in the absence of platelet agonist when compared with the positive control (thrombin 0.25 U/mL). Upon agonist stimulation, analysis of dense granule secretion revealed the dose dependency of ponatinib, imatinib and nilotinib had no detectable effect on dense granule exocytosis over a range of platelet agonists including thrombin (0.25 U/mL). In contrast, the secretion of platelet dense granules was significantly inhibited (*P<0.05; n=4) at different concentrations of dasatinib in response to thrombin (0.25 U/mL), TRAP peptide (1.25 µM) and CRP (1.25 µg/mL) when compared to untreated platelets (Figure 25, 26 and 27). These data indicate that ponatinib, imatinib and nilotinib treatment results in normal dense granule release in human platelets in the presence of soluble agonists, while dasatinib has an inhibitory effect on agonist-induced dense granule release (***P<0.001; n=4).
Figure 25. The effect of TKIs on thrombin-mediated dense granule release as determined using flow cytometry. Washed human platelets (platelet count normalised to 100×10^9/L) were labelled with 100 μM quinacrine at 37°C. Labelled platelets were either untreated or pre-incubated with increasing concentrations of ponatinib, nilotinib, dasatinib or imatinib (0.05-10 μM) for 10 minutes at 37°C and then stimulated by of 0.25 U/mL thrombin for 15 minutes 37°C. The results of dose response-of various drugs were compared with baseline. Results are representative of four independent experiments and are shown as the mean±SEM (** P<0.01 and *** P<0.001; n=4) using unpaired student’s t test.
Figure 26. The effect of TKIs on TRAP peptide-mediated dense granule release was assessed using flow cytometry. Quinacrine-labelled platelets (platelet count normalised to 100×10^9/L) were either untreated or treated with ponatinib, nilotinib, dasatinib or imatinib in a dose-dependent manner (0.05-10 µM) for 10 minutes at 37°C and stimulated with 1.25 µM TRAP peptide for 15 minutes 37°C. Data were expressed as the mean±SEM from four independent experiments (*P<0.05 and ** P<0.01; n=4) using unpaired student’s t test.
Figure 27. The effect of TKIs on CRP-agonist peptide-mediated dense granule exocytosis was determined by flow cytometry. Washed platelets from normal human donors (platelet count normalised to 100×10⁹/L) were stained with quinacrine (100 µM) for 30 minutes at 37°C. Quinacrine-labelled platelets were either untreated or treated with ponatinib, nilotinib, dasatinib or imatinib (0.05-10 µM) in a dose-dependent range and activated with 1.25 CRP µg/mL (B). Results are expressed as the mean±SEM from four independent experiments (*P<0.05 and **P<0.01; n=4) using unpaired student’s t test.
As shown in Figure 28, thrombin induced activation of platelets derived from imatinib-treated, ponatinib-treated, nilotinib-treated or untreated human platelets displayed a similar ability to bind with the fibrinogen mimetic PAC-1 mAb that recognises the active conformation of integrin αIIbβ3 on platelets. These results indicated that platelets from ponatinib-treated human platelets had normal conversion from a resting state of integrin αIIbβ3 to an activated conformation. In contrast, dasatinib-treated platelets showed reduced conversion from a resting to activated conformation of integrin αIIbβ3 (**P<0.01; n=4).
Figure 28. The effect of TKIs on PAC-1 mAb binding. Washed platelets (100×10^9/L) incubated with dose responses of ponatinib, imatinib, nilotinib or dasatinib for 10 minutes at 37°C following agonist activation of thrombin (0.25 U/mL) or unstimulated (resting). The platelets were then labelled with PAC-1-FITC monoclonal antibody. Results are represented as MFI±SEM from four independent experiments (**P<0.01; n=4) using unpaired student’s t test.
3.2.3 Impact of TKIs on platelet aggregation

A platelet aggregation study was performed to investigate the effect of ponatinib on human platelet function. Concentrations were determined for different aggregating agents in order to induce irreversible and sub-optimal platelet aggregation. ADP is considered a weak agonist due to reversible nature of the platelet aggregation. As shown in Figure 29, a dose-dependent increase in 5 µM ADP-induced platelet-aggregation was observed from 1-10 µM ponatinib concentrations. Ponatinib TKI promoted ADP-mediated platelet aggregation in 1 µM (37±2.6%, **P<0.01; n=4), 2.5 µM (38±3.84%, **P<0.01; n=4), 5 µM (36±0.88%, **P<0.01) and 10 µM (39±0.57%, **P<0.01; n=4) in a dose-dependent manner (0.05-10 µM), indicated that higher concentrations of ponatinib induced more pronounced aggregation potentiating effect. In contrast, both imatinib and dasatinib showed efficiently inhibited platelet aggregation at different concentrations (Figure 29).

Danatinib demonstrated a more potent inhibitor shown by a significant inhibitory effect at all concentrations tested from 0.05 to 10 µM for all agonists including ADP, collagen and CRP (Figure 29-31). As showed in the Figure 30, ponatinib showed enhancement of collagen induced platelet aggregation at 5 and 10 µM drug concentrations.

GPVI selective ligand, collagen-related peptide (CRP), on the other hand, is a stronger agonist that only required 0.5 µg/ml to induce platelet aggregation (Figure 31). From the experimental results, 5 and 10 µM ponatinib demonstrated a modest potentiation in platelet aggregation mediated by CRP 0.5 µg/ml (26±0.8%, *P<0.05; n=4) but did not achieve a statistically significant difference at lower concentrations of ponatinib.
Figure 29. The effect of TKIs on ADP-induced platelet aggregation. PRP from normal human blood was normalised to 100×10⁹/L (platelet count). Ponatinib-treated PRP, nilotinib-treated PRP, imatinib-treated PRP and dasatinib-treated PRP (dose-dependent drug response 0.05-10 µM) for 10 minutes at 37°C. Aggregation responses of PRP were determined following agonist stimulation by ADP (5 µM). Note that ADP stimulated drug-treated PRP are compared relative to untreated control PRP. These results represent the mean±SEM from four independent experiments (** P<0.01 and *** P<0.001; n=4) using unpaired student’s t test.
Figure 30. The effect of TKIs on collagen-induced platelet aggregation. PRP from normal human blood was normalised to 100×10^9/L (platelet count). Ponatinib-treated PRP, nilotinib-treated PRP, imatinib-treated PRP and dasatinib-treated PRP (dose-dependent drug response 0.05-10 µM) for 10 minutes at 37°C. Aggregation responses of PRP were determined following agonist stimulation by collagen (1 µg/mL). Note that collagen induced drug-treated PRP are compared relative to untreated control PRP. These results represent the mean±SEM from four independent experiments (* P<0.05; ** P<0.01 and *** P<0.001; n=4) using unpaired student’s t test.
Figure 31. The effect of TKIs on CRP-induced platelet aggregation. PRP from normal human blood was normalised to 100×10⁹/L (platelet count). Ponatinib-treated PRP, nilotinib-treated PRP, imatinib-treated PRP and dasatinib-treated PRP (dose-dependent drug response 0.05-10 µM) for 10 minutes at 37°C. Aggregation responses of PRP were determined following agonist stimulation by CRP (0.5 μg/ml). Note that CRP induced drug-treated PRP are compared relative to untreated control PRP. These results represent the mean±SEM from four independent experiments (* P<0.05 and *** P<0.001; n=4) using unpaired student’s t test.
Compared to other TKIs that were tested, dasatinib acted as a potent inhibitor and achieved a significant reduction in ADP, collagen and CRP mediated platelet aggregation at different drug concentrations (Figure 29-31). This is consistent with the previous studies that dasatinib acts as a potent inhibitor [223].

3.2.4 Assessment of platelet thrombus growth in ponatinib-treated normal human whole blood over type I collagen under in vitro arterial flow conditions.

We hypothesised that the Bcr-Abl tyrosine kinase inhibitor, ponatinib modulates platelet activation, adhesion and aggregation for fibrillar collagen surface under arterial flow. As platelet adhesion and activation on collagen is driven by GPVI and α2β1 that activates integrin αIIbβ3, it was important to examine the effect of ponatinib TKI on this process. Assays were conducted to examine the dose-dependent effect of TKIs on in vitro platelet thrombus formation in blood samples from healthy humans and C57BL/6 mice model. Citrated whole blood was treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM) for 30 minutes at 37°C. Whole blood was fluorescently labelled with rhodamine 6G [untreated whole blood or whole blood treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM)] was perfused through a collagen-coated microcapillary tube flow chamber at an arterial shear rate of 1800s⁻¹. After six minutes of sample perfusion, thrombus images for ponatinib, imatinib, nilotinib and dasatinib-treated whole blood were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated whole blood samples.
As shown in Figure 32 and 33, ponatinib-treated human whole blood displayed increased thrombus height, thrombus surface coverage (area) and thrombus volume when compared to untreated whole blood at all ponatinib concentrations of 0.1 and 1 μM.
<table>
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**Figure 32. Ponatinib effect on *in vitro* human thrombus formation and growth on Type I collagen under arterial shear flow.** Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), PON (1 µM) or vehicle control was perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800 s⁻¹ in real time over a 6 minute time period.
Figure 33. Kinetics of ponatinib effect on *in vitro* human thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height. (B) Thrombus area. (C) Thrombus volume. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), PON (1 µM) and vehicle control were perfused across 500 µg/ml type I collagen at arterial flow rate 1800s⁻¹ in real time over a 6 minute time period. Results are presented as the mean ± SEM. (*P < 0.05 and ** P < 0.01; n=4) using unpaired student’s t-test.
3.2.5 Assessment of platelet thrombus growth in TKI-treated normal human whole blood over type I collagen under *in vitro* arterial flow conditions

To determine the effect of Bcr-Abl inhibition on human platelet adhesion and aggregation on type I fibrillar collagen under arterial flow, a comparison of ponatinib was done with other TKIs. As shown in Figure 34 and 35, human whole blood treated with nilotinib showed increased thrombus formation for *in vitro* thrombus growth over type I collagen and the surface area covered by platelet thrombi and the thrombus volume were similar to ponatinib compared to control. In contrast, smaller thrombus formation was observed with dasatinib and imatinib compared to untreated control as shown in the thrombus height, area and volume. These results demonstrate different characteristics of TKIs including ponatinib versus control. Assays were conducted to examine the dose-dependent effect of TKIs on *in vitro* platelet thrombus formation in blood samples from healthy humans and C57BL/6 mice model. Citrated whole blood was treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM) for 30 minutes at 37°C. Whole blood containing rhodamine 6G fluorescently labelled platelets [untreated whole human blood or whole human blood treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM)] was perfused through a collagen-coated microcapillary flow chamber at an arterial shear rate of 1800s⁻¹. After six minutes of sample perfusion, thrombus images for ponatinib, imatinib, nilotinib and dasatinib-treated whole blood were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated whole blood samples.
Figure 34. TKI effect on in vitro human thrombus formation and growth on Type I collagen under arterial shear flow. Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), NIL (5 µM), IM (5 µM), DAS (0.1 µM) and vehicle control were perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800 s\(^{-1}\) in real time over a 6 minute time period.
Figure 35. Kinetics of TKI effect on in vitro human thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), NIL (5 µM), IM (5 µM) and DAS (0.1 µM) were perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800s⁻¹ in real time over a 6 minute time period. (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4) using unpaired student’s t-test.
3.2.6 Assessment of platelet thrombus growth in ponatinib-treated murine blood over type I collagen under in vitro arterial flow conditions.

We hypothesised that Bcr-Abl inhibitor ponatinib would modulate murine platelet adhesion and aggregation on type I fibrillar collagen surface under arterial flow conditions. Assays were conducted to examine the dose-dependent effect of TKIs on in vitro platelet thrombus formation in blood samples from C57BL/6 mice model. Citrated whole murine blood was treated with ponatinib (0.1 and 1 μM), nilotinib (5 μM), imatinib (5 μM) or dasatinib (0.1 μM) for 30 minutes at 37°C. Whole murine blood containing rhodamine 6G fluorescently labelled platelets [untreated whole murine blood or whole blood treated with ponatinib (0.1 and 1 μM), nilotinib (5 μM), imatinib (5 μM) or dasatinib (0.1 μM)] was perfused through a collagen-coated microcapillary flow chamber at an arterial shear rate of 1800s⁻¹. After six minutes of sample perfusion, thrombus images for ponatinib, imatinib, nilotinib and dasatinib-treated whole blood were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated whole blood samples.

As shown in Figure 36 and 37, ponatinib-treated murine whole blood displayed increased thrombus growth in terms of thrombus height, surface area and thrombus volume when compared to untreated whole blood at all ponatinib concentrations tested at 0.1 and 1 μM.
Figure 36. Ponatinib effect on *in vitro* murine thrombus formation and growth on Type I collagen under arterial shear flow. Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from C57BL/6 mice in the presence of PON 0.1 μM, PON 1 μM and vehicle control were perfused across 500 μg/ml type I collagen at an arterial flow rate of 1800 s⁻¹ in real time over a 6 minute time period.
Figure 37. Kinetics of ponatinib effect on *in vitro* murine thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from C57BL/6 mice in presence of PON (0.1 µM), PON (1 µM) or vehicle control were perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800 s⁻¹ in real time over 6 minute time period. (*P < 0.05 and **P < 0.01; n=4) using unpaired student’s t-test.
3.2.7 Assessment of platelet thrombus growth in TKI-treated C57BL/6 mouse blood over type I collagen under \textit{in vitro} arterial flow conditions.

To determine the effect of Bcr-Abl inhibitors on murine platelet adhesion and aggregation on fibrillar collagen under arterial flow conditions, a comparison of ponatinib with other TKIs was performed. Assays were conducted to examine the dose-dependent effect of TKIs on \textit{in vitro} platelet thrombus formation in blood samples using C57BL/6 mice model. Citrated whole murine blood was treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM) for 30 minutes at 37°C. Whole murine blood containing rhodamine 6G fluorescently labelled platelets [untreated whole blood or whole murine blood treated with ponatinib (0.1 and 1 µM), nilotinib (5 µM), imatinib (5 µM) or dasatinib (0.1 µM)] was perfused through a collagen-coated microcapillary flow chamber at an arterial shear rate of 1800s$^{-1}$. After six minutes of sample perfusion, thrombus images for ponatinib, imatinib, nilotinib and dasatinib-treated whole blood were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated whole blood samples.

As shown in Figures 38 and 39, both ponatinib and nilotinib-treated murine whole blood displayed increased thrombus growth in terms of thrombus height, thrombus surface area and thrombus volume when compared to untreated whole blood with increased development of thrombus growth over type I collagen under flow conditions. In contrast, the thrombus growth was reduced with imatinib and dasatinib revealing inhibition in thrombus formation when compared to thrombi formed by untreated control blood (Figure 38 and 39).
Figure 38. TKI effect on *in vitro* murine thrombus formation and growth on Type I collagen under arterial shear flow. Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from C57BL/6 mice in the presence of PON (0.1 μM), NIL (5 μM), IM (5 μM), DAS (0.1 μM) and vehicle control were perfused across 500 μg/ml type I collagen at arterial flow rate 1800s⁻¹ in real time over a 6 minute time period.
Figure 39. Kinetics of TKI effect on in vitro murine thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from mouse C57BL/6 in the presence of PON (0.1 µM), PON (1 µM), NIL (5 µM), IM (5 µM) and DAS (0.1 µM) were perfused across 500 µg/ml type I collagen at arterial flow rate 1800s⁻¹ in real time over 6 minute time period. (*P < 0.05 and ** P < 0.01; n=4) using unpaired student’s t-test.
3.2.8 Assessment of platelet thrombus growth in TKI-treated normal human whole blood of blinded studies over type I collagen under in vitro arterial flow conditions

In order to remove any operator bias, blinded studies of two unknown TKIs compounds was performed for in vitro human thrombus formation on type I collagen. Assays were conducted to examine the dose-dependent effect of TKIs on in vitro platelet thrombus formation in blood samples from healthy humans and C57BL/6 mice model. Citrated whole blood was treated with compound A (0.1 and 1 µg/ml) and compound B (0.1 and 1 µg/ml) for 30 minutes at 37°C. Whole blood containing rhodamine 6G fluorescently labelled platelets (untreated whole blood or whole blood treated with compound A and B) was perfused through a collagen-coated microcapillary flow chamber at an arterial shear rate of 1800s⁻¹. After six minutes of sample perfusion, thrombus images for compound A and B-treated whole blood were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated whole blood samples.

Two unknown compounds A and B were supplied from ARIAD/Takeda pharmaceutical company, USA. As shown in Figure 40 and 41, compound A with different concentrations (0.1 and 1 µg/ml) showed increased thrombus formation compared with the vehicle control. In contrast, the compound B (0.1 and 1 µg/ml) displayed reduced thrombus formation as shown in the decreased thrombus height, area and volume compared to vehicle human control sample. Note compound A and B samples that were received for testing for ARIAD and they confirmed identity of TKIs by LC-ms studies. They decoded compound A as ponatinib and compound B as imatinib.
Figure 40. Blinded studies of TKI effect on \textit{in vitro} thrombus formation and growth on Type I collagen under arterial shear flow. Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from human in the presence of compound A (0.1 $\mu$g/ml), compound A (1 $\mu$g/ml), compound B (0.1 $\mu$g/ml), compound B (1 $\mu$g/ml) and vehicle control were perfused across 500 $\mu$g/ml type I collagen at arterial flow rate of 1800s$^{-1}$ in real time over a 6 minute time period.
Figure 41. Blinded studies of TKI effect on *in vitro* human thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from human in the presence of compound A (0.1 µg/ml), compound A (1 µg/ml), compound B (0.1 µg/ml), compound B (1 µg/ml) and vehicle were perfused across 500 µg/ml type I collagen at arterial flow rate 1800 s⁻¹ in real time over a 6 minute time period. Results are presented as the mean ± SEM. (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4) using unpaired student’s t-test.
3.3 DISCUSSION

Clinical studies have suggested that ponatinib and nilotinib may have an effect on arterial thrombotic event of the blood vessels and initiation of PAOD events over time. However, neither imatinib nor dasatinib have this effect. This study provides one of the first insights into the effect of ponatinib in platelet activation in vitro, also highlighting the possible mechanism by which the risk of vascular events is increased. We found that an acute single dose of ponatinib treatment contributed to platelet activation.

ADP is known to be a weak agonist to induce platelet aggregation compared to collagen and CRP. At 5 µM ADP, ponatinib potentiated the ADP-mediated platelet aggregation response consistent with modulation of a weak agonist induced platelet aggregation.

Platelet activation occurs in response to vessel injury and is triggered by different receptors, including thrombin, thrombin receptors, PAR-1 and PAR-4 [168]. It has previously found that PAR-1 is the primary thrombin receptor with high affinity on human platelets and couples to distinct sets of G-proteins for the regulation of signalling pathway [224]. Ponatinib and nilotinib appear to potentiate PAR-1 mediated α-granule release in normal healthy donor platelets. Although the mechanism remains unclear, it is possible that it has off targeting effects that selectively act on PAR-1 mediated α-granule pool release of P-selectin and impacts on haemostasis. In these studies, ponatinib was demonstrated to significantly promote platelet aggregation in response to adenosine diphosphate (ADP) in a dose-dependent manner, which was not seen in imatinib, nilotinib or dasatinib. In addition, both ponatinib and nilotinib were shown to potentiate exposure of platelet activation marker, P-selectin (CD62) from alpha granule (α-granule) in vitro upon PAR-1 stimulation, but it had no effect following thrombin and CRP stimulation.
Upon platelet activation, α-granules release their contents and P-selectin is rapidly translocated and expressed on platelet surface that influence the integrity of blood vessel wall [25]. It plays a role in triggering inflammation by mediating the adherence of leukocyte to endothelial cells, procoagulant release and tissue factor production [225]. Ponatinib potentiated P-selectin exposure suggesting platelet activation and is associated with the responses to endothelial injury or stimulation of vascular tissue. Interestingly, α-granule release was not potentiated in PAR-4 mediated platelet activation. Previous studies have indicated that human platelets contain different α-granule pools; PAR-1 mediated α-granule release of vascular endothelial growth factor (VEGF) and fibrinogen containing pro-angiogenic proteins whereas PAR-4 mediated α-granule release of Endostatin and vWFContaining anti-angiogenic proteins [226]. This highlights the differential release of α-granule protein pools from platelets in these two independent groups. It has been well established that thrombin is the most potent physiological stimuli and has a pronounced pro-inflammatory character [227].

However, ponatinib demonstrated no potentiating effect on α-granule release by thrombin-mediated exposure of platelet activation. This could be explained by the fact that thrombin are coupled to multiple receptors, including PAR-1, PAR-4 and platelet glycoprotein (GP)1b-IX-V receptor. It has to be taken into consideration that GP1b binding site for thrombin is abundant on platelet membrane and full platelet activation stimulated by thrombin is required. The stimulation effect is possibly counteracted to different receptors, hence minimising or masking the potentiation [228].

It is widely accepted that ruptured atherosclerotic lesions expose high concentrations of type I fibrillar collagen which initiates localised thrombosis through recruitment and adherence of platelets to exposed ECM constituents within the vascular wall followed by activation of coagulation that intensify thrombus formation. To examine the contribution of TKIs, specifically ponatinib in modulating platelet aggregation and arterial thrombus growth several complementary approaches were used. The findings achieved in this Chapter provided some
novel observations into the effect of ponatinib, imatinib, nilotinib and dasatinib on platelet thrombus formation *in vitro* from both normal humans and wild-type C57BL/6 mice.

Unlike nilotinib, dasatinib and imatinib, the effect of ponatinib on platelet thrombus formation under *in vitro* flow conditions had not been extensively examined. In this Chapter, it was of interest to test and compare the ability of ponatinib and other TKIs to modulate platelet thrombus growth under arterial *in vitro* flow conditions by intravital microscopy that allowed capturing of real time images from human and C57BL/6 mouse samples. These experiments demonstrated that ponatinib and nilotinib significantly potentiated thrombus growth under *in vitro* arterial flow conditions over immobilised type I collagen at all doses tested. By contrast, imatinib and dasatinib inhibited platelet adhesion on type I fibrillar collagen and the thrombus area and volume was significantly reduced at 4 and 6 minutes of perfusion when compared with vehicle control of whole blood.

Previous work by Loren *et al.*, appears to contradict the results of my *in vitro* thrombus formation analysis induced by ponatinib [222]. The reason for this difference likely reflects differences in assay systems. These workers used a shear flow rate of 2200s⁻¹ on type I collagen and coated surface was blocked with denatured BSA whereas in this study arterial shear flow rate of 1800s⁻¹ (average shear flow rate in healthy arteries) on type I collagen without surface blocked by BSA [222]. Another factor relates to solubilisation of ponatinib. In this study, 25 mM citrate buffer pH 2.75 achieved maximal solubilisation of ponatinib for the experiments which would provide optimal bioactive ponatinib for stimulation of platelet activation and thrombus formation under arterial flow conditions [222].

Previous study by Gratacap *et al.*, demonstrated that imatinib-treated patients significantly reduced thrombus formation on immobilised type I collagen under physiologic shear rate while dasatinib-treated patients formed smaller and less stable thrombi that was much more pronounced than imatinib. The same research group also reported that blood derived from
dasatinib-treated mice formed much smaller thrombi over type I collagen under arterial flow shear flow rate [47].

In conclusion, clinical data have reported that treatment of CML patients with ponatinib is associated with increased rates of atherothrombotic events [229]. Data in this Chapter revealed insights and differential effects of various TKIs on platelet function profiles derived from healthy human donors and murine samples. The laboratory experiments demonstrated that imatinib and dasatinib treatments have inhibitory effects on platelet aggregation, platelet granule exocytosis and in vitro thrombus formation. While these studies revealed that ponatinib and nilotinib selectively potentiated platelet alpha granule release (P-selectin exposure) in response to PAR-1 in human platelets suggesting that ponatinib and nilotinib TKIs potentiate agonist induced platelet activation.

This Chapter confirms a potential involvement of ponatinib treatment in the potentiation of in vitro thrombus formation for both human and murine on type I collagen under arterial flow conditions. In addition, these data provide strong evidence that ponatinib and nilotinib induced increased arteriolar thrombus development associated with fibrillar collagen as a thrombogenic substance exposed from the endothelium. These results support a direct effect of ponatinib and nilotinib TKI’s in modulating platelet activation, adhesion and aggregation events. In addition, it indicates a potential mechanism for supporting an increased rate of thrombus formation in vascular disease and atherosclerosis. Future strategies that involve pre-treatment or concurrent treatment to reduce prothrombotic effect of nilotinib or ponatinib therapy should be considered.
Chapter 4: INVESTIGATION THE ACUTE EFFECT OF TYROSINE KINASE INHIBITORS TREATMENT ON ARTERIAL THROMBUS FORMATION IN AN EX VIVO & IN VIVO MODEL
4.1 INTRODUCTION

A retrospective analysis of CML patients treated with nilotinib showed 2.1% of nilotinib-associated vascular events of acute cases were vasospastic in nature with no evidence of atherosclerotic lesions [230]. It is also likely that ponatinib TKI has a similar effect on induction of vasospasm. Consistent with this possibility is that ponatinib has been shown to augment vasoconstriction and attenuate vasorelaxation in mouse aortic rings [231]. Furthermore, the rapid onset of arterial events in CML patients on ponatinib suggests an acute vascular toxicity within a few weeks of drug exposure. This suggested that ponatinib could have a direct effect on the blood vessel wall potentiating vasospasm leading to endothelial and platelet activation. To investigate these possibilities, the acute effect of various TKIs was examined ex vivo and in vivo thrombus formation in C57BL/6 mouse model to examine thrombus characteristics.

Platelet activation and aggregation can have a significant involvement in the development of arterial thrombotic occlusion and ultimately leading to myocardial infarction or cerebral ischaemic stroke [232]. Thrombosis related to cancerous lesions is the second major cause of death among leukaemic patients; therefore, platelets are an important blood component to target for treatment [233].

Primarily, the adhesion is facilitated by binding P-selectin from platelets and endothelial cells to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, which eventually endorses the stability of thrombus in vivo after a vascular damage [234]. The arterial thrombi formed at the sites of the ruptured atherosclerotic plaque are rich in platelets and exposed to blood flow forces.

Platelets easily move in vivo in the bloodstream and do not interact with the non-activated endothelium. Nevertheless, in a damaged or diseased endothelial surface, the platelets respond
by tethering, adhering, and aggregating, resulting in the propagation of arterial thrombosis. Similarly, exposed tissue factor induces the stimulation of clotting cascade, leading to thrombin and fibrin accumulation that encourages the activation of platelets and potentiates the development of thrombus [235].

However, using intravital microscopy analysis in real-time in vivo thrombus formation, have confirmed platelet aggregation to be an active process that has a major role in areas of vascular injury.

Although, ponatinib was temporarily withdrawn from the market in 2013 due to arterial vascular occlusive events, it returned again in 2014 by FDA with new safety measures including dose reduction strategies from 45 mg QD to 30 to 15 mg QD [36]. However, a prior study indicated that blood from dasatinib-treated mice inhibited the formation of thrombus in vitro, ex vivo, and in vivo [236]. Moreover, the research also determined that primary haemostasis in vivo was impaired after treatment with dasatinib about four hours after ingestion [237]. A previous study in our laboratory examined the influence of TKI, imatinib, dasatinib or nilotinib but not ponatinib in mediating in vivo thrombus development in real time [238].

In this Chapter, the aim is to test the ability of TKIs, specifically ponatinib, to modulate thrombus formation under ex vivo physiological flow conditions in a C57BL/6 model. In addition, cohorts of TKI-treated mice were subjected to FeCl₃-mediated vascular induced injury of the mesenteric arterioles (80-100 μM) and in vivo microvascular thrombus growth was evaluated. Moreover, cohorts of TKI-treated mice were subjected to FeCl₃-mediated vascular induced injury of the carotid artery as a mechanism for inducing arteriolar thrombosis in vivo. These approaches were taken to determine the effect of Bcr-Abl TKIs on platelet phenotype and ex vivo thrombus formation under arterial flow, and to assess microvascular and arteriolar thrombosis in vivo in a C57BL/6 mouse model.
4.2 RESULTS

4.2.1 Impact of TKIs on thrombus growth under conditions of *ex vivo* arterial flow on immobilised type I collagen

The effect of TKIs on platelet function was further examined by comparing *ex vivo* thrombus formation by pre-treating C57BL/6 mice with various TKI for four hours followed by examination of thrombus growth characteristics under arterial flow conditions of mouse whole blood. Briefly, blood was collected from C57BL/6 mice which were treated with either 3 mg/kg, 10 mg/kg or 30 mg/kg ponatinib, 25 mg/kg imatinib, 25 mg/kg nilotinib, 5 mg/kg dasatinib or 25 mM citrate pH 2.75 (vehicle control) by oral gavage and left for four hours from the initial dose (C<sub>max</sub> peak drug concentration). Whole blood with fluorescently labelled platelets was perfused onto immobilised type I collagen at an arterial shear rate of 1800s<sup>-1</sup> for six minutes.

The blood from ponatinib-, imatinib-, nilotinib- and dasatinib-treated mice were compared to the sham control and the images of thrombi formed were recorded in real time. Analysis of 3D deconvolved images revealed that surface coverage of platelet adhesion, thrombus height and the thrombus volume were significantly enhanced with ponatinib doses versus sham control. As shown in Figure 42 and 43, all doses of ponatinib showed a significant potentiation of thrombus height, area and thrombus volume over time compared vehicle control.
Figure 42. The effect of a dose response of ponatinib on *ex vivo* murine thrombus formation and growth. Fluorescently-labelled whole blood of C57BL/6 mice untreated or treated with 3, 10 or 30 mg/kg ponatinib was perfused over 500 µg/mL type I collagen under arterial flow conditions at shear rate of 1800 s\(^{-1}\). Z-stack images were captured over 6 minutes duration with a digital Axiocam mRm camera (Carl Zeiss) and analysed with Zeiss Axiovision Rel4.6 software.
Figure 43. Kinetics of a dose response of ponatinib effect on *ex vivo* thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from mouse in the presence of PON (3 mg/kg), PON (10 mg/kg), PON (30 mg/kg) and vehicle after treatment for 4 hours were perfused across 500 µg/ml type I collagen at arterial flow rate 1800s⁻¹ in real time over a 6 minute time period. Results are presented as the mean ± SEM. (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4) using unpaired student’s t-test.
Similarly, mice treated with 3 mg/kg of ponatinib or 25 mg/kg nilotinib displayed a significant enhancement of thrombus growth in thrombus height, area and volume compared to vehicle control. In contrast, imatinib and dasatinib caused a significant inhibition of thrombus formation (Figure 44 & 45). 3 mg/kg treated mice correlates with 45 mg QD given to human CML patients.

Based upon these results, C57BL/6-treated mice with ponatinib and nilotinib doses shows strong potentiation of platelet adhesion and thrombus growth in real time, while imatinib or dasatinib treatment had an inhibitory effect on platelet thrombus formation on immobilised type I collagen matrix under ex vivo physiologic arterial shear flow. These results indicate that the prothrombotic effect of ponatinib and nilotinib treatment of wild-type mice required the presence of the endothelium to prime the platelets in vivo to potentiate ex vivo thrombus growth under arterial flow conditions.
Figure 44. The effect of TKIs on ex vivo murine thrombus formation and growth. Fluorescently-labelled whole blood of C57BL/6 mice untreated or treated with 3 mg/kg ponatinib, 25 mg/kg imatinib, 25 mg/kg nilotinib and 5 mg/kg dasatinib was perfused over 500 µg/mL type I collagen under arterial flow conditions at shear rate of 1800 s⁻¹. Z-stack images were captured over a 6 minute duration with a digital Axiocam mRm camera (Carl Zeiss) and analysed with Zeiss Axiovision Rel4.6 software.
Figure 45. Kinetics of TKI effect on ex vivo murine thrombus formation and growth on Type I collagen under arterial shear flow. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from mouse in the presence of PON (3 mg/kg), NIL (25 mg/kg), IM (25 mg/kg), DAS (5 mg/kg) and vehicle control after treatment for 4 hours were perfused across 500 µg/ml type I collagen at arterial flow rate 1800 s⁻¹ in real time over a 6 minute time period. Results are presented as the mean ± SEM. (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4) using unpaired student’s t-test.
4.2.2 The effect of ponatinib on platelet glycoprotein surface expression

Platelet glycoprotein receptors are known to play an important role in the regulation of platelet function and haemostatic mechanisms. In this study, this assay was conducted to examine whether ponatinib treatment would alter the expression of glycoproteins on the surface of resting platelets in wild-type C57BL/6 mice. Several glycoproteins, including GPVI, GPIb-IX-V complex, integrin α2β1, integrin αIIbβ3 and CD9, were detected on resting platelets using well-characterised specific monoclonal antibodies. Wild-type C57BL/6 mice were orally administered with 3 mg/kg ponatinib and blood samples were collected by cardiac puncture four hours later. Washed platelets in the resting state derived from untreated or ponatinib treated mice were incubated with respective anti-mouse platelet glycoprotein-FITC conjugated. As shown in Figure 46, the surface expressions of all glycoproteins GPVI, GPIb-IX-V complex, integrin α2β1, integrin αIIbβ3 and CD9 on platelets derived from ponatinib-treated mice were normal when compared to untreated mice. Results were derived from four independent experiments and are represented as mean fluorescence intensity MFI±SEM. Therefore, a single acute dose of ponatinib TKI did not affect platelet glycoprotein expression despite inducing an 

*ex vivo* prothrombotic state under arterial flow conditions.
**Figure 46. The effect ponatinib on surface expression of platelet glycoproteins in mice.**

Flow cytometric analysis on a FACS Canto II analyser of GPVI, Integrin α2β1, Integrin αIIbβ3, GPIb-IX-V and CD9 expression on resting platelets from C57BL/6 mice treated with 3 mg/kg ponatinib versus vehicle control using specific fluorescently labelled monoclonal antibodies for wild-type mouse platelets. The assay samples were performed in triplicate, and the results shown are representative of four independent experiments and presented as MFI±SEM (P>0.05; n=4) using unpaired student’s t test.
4.2.3 The effect of TKI on the conversion of integrin αIIbβ3 to its active conformation on platelets

The formation of stable platelet aggregation and thrombus formation is dependent upon the conversion of integrin αIIbβ3 from its resting to an activated state where it will bind its soluble ligand fibrinogen. We studied the effect of ponatinib and other TKIs on the conversion of integrin αIIbβ3 to its active conformation in wild-type platelets detected using JON/A mAb-PE binding. Washed platelets derived from ponatinib-treated (3 mg/kg), nilotinib-treated (25 mg/kg), imatinib-treated (25 mg/kg), dasatinib-treated (5 mg/kg) or untreated C57BL/6 mice showed minimal binding of JON/A-PE under resting conditions, compared with agonist-induced activation with thrombin (0.5 U/mL). As shown in Figure 47, thrombin induced activation of platelets derived from ponatinib-treated, nilotinib-treated, imatinib-treated or untreated mice displayed a similar ability to bind with the fibrinogen mimetic JON/A mAb that recognises the active conformation of integrin αIIbβ3 on platelets. In contrast, platelets from dasatinib-treated C57BL/6 mice displayed a reduced ability to bind the fibrinogen mimetic JON/A mAb. These results indicated that platelets from ponatinib and nilotinib-treated mice had normal conversion from a resting state of integrin αIIbβ3 to an activated conformation on platelets.
**Figure 47. The effect of TKI on JON/A mAb binding in murine platelets.** Flow cytometric analysis on a FACS Canto II analysis resting versus agonist induced active conformation of integrin αIIbβ3 on platelets from wild-type mice treated with 3 mg/kg ponatinib, 25 mg/kg nilotinib, 25 mg/kg imatinib, 5 mg/kg dasatinib or PBS using JON/A-PE mAb binding to platelets stimulated with thrombin (0.5 U/mL), or unstimulated (vehicle control). The assay samples were performed in triplicate, and the results shown are representative of four independent experiments and presented as MFI±SEM (*P < 0.05; **P < 0.01; n=4) using unpaired student’s t test.
4.2.4 Assessment of microvascular thrombosis formation and stability in BCR-Abl TKI-treated C57BL/6 mice subjected to FeCl₃-induced vascular injury of the mesenteric arterioles.

As the Bcr-Abl inhibitor, ponatinib and nilotinib displayed a potentiating effect on *in vitro* thrombosis formation under arterial flow, we wanted to examine thrombosis characterisation in an *in vivo* FeCl₃-induced vascular injury of mesenteric arterioles in C57BL/6 mice. It was clear that ponatinib and nilotinib TKI displayed a potentiating effect on *in vitro* and *ex vivo* thrombus formation, whereas imatinib and dasatinib exhibited an inhibitory role on *in vitro* and *ex vivo* thrombus growth under arterial flow conditions. Therefore, the effect of TKI’s (ponatinib, nilotinib, imatinib and dasatinib) drugs was examined on the formation of *in vivo* microvascular thrombi in mesenteric arterioles using FeCl₃-induced vascular injury in a C57BL/6 mouse model. Thrombus formation and stability characteristics were analysed in real time by measuring the number of fluorescently-labelled platelets bound to injured endothelium. In this model, wild-type mice were treated with ponatinib (3, 10, 30 mg/kg), 25 mg/kg imatinib, 25 mg/kg nilotinib, 5 mg/kg dasatinib or PBS pH 7.4 or 25 mM citrate buffer pH 2.75 by oral administration at four hours before surgery. The mice were then completely anaesthetised, the ideal diameter of the mesenteric arterioles (80-100 µm) was determined, and the animals were administered rhodamine 6G dye. Thrombus growth was observed and compared between TKI-treated mice and citrate buffer pH 2.75-treated mice for 10 minutes in real time *in vivo* through intravital microscopy. The rendered Z-stack images were deconvolved with Axiovision Rel 4.6 software, to construct 3D-images, and the volume of the thrombi was calculated from the area multiplied by the height of each thrombus. As shown in the representative images in Figure 48 & 49, enhanced thrombus growth was observed in FeCl₃-injured mesenteric arterioles of 3, 10, 30 mg/kg of ponatinib-treated mice in four hours after ingestion when compared to vehicle control at each time point beyond four minutes.
Figure 48. Ponatinib dose response on *in vivo* thrombus growth characteristics using FeCl$_3$ injured mesenteric arterioles. Representative images of thrombus formation in mesenteric arterioles (80-100 μm) at 2,4,6,8,10 minutes after 7.5% FeCl$_3$-induced vascular injury with ponatinib (3, 10 and 30 mg/kg) treated mice at 4 hours compared to vehicle control mice (n=4).
Figure 49. Kinetics of thrombus height, thrombus area and thrombus volume with different doses of Ponatinib TKI. (A) Thrombus height, (B) Thrombus area (C) Thrombus volume were determined over time for thrombi formed in mesenteric arterioles of ponatinib (3, 10, 30 mg/kg) treated mice after 4 hours. Results are presented as the mean ± SEM. (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4/group) using unpaired student’s t-test.
As shown in Figure 50, in contrast to the effects seen with ponatinib (3 mg/kg) and nilotinib (25 mg/kg), thrombus growth was reduced in mice treated with imatinib (25 mg/kg) and significantly diminished over a ten-minute period in 5 mg/kg dasatinib treated-mice following FeCl$_3$-induced injury, when compared with the mouse vehicle control. The Z-stack analysis of thrombus growth over all time-points revealed that imatinib-treated mouse arterioles displayed slightly decreased thrombus formation. No statistically significant differences were observed for any thrombus formation parameters when compared with vehicle-treated mouse arterioles (Figure 50). At 6 minutes, the kinetics of thrombus volume in imatinib-treated mice was (26421±3256 µm$^3$). However, mice treated with ponatinib exhibited a significant increase in the kinetics of thrombus volume at 6 minutes when compared to the control (70520±3352 µm$^3$). Also, there was a significant increase in the kinetics of thrombus volume with nilotinib (65411±3327 µm$^3$). Nevertheless, significant inhibition was observed in the kinetics of the thrombus volume (22544±3215 µm$^3$) at 6 minutes in mice treated with 5 mg/kg dasatinib compared to the wild-type vehicle control. Overall, an increase in the kinetics of thrombus volume for mice treated with either ponatinib or nilotinib was observed at 6, 8 and 10 minute time points, whereas the inhibition of thrombus formation with both imatinib and dasatinib was observed when compared to the vehicle control at 6, 8 and 10 minute time points (Figure 51).
Figure 50. TKI effect on *in vivo* thrombus growth using FeCl$_3$ injured mesenteric arterioles in C57BL/6 mice model. Representative images of thrombus formation in mesenteric arterioles (80-100 μm) at 2, 4, 6, 8, 10 minutes after 7.5% FeCl$_3$-induced vascular injury in ponatinib (3 mg/kg), nilotinib (25 mg/kg), imatinib (25 mg/kg) and dasatinib (5 mg/kg) treated mice at 4 hours compared to vehicle control mice. All microvessels are viewed at 200x magnification, and images acquired at a 1280x1024 pixel array using Axiovision Rel 4.6 version software attached to an Axiovert 135 M1 microscope (Carl Zeiss).
Figure 51. Kinetics of TKI effect on in vivo thrombus growth and stability using FeCl₃ injured mesenteric arterioles in C57BL/6 mouse model. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume were determined over time for thrombi formed in mesenteric arterioles of TKIs treated mice after 4 hours. Results are presented as the mean ± SEM. (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; n=4) using unpaired student’s t-test.
4.2.5 Assessment of arterial thrombosis formation in TKI-treated C57BL/6 mice subjected to FeCl₃-induced vascular injury of the mesenteric in blinded studies.

In order to remove operation bias, ARIAD/Takeda pharmaceutical company supplied two unknown TKI compounds to test the *in vivo* thrombus formation characteristics in mesenteric arterioles of C57BL/6 mice. The mice were anaesthetised, the ideal diameter of the mesenteric arterioles (80-100 µm) was determined, and the animals were administered rhodamine 6G dye. Thrombus growth was observed and compared between TKI-treated mice and vehicle-treated mice for 10 minutes in real time *in vivo* through intravital microscopy. The rendered Z-stack images were deconvolved with Axiovision Rel 4.6 software, to construct 3D-images, and the volume of the thrombi was calculated from the area multiplied by the height of each thrombus. The percentages of vessel occlusion and stability scores were also analysed for untreated wild-type mice or those treated with TKIs. Thrombi formed were analysed at all time points (0-2, 2-4, 4-6, 6-8 and 8-10 min).

As shown in the Figure 52 and 53, the kinetics of thrombus height, area and volume in compound A (5 and 25 mg/kg) -treated mice at 6, 8 and 10 minutes showed a significant increase in the thrombus formation when compared to the vehicle control. In contrast, significant inhibition was observed in the kinetics of the thrombus volume at 6, 8 and 10 minutes in mice treated with compound B (5 and 25 mg/kg) compared to the wild-type control. Overall, changes were observed in the kinetics of thrombus height, area and volume for mice treated with both concentration 5 and 25 mg/kg of compound A, whereas the inhibition with both concentration 5 and 25 mg/kg of compound B were observed when compared to the 25 mM citrate buffer pH 2.75 control (Figure 52). The compounds A and B were tested by LC-MS by ARIAD/Takeda pharmaceutical company. They recorded that compound A is ponatinib and compound B is imatinib.
Figure 52. TKI effect on in vivo thrombus growth and stability using FeCl₃ injured mesenteric arterioles in blinded studies. Representative images of thrombus formation in mesenteric arterioles (80-100 μm) at 2, 4, 6, 8, 10 minutes after 7.5% FeCl₃-induced vascular injury in compound A (5 mg/kg), compound A (25 mg/kg), compound B (5 mg/kg) and compound B (25 mg/kg) treated mice at 4 hours compared to vehicle control mice (25 mM citrate buffer pH 2.75).
Figure 53. Kinetics of TKI effect on \textit{in vivo} thrombus growth and stability using FeCl$_3$ injured mesenteric arterioles in blinded studies. (A) Thrombus height, (B) Thrombus area and (C) thrombus volume were determined over time for thrombi formed in mesenteric arterioles of TKIs treated mice after 4 hours. Results are presented as the mean ± SEM. (*$P < 0.05$ and **$P < 0.01$; n=3/group) using unpaired student’s $t$ test.
4.2.6 Evaluation of arterial thrombosis formation and time to vessel occlusion in TKI treated C57BL/6 mice through FeCl₃-induced vascular injury of the carotid artery

In order to investigate the effect of Bcr-Abl inhibitor ponatinib compared to other TKI’s in arteriolar thrombosis, we treated cohorts of C57BL/6 with respective doses of TKI’s for 4 hours then examined time to 95 % vessel occlusion by doppler laser flow probe.

In the microvascular thrombosis studies, mice treated with a single dose of ponatinib and nilotinib exhibited more rapid vessel occlusion in vivo following FeCl₃-induced injury of the mesenteric arterioles, whilst those treated with imatinib and dasatinib displayed reduced vessel occlusion compared to vehicle control mice. In this study, the possibility that ponatinib and nilotinib have comparable prothrombotic phenotypes, and that imatinib and dasatinib can inhibit thrombus formation in vivo were determined by measuring the time to achieve 95% vessel occlusion in real time. In this model, platelet recruitment and adhesion to form thrombi at injured sites is initiated by FeCl₃-induced vascular injury of the carotid artery and monitored blood flow using a Doppler laser flow probe.

As shown in Figure 54, the mean time to reach >95% vessel occlusion four hours after both ponatinib-treatment (3 mg/kg) of C57BL/6 mice was 888±22.51 seconds and nilotinib-treatment (25 mg/kg) was 848.4±21.74 seconds which were significantly shorter than the time observed for imatinib-treated (25 mg/kg) (1453.2±34.26 seconds) and dasatinib-treated (5 mg/kg) (1573.8±53.74 seconds) versus vehicle-treated (1113.8±28.39 seconds) C57BL/6 mice (Figure 54 & 55). These results suggest that a single acute dose of ponatinib and nilotinib can potentiate arteriolar thrombosis, whereas imatinib and dasatinib reduced arteriolar thrombosis in the carotid artery in vivo.
Figure 54. Examination of thrombus formation in the injured carotid artery in TKI-treated C57BL/6 mice. (A-E) Representative graphs of blood flow restriction in the carotid artery due to thrombus formation following 20% (w/v) FeCl$_3$ induced injury. (A) Vehicle-treated mice, (B) imatinib-treated mice, (C) nilotinib-treated mice, (D) dasatinib-treated mice and (E) ponatinib-treated mice. The vertical lines indicate the time when the carotid artery reached 95% occlusion, and the arrows indicate when FeCl$_3$ injury was initiated.
Figure 55. Time to 95% vessel occlusion in the injured carotid artery in TKI-treated C57BL/6 mice. Time to 95% carotid vessel occlusion was determined from the initiation of FeCl$_3$-induced injury in various TKI-treated mice. Each symbol represents one mouse, and all mice were sex-matched and weight-matched (20±2g). Data represent as mean±SEM and are representative of n=4 mice/group. (*P<0.05 and **P<0.01) using unpaired student’s t-test.
4.3 DISCUSSION

In the recent research on TKIs, it was observed that TKIs bear differential influence on the formation of platelet thrombus as an emerging subject matter. Furthermore, there are clinical studies which indicate that ponatinib might directly impact arterial thrombotic and trigger PAOD events in a dose dependent manner during CML treatment [239]. Nevertheless, there has been no other direct evidence that shows the role of ponatinib treatment in moderating pathological thrombus growth. We utilised a range of methods in this Chapter to assess platelet thrombi growth over time in C57BL/6 mice which are treated with an acute single dose 3, 10 or 30 mg/kg ponatinib, 25 mg/kg nilotinib, 25 mg/kg imatinib, 5 mg/kg dasatinib or vehicle control. The methods included assessment of the formation of platelet thrombus after FeCl$_3$-induced vascular injury in either the carotid artery or mesenteric arterioles.

In this Chapter, it was of interest to test and compare the ability of ponatinib and other TKIs to modulate platelet thrombus growth under ex vivo arterial flow conditions by intravital microscopy that allowed capturing of real time images from C57BL/6 mice. These experiments demonstrated that ponatinib and nilotinib had significant effects on thrombus growth under ex vivo arterial flow conditions over immobilised type I collagen at all doses tested. By contrast, dasatinib and imatinib inhibited platelet adhesion on type I collagen and the thrombus volume was significantly reduced at 4 and 6 minutes of perfusion when compared with untreated vehicle control. Furthermore, the ex vivo studies used drug concentrations that were optimised based on dose response curves, and the drug concentrations were similar to those used previously. As demonstrated in this Chapter, all concentrations 3, 10 and 30 mg/kg of ponatinib and 25 mg/kg of nilotinib significantly increased ex vivo thrombus growth on immobilised type I collagen over time when compared to vehicle control in real time. By contrast, thrombus
growth on type I collagen under \textit{ex vivo} flow shear conditions was inhibited by imatinib and dasatinib TKIs.

The existence of glycoprotein receptors on the platelet surface, such as GPIb-IX-V, GPVI, integrin α2β1, integrin αIIbβ and CD9, are important participants in the regulation of platelet function in both humans and mice. The expression of these receptors contributes directly to haemostatic processes through involvement by either coordinating adhesive receptors with exposed subendothelial molecules at vascular trauma, platelet stimulation in response to soluble agonists or aggregating growth for associating with other platelets or with other blood components, including leukocytes, which leads to formation of the haemostatic plug. However, up-regulation of receptor expression on platelet surfaces has been shown to play a critical role in platelet arterial thrombosis in both humans and mice [5, 64]. Previous research showed that dasatinib impaired the collagen/GPVI association and created subsequent defects in the ITAM-mediated signaling pathways in platelets, particularly for FcγRIIa and collagen GPVI/FcR gamma chain that involve tyrosine kinase phosphorylation, PI3-kinase activation and PLC/DAG kinase activation, with the end result of increased bleeding events and prevention of thrombus growth [47].

Following identification that ponatinib treatment of C57BL/6 mice resulted in a prothrombotic phenotype under \textit{ex vivo} arterial flow conditions. The next step therefore was to investigate whether this could be attributed to increased platelet glycoprotein expression or induced activated conformation of integrin αIIbβ3 \textit{in vivo}. This Chapter’s results demonstrated that 3 mg/kg ponatinib did not show a direct effect on the surface expression of glycoproteins on resting platelets in C57BL/6 mice. In addition, the results here demonstrated that ponatinib did not directly alter conversion of integrin αIIbβ3 to its active conformation in murine platelets upon agonist stimulation.
Platelets from ponatinib-treated mice had a similar ability for binding with JON/A mAb as platelets from untreated mice, resulting in normal conversion of integrin αIIbβ3 from resting to activated conformation to bind its fibrinogen mimetic JON/A mAb. Collectively, these findings of the initial characterisation of ponatinib treatment suggest that ponatinib had no effect on modulation of platelet glycoprotein expression or conformation of integrin αIIbβ3 at a single acute dose. However, further studies could be of interest to ascertain the possibility of potential effects of multiple treatments of ponatinib (chronic model) on regulation of platelet glycoprotein receptors.

In this study, the intravital microscopy was used to examine FeCl₃ induced injury of mesenteric arterioles or carotid artery. The results show that the mice treated with ponatinib and nilotinib increased thrombus growth in vivo at 6, 8 and 10 minutes. This study and others have shown that dasatinib-treated mice yielded smaller thrombi [240]. Therefore, these studies highlight that dasatinib has the potential to inhibit the growth of platelet thrombus in vivo. In addition, mice treated with imatinib had smaller thrombi.

Moreover, FeCl₃ induced oxidation of haemoglobin, resultant in erythrocyte haemolysis that demanded denudation of the endothelium and subsequent exposure of ECM molecules including collagen [241]. Platelets circulating in the blood will tether and stick to subendothelial collagen at the vascular injury sites by binding with their receptors (the GPVI and GPIb-IX-V complexes). The receptors are expressed on the surface membrane of the platelet, thus engaging platelet attachment, activation, and promotion of platelet aggregation and growth of the thrombus [242].

Once the platelets are stimulated, adhesive proteins, such as CD40L and P-selectin are generated to support the activation of platelets and enhance the formation of arterial thrombus.
In this study, through the use of intravital microscopy, there was an enhanced extent of FeCl₃-induced injury of mesenteric arterioles ponatinib treated mice showed increased thrombus growth compared to those in the vehicle control. However, the differences were statistically significant (Figure 48 & 49). This proves that ponatinib and nilotinib TKIs potentiate the interaction of platelets and endothelium together with platelet-platelet interaction the formation of thrombus \textit{in vivo}. Conversely, a FeCl₃-induced vascular injury resulted in smaller thrombus growth among mice treated with imatinib as compared to those in vehicle control. As anticipated, there were smaller thrombi in dasatinib-treated mice than those in the sham control group after the FeCl₃-induced injury which showed a significant difference compared to ponatinib, nilotinib or vehicle control (Figure 50 & 51).

This study showed a ponatinib-mediated prothrombotic phenotype using a substitute experimental \textit{in vivo} model that engaged a different type of vascular bed that contained a higher rate of shear. There was a measurement of time to achieve >95% C57BL/6 mouse carotid arteries vessel occlusion following treatment of ponatinib, imatinib, nilotinib, dasatinib, or vehicle (control). The process was preceded by FeCl₃-induced injury of carotid arteries followed by monitoring with the use of a Doppler laser flow probe. Following TKI treatment, the time required to achieve >95% vessel occlusion was significantly shorter in ponatinib or nilotinib-treated mice than in the vehicle control, dasatinib or imatinib-treated mice. Although there was a delay in the mean time to 95% vessel occlusion, that was statistically significant in the imatinib or dasatinib-treated mice when compared with shorted time ponatinib, nilotinib compared to the vehicle control (Figure 54 & 55).

The study proves the hypothesis, which indicated that treatment of mice with imatinib and dasatinib causes a delay in achieving 95% vessel occlusion following the FeCl₃-induced injury of the carotid artery in comparison to the vehicle control. The FeCl₃-induced thrombosis primarily happens as a result of the platelet activation at the spot where there is vascular injury.
through contact between circulating platelets and the bare subendothelial adhesive proteins, like vWF and type I collagen, which attach themselves to their receptors (GPIb-IX-V and GPVI complexes correspondingly) [243]. The findings here are parallel to the observations made above as established from the model in the first experimental model indicating that ponatinib and nilotinib dose and not of dasatinib or imatinib enhance in vivo thrombus growth following the mesenteric arterioles’ FeCl₃-induced injury. Consequently, the integration of the two result findings indicates that ponatinib and nilotinib therapy play a key role in increasing the recruitment of platelets at FeCl₃-induced vascular injury sites, thus promoting progressive prothrombotic growth over time.

Previous studies in our lab, Alhawiti et al., showed that nilotinib induced a prothrombotic state in vitro, ex vivo and in vivo following FeCl₃-induced injury of mesenteric arterioles, while both imatinib and dasatinib showed inhibition of thrombus growth [238].

A recent study demonstrated that ponatinib induced thrombus formation using Rose Bengal photoactivation technique in an in vivo mouse model. These studies correlated with my study despite using a photoactivation model that induces in vivo thrombus formation but also causes vessel blood cells to stagnate [244].

In conclusion, this Chapter has provided novel insights into the influence of TKIs on arteriolar thrombus formation in vivo using different models. Ponatinib or nilotinib selectively induced a prothrombotic state in vivo four hours after a single dose (C_max) of the drug in the context of blood vessels with different vascular beds. This included examining platelet thrombus formation following FeCl₃-induced vascular injury in either mesenteric arterioles (80-100 µm in diameter) or in the carotid artery.
Chapter 5: EXAMINING THE AMELIORATION OF THE PONATINIB AND NILOTINIB INDUCED PROTHROMBOTIC EFFECTS ON HUMAN AND MICE USING DIFFERENT AGENTS INCLUDING CALCIUM CHANNEL BLOCKER, DILTIAZEM, COX2 INHIBITOR, DICLOFENAC AND eNOS SYNTHASE INHIBITOR, L-NNA.
5.1 INTRODUCTION

The endothelium refers to a monolayer of endothelial cells, which form the inner blood vessel lining and the lymphatic system. Moreover, the endothelium functions by regulating blood flow and preventing platelets from adhesion because of the anti-thrombotic surface that is created by the endothelial cells [245]. The functioning of the endothelium is controlled by several vasoactive substances, a mechanical network, and other mechanical forces such as pressure [246]. From this perspective, it is evident that the endothelium serves a primary role in ensuring enough flow of blood, which is regulated by other substances. Key vasodilators include nitric oxide and prostaglandin I\(_2\) that are aided by the secretion of other substances including C-type natriuretic peptide and the hyperpolarising factor that is derived from the endothelium [247]. NO and PGI\(_2\) serve as key bioactive secretions from endothelium that inhibit platelet aggregation. Anti-thrombotic and anti-aggregation of platelets is achieved through the increased secretion of the vasodilators, which increase adenylate cyclase and guanylate cyclase activity that are responsible for raising the levels of c-AMP and c-GMP in the body [248].

The endothelium also secretes vasoconstrictors namely thromboxane A\(_2\), angiotensin II, reactive oxygen species (ROS), and endothelin-1 [249]. The natural anticoagulant nature of the endothelial cells is achieved through their cell surface expression of thrombomodulin that converts thrombin into an anticoagulant enzyme by binding and activating protein C that down regulates FVa and FVIIIa. [92].
Endothelial cell injury following vascular inflammation leads to shedding of endothelial micro particles into the blood circulating system, which can be measured with increased fragments of the endothelial cells in association with endothelial dysfunction and unstable coronary vasculitis. Imbalanced release of vasodilators and vasoconstriction-related elements result in endothelial dysfunction [250].

Calcium flux plays an important role during both platelet and endothelial activation [251]. In addition, the intracellular calcium communication mediates by co-operative interplay between integrin αIIbβ3 engagement and ADP activation [252]. Hence the co-ordination of calcium flux through platelet activation leads to an increase in the calcium signal contributing to the development of thrombus formation [253]. Since GPIb and integrin α2β1 aid in platelet adhesion that is essential for the growth of thrombus, the research findings unearth the possibility that calcium is responsible for signaling the downstream of the receptors and in the aggregation of platelets, which is vital in fluxing of calcium that facilitates the development of thrombus and aggregation of platelets on vWF [254].

Recent myograph studies of mouse aortic rings have demonstrated that ponatinib augmented norepinephrine enhanced vasoconstriction of the aortic rings compared to vehicle control treated [231]. Notably, vascular function is facilitated by two major regulating components that are produced by endothelial cells including endothelial nitric oxide synthase (eNOS) and cyclooxygenase-2 (COX-2). In normal circumstances, PGI2 synthesis and the expression of COX-2 is promoted by laminar flow, which acts as an eNOS stimulant [255]. In effect, optimal vascular control is attained coupled with balanced homeostasis that are essential in preventing the activation and aggregation of platelets. The imbalance between the endothelium relaxers
and contractors are the underlying mechanisms for endothelial dysfunction. This occurs when there is continuous reduction of NO caused by changes in eNOS activity and expression, increased COX factors, and increased oxygen-free radicals [256].

In this chapter, cohorts of TKI-treated mice were subjected to FeCl₃-mediated vascular induced injury of the mesenteric arterioles (80-100 µM) and in vivo microvascular thrombus growth was evaluated to examine the effect of calcium channel blocker (CCB) or COX-2 inhibitor, diclofenac or eNOS synthase inhibitor, L-NNA on ponatinib and nilotinib treated-mice. Moreover, an in vivo study was performed to evaluate the effect on soluble cytokines levels. The use of these approaches would provide an insight into the effect of pretreatment of CCB diltiazem, COX-2 inhibitor, diclofenac and eNOS synthase inhibitor, L-NNA on ponatinib and nilotinib platelet phenotype and thrombus formation in vivo under arterial flow in a C57BL/6 mouse model.
5.2 RESULTS

5.2.1 The effect of pretreatment with calcium channel blocker, diltiazem before ponatinib or nilotinib-treated normal human whole blood over type I collagen under in vitro arterial flow conditions

To determine the effect of calcium channel blocker prior of Bcr-Abl inhibition on human platelet adhesion and aggregation on type I fibrillar collagen under arterial flow, a comparison of ponatinib and nilotinib after pretreatment with calcium channel blocker was done. Assays were conducted to examine the dose-dependent effect of TKIs on in vitro platelet thrombus formation in blood samples +/- CCB from healthy humans and C57BL/6 mice model. Citrated whole blood was treated with ponatinib (0.1 µM), ponatinib (0.1 µM) & Diltiazem (5 µM), nilotinib (5 µM) or nilotinib (5 µM) & Diltiazem (5 µM) for 30 minutes at 37°C. Whole blood containing rhodamine 6G fluorescently labelled platelets was perfused through a collagen-coated microcapillary tube flow chamber at an arterial shear rate of 1800s⁻¹. After six minutes of sample perfusion, thrombus images for TKIs-treated whole blood +/- CCB were recorded and determined in real time. Deconvolved Z-stack images of thrombi recorded in real time were analysed for thrombus parameters including thrombus area, thrombus height and thrombus volume in the TKI-treated +/- CCB whole blood samples.

As shown in Figure 56 and 57, human whole blood treated with ponatinib and nilotinib showed increased thrombus formation for in vitro thrombus growth over type I collagen and the thrombus height, surface area covered by platelet thrombi and the thrombus volume compared to vehicle control. In contrast, the pretreatment with calcium channel blocker showed 22% reduction of thrombus formation with either nilotinib or ponatinib (Figure 56 & 57). These results demonstrate that calcium channel blockade prior to ponatinib and nilotinib TKI treatment reduced platelet: ECM and platelet: platelet interactions on type I collagen.
Figure 56. Calcium channel blockade prior to TKI effect on in vitro thrombus formation and growth on Type I collagen under arterial shear flow. Representative images of thrombus formation over type I collagen under arterial flow conditions. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), PON (0.1 µM) & Diltiazem (5 µM), NIL (5 µM), NIL (5 µM) & Diltiazem (5 µM) and wild type (WT) vehicle control were perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800s⁻¹ in real time over a 6 minute time period.
Figure 57. Pre-treatment of whole blood from humans with calcium channel blocker, Diltiazem reduces *in vitro* thrombus formation in the presence of ponatinib or nilotinib. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. Fluorescently labelled whole blood from human in the presence of PON (0.1 µM), PON (0.1 µM) & Diltiazem (5 µM), NIL (5 µM), NIL (5 µM) & Diltiazem (5 µM) and vehicle control were perfused across 500 µg/ml type I collagen at an arterial flow rate of 1800s⁻¹ in real time over a 6 minute time period. (*P < 0.05, **P < 0.01 and ***P < 0.001; n=4) using unpaired student’s t-test.
5.2.2 The effect of pretreatment with calcium channel blocker before ponatinib or nilotinib treatment subjected to FeCl₃-induced vascular injury of the mesenteric arterioles to assess in vivo thrombus formation

Ponatinib and nilotinib TKIs induced a potentiating effect on in vivo thrombus formation under arterial flow. In this study, we examined thrombus characteristics in an in vivo FeCl₃ induced vascular injury of mesenteric arterioles in C57BL/6 mice after pretreatment with calcium channel blocker prior to ponatinib or nilotinib treatment. Thrombus formation characteristics were analysed in real time by measuring the number of fluorescently-labelled platelets bound to injured endothelium. In this model, wild-type mice were treated with ponatinib (3 mg/kg) with and without pretreatment of CCB, diltiazem 30 mg/kg and 25 mg/kg nilotinib with and without CCB, diltiazem 30 mg/kg or vehicle control 25 mM citrate buffer pH 2.75 by oral administration at four hours before surgery. The mice were then completely anaesthetised, the ideal diameter of the mesenteric arterioles (80-100 µm) was determined, and the animals were administered rhodamine 6G dye. Thrombus growth was observed and compared between TKI-treated mice with and without diltiazem 30 mg/kg and citrate-treated mice for 10 minutes in real time in vivo through intravital microscopy. The rendered Z-stack images were deconvolved with Axiovision Rel 4.6 software, to construct 3D-images, and the volume of the thrombi was calculated from the area multiplied by the height of each thrombus. Thrombi formed were analysed at all time points (0-2, 2-4, 4-6, 6-8 and 8-10 min). As shown in Figure 58 and 59, the effect of diltiazem with ponatinib treatment reduced the thrombus volume over time compared to ponatinib 3 mg/kg treated-mice by 48% at 10 minutes.
Figure 58. Pre-treatment of C57BL/6 mice with calcium channel blocker, Diltiazem reversed the prothrombotic effect of ponatinib. Representative images of thrombus formation in mesenteric arterioles (80-100 µm) at 2, 4, 6, 8, and 10 minutes after 7.5% FeCl₃ induced vascular injury. Vehicle control, WT+ Diltiazem (30 mg/kg), ponatinib (3 mg/kg) or PON (3 mg/kg) & Diltiazem (30 mg/kg) treated mice for 24 hours followed by ponatinib or vehicle for 4 hours were appropriate (n=4).
Figure 59. Kinetics of thrombus growth in C57BL/6 mice pretreated with calcium channel blocker, Diltiazem reversed the prothrombotic effect of ponatinib. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. The kinetics of thrombus volume were determined over time for thrombi formed in mesenteric arterioles of various treatment groups of C57BL/6 mice. 3 dimensional deconvolved thrombi were analysed for volume using unpaired student’s t test (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4).
In addition, the same effect is observed with nilotinib treated-mice by pretreatment with diltiazem. The result showed reduced thrombus volume over time when mice were pretreated with calcium channel blocker before ingestion of nilotinib reversed the prothrombotic phenotype by 44% at 10 minutes (Figure 60 & 61). In contrast, the nilotinib-treated mice alone showed enhancement of thrombus growth.
Figure 60. Pre-treatment of C57BL/6 mice with calcium channel blocker, Diltiazem reversed the prothrombotic effect of nilotinib. Representative images of thrombus formation in mesenteric arterioles (80-100 µm) at 2, 4, 6, 8 and 10 minutes after 7.5% FeCl₃ induced vascular injury. Vehicle control, WT+ Diltiazem (30 mg/kg), NIL (25 mg/kg) or NIL (25 mg/kg) & Diltiazem (30 mg/kg) treated mice for 24 hours followed by nilotinib or vehicle control for 4 hours were appropriate (n=4).
Figure 61. Kinetics of thrombus growth in C57BL/6 mice pretreated with calcium channel blocker, Diltiazem reversed the prothrombotic effect on nilotinib. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. The kinetics of thrombus volume were determined over time for thrombi formed in mesenteric arterioles of various treatment groups of C57BL/6 mice. 3 dimensional deconvolved of the thrombi were analysed for volume using unpaired student’s t test (* P < 0.05; ** P < 0.01; n=4).
5.2.3 The effect of pretreatment with calcium channel blocker, Diltiazem before ponatinib and nilotinib treatment for soluble P-selectin level

P-selectin translocated to the surface and is rapidly cleaved in vivo to create soluble P-selectin as marker of endothelial and platelet activation [257]. Soluble P-selectin plays an important role in the promotion of thrombotic events by exerting procoagulant reactivity [258]. This ELISA experiment was used to measure the levels of sP-selectin in mouse plasma. Plasma samples were collected after pretreatment with diltiazem for 24 hours prior to ponatinib and nilotinib treatment for 4 hours. As shown in Figure 62, both ponatinib and nilotinib showed increased in plasma level of sP-selectin compared to vehicle control which reflects proteolytic shedding of P-selectin from activated platelets and endothelial cells in vivo in response to ponatinib or nilotinib treatment. By contrast, the pretreatment with CCB before treatment with ponatinib and nilotinib showed reduced plasma levels of sP-selectin compared to ponatinib and nilotinib treatment alone without pretreatment with diltiazem or vehicle control. Thus, these results support that ponatinib and nilotinib treatment positively induced platelet and endothelial activation via potentiation of P-selectin exposure and cleavage from platelet-endothelial cell surface in wild-type mice. However, the pretreatment with CCB diltiazem prevented the increase in plasma sP-selectin (Figure 62).
Figure 62. Measurement of sP-selectin levels in plasma derived from C57BL/6 mice pretreated with diltiazem followed by ponatinib or nilotinib. Plasma obtained from C57BL/6 mice treated with 3 mg/kg ponatinib, 25 mg/kg nilotinib, 3 mg/kg ponatinib & 30 mg/kg diltiazem, 25 mg/kg nilotinib & 30 mg/kg diltiazem or vehicle control was collected after 4 hours of ingestion. Plasma sP-selectin (ng/mL) levels of C57BL/6 mice treated was determined by ELISA. Each experiment were performed in triplicate and represented as mean±SEM of four independent experiments (*P < 0.05, ** P < 0.01 and *** P < 0.001; n=4) using unpaired students’ t test.
5.2.4 The effect of pretreatment with calcium channel blocker, Diltiazem before ponatinib and nilotinib on IL-6, TNF-alpha and IFN-gamma levels in plasma.

Vascular endothelial cells and other cells including monocytes/macrophages produce TNF-alpha, IL-6 and IFN-gamma cytokines and secreted TNF-alpha is thought to be involved in an autocrine activation of endothelial cells with cell-bound TNF-alpha serving to activate target cells on endothelial cells [259]. Both TNF-alpha and IL-6 play an important role in the acute phase response, inflammation and infection [260]. In this study, ELISA test was used to measure the levels of cytokines in mouse plasma. Plasma samples were collected after pretreatment of C57BL/6 mice with diltiazem for 24 hours prior ponatinib 3 mg/kg or nilotinib 25 mg/kg treatment for 4 hours. As shown in Figure 63, 64 and 65, both ponatinib or nilotinib treated-mice alone showed increased plasma levels of TNF-alpha, IL-6 and IFN-gamma cytokines compared to the vehicle control. By contrast, the pretreatment with CCB, Diltiazem prior to treatment with ponatinib or nilotinib showed reduced plasma levels of IL-6, TNF-alpha and IFN-gamma. This result indicates that both ponatinib and nilotinib induced a pro-inflammatory response that can be ameliorated by blockage of calcium flux.
Figure 63. Measurement of sIL-6 level in plasma derived from C57BL/6 mice pretreated with diltiazem followed by ponatinib and nilotinib. Plasma obtained from C57BL/6 mice treated with 3 mg/kg ponatinib, 25 mg/kg nilotinib, 3 mg/kg ponatinib & 30 mg/kg diltiazem, 25 mg/kg nilotinib & 30 mg/kg diltiazem or vehicle control was collected after 4 hours of ingestion. Plasma IL-6 (pg/mL) levels of C57BL/6 mice treated was determined by ELISA. Each experiment were performed in triplicate and represented as mean±SEM of four independent experiments (** P < 0.01 and *** P < 0.001; n=4) using unpaired students’ t test.
Figure 64. Measurement of TNF-alpha level in plasma derived from C57BL/6 mice pretreated with diltiazem followed by ponatinib and nilotinib. Plasma obtained from C57BL/6 mice treated with 3 mg/kg ponatinib, 25 mg/kg nilotinib, 3 mg/kg ponatinib & 30 mg/kg diltiazem, 25 mg/kg nilotinib & 30 mg/kg diltiazem or vehicle control was collected after 4 hours of ingestion. Plasma TNF-alpha (pg/mL) levels of C57BL/6 mice treated was determined by ELISA. Each experiment were performed in triplicate and represented as mean±SEM of four independent experiments (** P < 0.01, *** P < 0.001 and **** P < 0.0001; n=4) using unpaired student’s t test.
Figure 65. Measurement of IFN-Gamma level in plasma derived from C57BL/6 mice pretreated with diltiazem followed by ponatinib and nilotinib. Plasma obtained from C57BL/6 mice treated with 3 mg/kg ponatinib, 25 mg/kg nilotinib, 3 mg/kg ponatinib & 30 mg/kg diltiazem, 25 mg/kg nilotinib & 30 mg/kg diltiazem or vehicle control was collected after 4 hours of ingestion. Plasma IFN-gamma (pg/mL) levels of C57BL/6 mice treated was determined by ELISA. Each experiment were performed in triplicate and represented as mean±SEM of four independent experiments (** P < 0.01 and *** P < 0.001; n=4) using unpaired student’s t test.
5.2.5 The effect of pretreatment of C57BL/6 mice with COX-2 inhibitor, diclofenac inhibitor before ponatinib treatment subjected to FeCl₃-induced vascular injury of the mesenteric arterioles to induce in vivo thrombus formation

In this study, we examined thrombosis characterisation in an in vivo FeCl₃ induced vascular injury of mesenteric arterioles in C57BL/6 mice after pretreatment with COX-2 inhibitor, diclofenac. Thrombus growth characteristics were analysed in real time by measuring the number of fluorescently-labelled platelets bound to injured endothelium. In this model, wild-type mice were treated with ponatinib 3 mg/kg with and without pretreatment of COX-2 inhibitor, diclofenac 10 mg/kg or vehicle control 25 mM citrate buffer pH 2.75 by oral administration at four hours before surgery. The mice were then completely anaesthetised, the ideal diameter of the mesenteric arterioles (80-100 μm) was determined, and the animals were administered rhodamine 6G dye. Thrombus growth was observed and compared between TKI-treated mice with and without diclofenac 10 mg/kg and citrate-treated mice for 10 minutes in real time in vivo through intravital microscopy. The rendered Z-stack images were deconvolved with Axiovision Rel 4.6 software, to construct 3D-images, and the volume of the thrombi was calculated from the area multiplied by the height of each thrombus. Thrombi formed were analysed at all time points (0-2, 2-4, 4-6, 6-8 and 8-10 min). As shown in Figure 66 and 67, the effect of COX-2 inhibitor, diclofenac reverses thrombus formation by 35% at 10 minute time point compared to ponatinib 3 mg/kg treated-mice only.
Figure 66. Pre-treatment of C57BL/6 mice with COX-2 inhibitor, diclofenac reversed the prothrombotic effect of ponatinib. Representative images of thrombus formation in mesenteric arterioles (80-100 µm) at 2, 4, 6, 8 and 10 minutes after 7.5% FeCl₃ induced vascular injury. Vehicle control, WT+ diclofenac (10 mg/kg), NIL (25 mg/kg) or NIL (25 mg/kg) & diclofenac (10 mg/kg) treated mice for 24 hours followed by ponatinib or vehicle for 4 hours were appropriate (n=4).
Figure 67. Kinetics of thrombus growth in C57BL/6 mice treated with COX-2 inhibitor, diclofenac reversed the prothrombotic effect of ponatinib. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. The kinetics of thrombus volume were determined over time for thrombi formed in mesenteric arterioles of various treatment groups of C57BL/6 mice. 3 dimensional deconvolved thrombi were analysed for thrombus volume using unpaired student’s t test (* P < 0.05, ** P < 0.01 and *** P < 0.001; n=4).
5.2.6 The effect of pretreatment of C57BL/6 mice with eNOS synthase inhibitor, L-NNA before ponatinib treatment subjected to FeCl₃-induced vascular injury of the mesenteric arterioles for \textit{in vivo} thrombus formation

In this study, we tested thrombosis characterisation in an \textit{in vivo} FeCl₃ induced vascular injury of mesenteric arterioles in C57BL/6 mice after pretreatment with eNOS synthase inhibitor, L-NNA prior to ponatinib treatment. Thrombus growth characteristics were analysed in real time by measuring the number of fluorescently-labelled platelets bound to injured endothelium. In this model, wild-type mice were treated with ponatinib 3 mg/kg with and without pretreatment with L-NNA 10 mg/kg or vehicle control 25 mM citrate buffer pH 2.75 by oral administration at four hours before surgery. The mice were then completely anaesthetised, the ideal diameter of the mesenteric arterioles (80-100 µm) was determined, and the animals were administered rhodamine 6G dye. Thrombus growth was observed and compared between TKI-treated mice with and without L-NNA 10 mg/kg and citrate-treated mice for 10 minutes in real time \textit{in vivo} through intravital microscopy. The rendered Z-stack images were deconvolved with Axiovision Rel 4.6 software, to construct 3D-images, and the volume of the thrombi was calculated from the area multiplied by the height of each thrombus. Thrombi formed were analysed at all time points (0-2, 2-4, 4-6, 6-8 and 8-10 min). As shown in Figure 68 and 69, the L-NNA reduces thrombus formation by 32% at 10 minute time point compared to ponatinib 3 mg/kg treated-mice only. In addition, pretreatment with L-NNA inhibitor prior to ponatinib TKI treatment reduce thrombus formation by 30% compared to L-NNA alone or ponatinib alone.
Figure 68. Pre-treatment of C57BL/6 mice with L-NNA prior to ponatinib treatment reduced the prothrombotic effect of ponatinib. Vehicle control, WT & L-NNA (10 mg/kg), treated mice for 24 hours followed by ponatinib 3 mg/kg or vehicle for 4 hours were appropriate. Representative images of thrombus formation in mesenteric arterioles (80-100 μm) at 2, 4, 6, 8 and 10 minutes after 7.5% FeCl₃ induced vascular injury.
Figure 69. Kinetics of thrombus growth in C57BL/6 mice treated with L-NNA prior to ponatinib treatment reduced the prothrombotic effect of ponatinib. (A) Thrombus height, (B) Thrombus area, (C) Thrombus volume. The kinetics of thrombus volume were determined over time for thrombi formed in mesenteric arterioles of various treatment groups of C57BL/6 mice. 3 dimensional deconvolved of the thrombi were analysed for volume using unpaired student’s t test (* P < 0.05 and ** P < 0.01; n=4).
5.3 DISCUSSION

Endothelial cell injury following vascular inflammation leads to shedding of endothelial microparticles into the blood circulating system, which can be measured with increased fragments of the endothelium cells in association with endothelial dysfunction [261]. Imbalanced release of vasodilators and vasoconstriction-related elements results in endothelial dysfunction. Notably, endothelial dysfunction is a core effect of hypertension. Normal functioning endothelial cells stiffen and lose vasorelaxation [262]. This makes it an important feature to identify hypertension. Studies on regulation of the vascular muscle that relies on nitric oxide with regards to physical and pharmacological response has been achieved in the past ten years [263]. Evidently, reduced availability of NO is caused by endothelial dysfunction, which causes the oxidative stress within the cells to increase. Disturbances in the endothelial physiology have been linked to certain cardiovascular risks [264]. Based on epidemiological studies, endothelial dysfunction can occur because of chronic inflammation or infection.

Currently, there are some proposals of how ponatinib promotes arterial occlusive events. For instance, the medication produces targeting effects on the multiple receptor pathways, which include fibroblast growth factors receptor kinases (FGFR), vascular endothelial growth factor receptor (VEGFR) and angiopoietin receptor (Tie-2), which produces some of targeting effects that can predispose to a condition of hypertension. The ponatinib has also been associated with thrombotic and proteinuria microangiopathy [265].

According to recent research, ponatinib inhibition of VEGFR is essential since it reduces migration, viability and the functionality of the human umbilical vein endothelial cells
(HUVEC) [266]. Ponatinib also uses blocking VEGFR to exert some anti-angiogenic activity in HUVEC and zebrafish model by delaying VEGFR signaling pathway in either an AKT or P13 kinase-dependent manner [267]. A study on ponatinib TKI further revealed that it controlled vasomotor function, endothelial cell growth, and blood flow recovery, as evident in an ischaemia reperfusion mouse model [268].

The blockade of Ang/Tie-2 pathway, using nilotinib or ponatinib, could upregulate the cytokine levels, resulting in pro-inflammatory effects [269]. These TKIs could promote cell-to-matrix or cell-to-cell interactions, which activates the platelets and advance the vascular effects over time. An analysis of the CML patients’ treated at MD Anderson with nilotinib indicated about 2.1% of vascular events was associated with vasospasm which did not show any evidence of atherosclerotic lesions [230]. This vasospasm effect was enhanced with higher doses of nilotinib of 400 mg QD compared to the previous 300 mg QD [270].

The early acute vascular effects point to ponatinib as the cause of vasospasm effects, which according to research, results from the dosage and later produce chronic effects of increased atherosclerosis, which majorly affects the CML patients with some known cardiovascular risk factors. Research also indicates that ponatinib cause attenuates vasorelaxation and vasoconstriction in the mouse aortic rings [231]. To reverse this, the use of either COX-2 or eNos synthase or calcium channel inhibitors, was applied to modulate vasoconstricting prostanoids to reduce the ponatinib effect.
L-type calcium channels mediate calcium flux from extracellular to intracellular space. Calcium influx is associated with release of calcium stores into the cytoplasm, resulting in cell contraction [271]. However, diltiazem is a L-type calcium channel blocker. It enhances vasodilatory tone by inhibiting Ca\(^{2+}\) flux into the vascular smooth muscle cells and enhances eNOS protein expression and production of endothelium derived NO [272]. Thus, bioactive NO is a potent inhibitor of platelet activation that could explain the reversal of ponatinib or nilotinib induced prothrombotic state.

The regulation of COX-1 and COX-2 enzymes is different despite sharing high homology levels of 65 percent [273]. The two COXs can work independently despite being in the same cell type. COX-1 preferred substrates include fatty acids including arachidonic acid as COX-2’s preferred substrate include 2-arachidonyl glycerol and fatty acids [274]. In addition, some products generated by COX-2 cannot be synthesised by COX-1. In both cases, lipid peroxides are required for activation purposes though COX-2 needs lower hydroperoxide concentrations by ten fold that its counterpart. This means that COX-2 can be used in the COX-I environments without being activated [275]. Moreover, Helliwell et al., identified that COX-2 is expressed on inflammation sites while COX-1 is expressed in most tissues even during shear stress [276].

Normal functioning blood vessels express COX-1 and COX-2 through the vascular smooth muscle cells and endothelial cells with the former COX-2 as the predominant isoform. Despite, the fact that COX-2 plays a significant role in the generation of prostacyclin in humans, COX-1 also contributes to the production of PGs in platelets. Thromboxane A\(_2\) within the cardiovascular system functions as a thrombogenic factor that aids in vasoconstriction through its generation from COX-1 and the endothelial cells. However, COX-1 disruption can result in
the decrease of PG synthesis though the inducible synthesis is not affected. This means normal survival can be achieved with severe consequences such as aggregation of impaired platelets and increased pain tolerance with airway hypersensitivity [277]. COX-2 deletion phenotype is arguably more severe since it is closely associated with an impaired inducible synthesis and normal PG synthesis [278]. Furthermore, prostacyclin is a powerful vasodilator and anti-aggregating factor in blood vessels. It also functions as the main arachidonic acid metabolite through synthesis at the endothelium site. Nonetheless, the spread of inflammation in most cardiovascular diseases is often accompanied with oxidative stress that facilitates eicosanoids production [279]. In effect, it changes production from anti-thrombosis to a prothrombotic state and vasodilation to vasoconstriction.

The exciting part of the study was pretreatment using L-type calcium channel blocker, diltiazem or COX-2 inhibitor, diclofenac or endothelial eNos synthase inhibitor, L-NNA before exposing to TKI treatment. The research revealed blockade of calcium flux was able to reverse the pro-inflammatory and prothrombotic phenotype for ponatinib. Similarly, calcium channel blockade also reduced the prothrombotic phenotype for nilotinib. The evidence supports the concept that nilotinib and ponatinib may produce a vasospastic effect, which activates dysregulation or contributes to endothelial and platelet activation. These actions suggest a direct impact on the blood vessel wall, which results in platelet and endothelial activation. Also, any dose of ponatinib, even at 15 mg QD, can still potentially produce fatal consequences [280]. The study examined ponatinib treatment in ex vivo and in vivo mouse models over a dose, dependent range from 3, 10 and 30 mg/kg. The study concluded that the 3 mg/kg of ponatinib was also sufficient to produce a prothrombotic phenotype, equivalent to 45 mg QD given to CML human patients.
The current safety measures were introduced in 2014 to counter ponatinib treatment’s adverse vascular effects in nilotinib and ponatinib treated patients. This will be a subject for future investigations. It is important to correlate the results from the single acute mouse model with chronically treated CML patients. Along with the safety measures were recommendations to reduce the amount of dosage from 45 to 30 to 15 mg QD for some patients to decrease dose-related prothrombotic risks. Ph+ refractory leukemias or CML patients using the drug may require concurrent anti-inflammatory or calcium channel anti-hypersensitive or antiplatelet medicines or anticoagulants that reduce reduction adverse vascular events in nilotinib and ponatinib treated patients in further investigation.
Chapter 6: THE EFFECT OF TKIs ON PLATELET AND ENDOTHELIAL ACTIVATION AND THROMBUS FORMATION IN CHRONICALLY TREATED CML PATIENTS
6.1 INTRODUCTION

There is an emerging occurrence in cardiovascular disease where the TKIs which are used in the treatment of patients with CML trigger events of arterial thrombosis and PAOD over time. While most CML patients who receive either imatinib or dasatinib for treatment have not shown any thrombotic side effects of such, there have been increasing reports of myocardial infarctions and PAOD on those who have received ponatinib or nilotinib treatment over time [281]. 12.5% of patients with CP-CML on nilotinib, as per a study by Kim et al, who used the treatment was reported to have developed PAOD [230].

Initial use of ponatinib was associated with increased incidence of severe arterial thrombotic events of 19%, thus requesting the need for careful patient selection, including consideration of co-morbidities and thromboembolic risks and dose reduction strategies, as the risk appeared to be dose dependent [282]. In addition to this, reports from different groups indicated that there was an increased occurrence of myocardial infarction and vascular adverse events on patients who were treated with 400 mg QD compared to those treated with 300 mg QD dose of nilotinib. This was indicated with a 12.5% and 6.1% occurrence rate on the respective doses [283]. Nilotinib therapy was reported to affect the lower limbs of the patients necessitating stent implantations, angioplasty and in severe cases, complete amputation [284]. However, incident reports of prothrombotic events are rare on patients receiving imatinib or dasatinib treatment when compared to untreated healthy patients [283].
The common events reported on patients during ponatinib or nilotinib treatment were associated with cardiovascular risk factors such as smoking, obesity, dyslipidaemia, and hypertension of the arteries [285]. There are heightened risks of vascular events on CP-CML patients who, through clinical trials, are switched from imatinib treatment to ponatinib or nilotinib therapy [282]. Other metabolic effects as a result of changing to nilotinib therapy included increased low density lipoprotein (LDL) and hypercholesterolemia [286].

One common cause of health issues that often lead to death in the western world today is arterial thrombosis. This can be associated with atherosclerosis caused by narrowing of blood vessels with the accumulation of fatty deposits and development of atherosclerotic lesions. Following rupture of atherosclerotic lesions, platelets stick to exposed thrombogenic proteins such as type I collagen to develop blood clots. Platelets have the ability to stick together and combine with the injured or diseased endothelium. This characteristic makes their activation an essential component for thrombus growth. This can better be clinically explained using the stages of formation of the atherosclerotic plaque. In the early stages of the atheroma process with narrowing of blood vessels with increased shear flow rate, platelets are activated [287]. They stick together and combine with the constituents of the ECM which have been exposed in arterial shear conditions. This gradually initiated the growth of local thrombus inside the walls of the blood vessels. An amplified arterial thrombosis is thereafter caused by an activated platelets in conjunction with activation and assembly of coagulation complexes.

According to various research studies, leukaemia treatment using anti-cancer therapy evidently affects the functioning of platelets. In some occasions, there is an increased aggregation of the platelets which end up creating pathologic thrombi [288]. Vast amounts of data obtained from
clinical trials indicate that there may exist other prothrombotic effects due to treatment using nilotinib, which is not evident with other TKIs. According to data, however, an adverse vascular risk profile does not fully explain the increased risk. This is because there is evidence of such events occurring on patients with low-risk profiles, contrary to expectations [289].

Hence, this study findings provides a first-hand demonstration, of how ponatinib or nilotinib affects the activation of platelets and endothelium to enhance thrombus formation in \textit{ex vivo} studies with CML patients. In contrast, the study shows how imatinib and dasatinib prevented the processes of platelet activation, aggregation and final formation of thrombus when administered to chronically TKI treated CML patients. The characteristics of these TKI-treated CML patients as shown in Table 4.

This study focuses on the effect of ponatinib and other TKIs on platelet and endothelial activation, pro-inflammatory cytokines and thrombus formation in both healthy humans and CML patients in order to determine the ability of TKIs to induce or inhibit a prothrombotic state.
Table 4. The characteristics of TKI-treated CML patients & normal human controls.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age years</th>
<th>Sex</th>
<th>Drug treatment</th>
<th>Platelets x 10^9/L</th>
<th>Medication</th>
<th>Vascular complications</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>67</td>
<td>M</td>
<td>Ponatinib 15 mg QD &amp; interferon</td>
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<td>Apixaban Amlodipine</td>
<td>PVD/CVA</td>
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<tr>
<td>2</td>
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<td>Irbesarten</td>
<td>Nil</td>
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<tr>
<td>3</td>
<td>83</td>
<td>F</td>
<td>Dasatinib 50 mg alt days</td>
<td>379</td>
<td>Clopidogrel Irbesarten</td>
<td>PVD/CVA</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>M</td>
<td>Nilotinib 300 mg QD</td>
<td>185</td>
<td>Aspirin</td>
<td>Nil</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>M</td>
<td>Dasatinib 100 mg QD</td>
<td>156</td>
<td>Irbesarten</td>
<td>PVD/IHD</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>M</td>
<td>Nilotinib 300 mg QD</td>
<td>186</td>
<td>Aspirin</td>
<td>Nil</td>
</tr>
<tr>
<td>7</td>
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<td>M</td>
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<td>Nil</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>M</td>
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<tr>
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<td>M</td>
<td>Nil</td>
<td>295</td>
<td>Nil</td>
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</tr>
</tbody>
</table>

PVD- prinzmetal’s variant angina (coronary artery vasospasm); CVA-cerebrovascular accident; IHD-ischaemic heart disease.
6.2 RESULTS

6.2.1 Assessment of ex vivo platelet thrombus growth in TKI-treated CML patients over type I collagen under arterial flow conditions

The effects of TKIs on human platelets were investigated further by comparing thrombus growth under arterial shear conditions by whole blood derived from CML patients treated with TKIs with blood from normal age matched healthy human donors (not on any antiplatelet or anticoagulant therapy). Rhodamine fluorescently labelled in whole blood were perfused over type I collagen at an arterial shear rate of 1800s\(^{-1}\) over a 6 minute time frame and real time imaging recorded.

As shown in Figure 70, blood from ponatinib or nilotinib-treated patients formed larger thrombi over time compared to blood from normal donors and from imatinib or dasatinib-treated patients. A Z-stack analysis undertaken at 2, 4 and 6 minutes of thrombus growth clearly showed that the thrombus height and area of blood from ponatinib or nilotinib-treated patients was significantly larger and thrombus volume was significantly increased when compared to blood from healthy donors (Figure 71).

By contrast, blood from patients treated with imatinib displayed a reduction in thrombus height, area and in thrombus volume when compared to the thrombi formed by blood from normal donors (Figure 71). Blood from dasatinib-treated patients showed a significant reduction in thrombus height, area and volume when compared to normal human donors, consistent with previous observations [238] (Figure 71).
Figure 70. The effect of TKI-treated CML patients on ex vivo thrombus formation and growth. Venous blood obtained from normal human donors or CML patients treated with ponatinib 15 mg QD, nilotinib 300 mg QD, imatinib 300 mg QD or dasatinib 100 mg QD prior to blood collection. Platelet count was normalised to 200×10⁹/L. Fluorescently-labelled platelets in whole blood from normal human donors or CML patients treated with ponatinib, nilotinib, imatinib or dasatinib were perfused through a Type I collagen-coated microcapillary (500 µg/mL) at a shear rate of 1800s⁻¹ for 6 minutes. Z-stack images were captured in real time with a digital Axiocam mRm camera (Carl Zeiss) and analysed with Zeiss Axiovision Rel4.6 software.
**Figure 71.** The kinetics of TKI-treated CML patients on *ex vivo* thrombus formation and growth. (A) Thrombus height, (B) Thrombus area (µm²) in real time was determined for thrombi formed on immobilised in type I collagen flow perfused with whole blood from healthy normal controls, ponatinib, nilotinib, imatinib or dasatinib treated CML patients. (C) Thrombus volume (µm³) over time was calculated from thrombus area (µm²)×thrombus height (µm) and determined at 2, 4, 6 minute time points for healthy normal controls, ponatinib, nilotinib, imatinib or dasatinib treated CML patients. 3D deconvolved reconstructions of thrombi formed were analysed for surface coverage of platelet aggregates (µm²), thrombus height (µm) and thrombus volume (µm³). Results shown are mean±SEM from three independent experiments. (*P<0.05, **P<0.01 and ***P<0.001) using unpaired student’s t test.
Collectively, whole blood derived from ponatinib or nilotinib-treated patients showed a significant increase in thrombus growth under flow conditions, which provided strong evidence that ponatinib and nilotinib treatment leads to an increased thrombus growth phenotype. By contrast, imatinib or dasatinib inhibited thrombus formation on immobilised type I collagen under *ex vivo* arterial shear flow rate. Taken together, these results indicate that the prothrombotic consequence of ponatinib or nilotinib treatment of CML patients is potentiated by the presence of the endothelium and priming of the platelets *in vivo*. 
6.2.2 Measurement of soluble P-selectin

P-selectin is translocated to the surface and is rapidly cleaved in vivo to create soluble P-selectin as a consequence of endothelial and platelet activation [290]. To examine whether ponatinib and other TKIs treatment altered plasma levels of sP-selectin in TKI-treated CML patients, the levels of sP-selectin were measured in patient plasma using sandwich ELISA. As shown in Figure 72, CML patients treated with ponatinib or nilotinib demonstrated a significant increase in plasma levels of sP-selectin, compared to normal control. By contrast, as shown in Figure 72, levels of sP-selectin in plasma derived from patients treated with either imatinib or dasatinib were decreased when compared to vehicle human control. Notably, the plasma levels of sP-selectin of normal control in this study is consistent with previous studies of random normal human donors (18.3-45.4 ng/ml) [291]. Based on these results, elevated plasma levels of sP-selectin in ponatinib or nilotinib-treated patients indicated that nilotinib induces the exposure of P-selectin in vivo, resulting in cleavage of P-selectin from activated platelets and/or endothelial cells leading to accumulation of circulating sP-selectin in patient’s plasma.
Figure 72. sP-selectin levels were determined in TKI-treated patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus human control. Venous blood was collected from CML patients chronically treated with various TKI’s. Plasma sP-selectin (ng/mL) levels of CML patients treated with ponatinib, nilotinib, imatinib or dasatinib TKIs was determined using commercial ELISA. Plasma samples were tested in triplicate and presented as mean±SEM comparing to control (**P<0.01; n=2) using unpaired student’s t test.
6.2.3 The effect of TKI-treated patients on soluble plasma IL-6, TNF-alpha, IFN-gamma sICAM-1 and sVCAM-1 levels.

Vascular endothelial cells and other cells including monocytes/macrophages produced TNF-alpha, IL-6, IFN-gamma, sICAM-1 and sVCAM-1 cytokines and secreted TNF-alpha is thought to be involved in an autocrine activation of endothelial cells with cell-bound TNF-alpha serving to activate target cells on endothelial cells [292]. Both TNF-alpha and IL-6 play an important role in the acute phase response, inflammation and infection [293]. In this study, ELISA test was used to measure the levels of cytokines in TKI-treated CML patient’s plasma. Whole blood was collected from TKI-treated CML patients and plasma by centrifugation. As shown in Figure 73, 74, 75, 76 and 77 both ponatinib and nilotinib treated-CML patients showed increased plasma levels of all cytokines compared to the normal control. By contrast, imatinib or dasatinib-treated patients showed a reduction in plasma levels of IL-6, TNF-alpha, IFN-gamma, sICAM-1 and sVCAM-1 compared to healthy normal controls. This result indicates that both ponatinib and nilotinib induced a pro-inflammatory response with platelet and endothelial activation and increased pro-inflammatory cytokines levels in chronically treated CML patients.
Figure 73. Measurement of IL-6 level in plasma derived from TKI-treated CML patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus normal human controls. Venous blood was collected from CML patients chronically treated with TKIs. Plasma IL-6 (pg/mL) levels of patients treated with ponatinib, nilotinib, imatinib or dasatinib vs healthy normal controls was determined using commercial ELISA. Plasma sample were tested in triplicate and presented as mean±SEM comparing to control (****P<0.0001; n=2) using unpaired student’s t test.
Figure 74. Measurement of TNF-alpha level in plasma derived from TKI-treated CML patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus normal human controls. Venous blood was collected from CML patients chronically treated with TKIs. Plasma TNF-alpha (pg/mL) levels of patients treated with ponatinib, nilotinib, imatinib or dasatinib vs healthy normal controls was determined using commercial ELISA. Plasma sample were tested in triplicate and presented as mean±SEM comparing to control (****P<0.0001; n=2) using unpaired student’s t test.
Figure 75. Measurement of IFN-gamma level in plasma derived from TKIs-treated patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus normal human controls. Venous blood was collected from CML patients chronically treated with TKIs. Plasma IFN-gamma (pg/mL) levels of patients treated with ponatinib, nilotinib, imatinib or dasatinib vs healthy normal controls was determined using commercial ELISA. Plasma sample were tested in triplicate and presented as mean±SEM comparing to control (****P<0.0001; n=2) using unpaired student’s t test.
Figure 76. Measurement of sICAM-1 level in plasma derived from TKI-treated CML patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus normal human controls. Venous blood was collected from CML patients chronically treated with TKIs. Plasma sICAM-1 (ng/mL) levels of patients treated with ponatinib, nilotinib, imatinib or dasatinib vs healthy normal controls was determined using commercial ELISA. Plasma sample were tested in triplicate and presented as mean±SEM compared to control (***P<0.001; n=2) using unpaired student’s t test.
Figure 77. Measurement of sVCAM-1 level in plasma derived from TKI-treated CML patients. Ponatinib-, nilotinib-, imatinib- or dasatinib-treated patients versus normal human controls. Venous blood was collected from CML patients chronically treated with TKIs. Plasma sVCAM-1 (ng/mL) levels of patients treated with ponatinib, nilotinib, imatinib or dasatinib vs healthy normal controls was determined using commercial ELISA. Plasma sample were tested in triplicate and presented as mean±SEM compared to control (**P<0.01; n=2) using unpaired student’s t test.
6.3 DISCUSSION

According to several research studies, ponatinib increased the adverse side effect of arterial thrombosis [294]. In addition, the administration of nilotinib over time causes development of PAOD possibly due its direct effect of vasospasm of the blood vessels. This is an effect which imatinib treatment has not displayed [295]. The induction of vasospasm maybe the reason as to why younger patients who have very low cardiovascular risk factors end up developing a PAOD form that is rapidly progressive over time.

This chapter sheds light on how the treatment of CML patients using ponatinib affects the functioning of platelets and endothelial cells. The first results of this study show that using intravital microscopy real-time imaging, it can be observed that unlike imatinib or dasatinib, ponatinib or nilotinib treatment significantly increased *ex vivo* thrombus formation on Type I collagen. In addition to this, results in this chapter confirmed that unlike the other TKIs, CML patients chronically treated with ponatinib and nilotinib increased the level of soluble plasma derived cytokines. It was evident that common cardiovascular risk factors significantly increased the chances of occurrence of vascular events. In addition, it confirmed the previous study of TKIs treated CML patients which showed increased of thrombus growth with nilotinib [238].

The *ex vivo* thrombus formation, as discussed in this chapter, was analysed using blood samples derived from healthy normal donors and CML patients chronically treated with ponatinib, nilotinib, imatinib or dasatinib TKI’s. This was an approach taken to correlate with TKI-treated C57BL/6 mice that received a single acute dose of ponatinib or nilotinib produced the same prothrombotic phenotype. The results showed that under *ex vivo* flow conditions, increased
adhesion of the platelets and thrombi formed on the fibrillar Type I collagen under arterial shear flow in ponatinib and nilotinib- treated CML patients. There was a two-fold increase in surface coverage of platelets, and the volume of the thrombus in CML patients who were chronically treated using ponatinib and nilotinib. However, in the \textit{ex vivo} adhesion of the platelets and thrombus growth, there was an evident limited thrombus formation on CML patients chronically treated using either imatinib or dasatinib TKIs. This was done in comparison to samples from healthy humans who acted as normal controls. Similar observations were also made in the fourth chapter of the study where C57BL/6 mice treated with ponatinib and nilotinib TKIs, showed significantly increased thrombus formation, unlike those under imatinib and dasatinib treatment, which was seen to act as inhibitory modulators of thrombus growth.

Results from the previous studies relate to our initial findings which showed that chronically treating CML patients with ponatinib and nilotinib increased under arterial flow \textit{ex vivo} thrombus growth Type 1 collagen. In contrast, treatment using imatinib and dasatinib evidently reduced thrombus formation. Together, both these findings show that there is a very close relationship between the effects of TKIs on a single acute dose TKI treatment of wild-type C57BL/6 mice and on chronically treated CML patients. Both subjects showed that the TKIs influenced their platelet adhesion and \textit{ex vivo} thrombus formation in real time under arterial flow conditions.

The uncontrolled accumulation of the activated platelets in situations of damaged endothelial or ruptured atherosclerotic lesions exposed type I collagen, triggering pathologic thrombosis and the resultant responses could include myocardial infarction, PAOD or cerebral ischaemic
stroke. This happens due to the leukocytes’ adhesion to the endothelium and the rolling that is mediated by P-selectin which enhances the condition of atherothrombosis and increased inflammation [296].

Various subclasses of cardiovascular diseases have described increased levels of soluble plasma P-selectin in vivo. In this chapter, the focus was to define in vivo endothelial and platelet activation by various biomarkers including sP-selectin, sICAM-1 and sVCAM-1. Plasma was isolated from CML patients who were treated using the four TKIs namely ponatinib, nilotinib, imatinib and dasatinib. Our data indicated that there was an elevated soluble plasma P-selectin levels in the plasma of CML patients who were treated using ponatinib or nilotinib. On the other hand, soluble plasma P-selectin levels showed no significant changes in CML patients chronically treated using imatinib or dasatinib. Most patients with PAOD have been reported to have high levels of sP-selectin in their plasma [297]. This was the same for those suffering from thrombosis, where sP-selectin is derived primarily from activated platelets and to a lesser extent activated endothelial cells [298]. The tethering role of GPIbα in capturing P-selectin on activated endothelium is clearly shown by the interaction between platelets and the endothelium [136]. The results of this chapter correspond to those of chapter four which showed that through cleavage of P-selectin and the resultant increase in soluble plasma P-selectin of wild-type C57BL/6 mice, ponatinib and nilotinib TKIs induced activation of the platelets and endothelium in vivo.

From recent observations, it has been confirmed that ponatinib and nilotinib increased production and secretion of pro-inflammatory cytokines. These TKI’s also increased release of soluble adhesion molecules including sICAM-1 and sVCAM from activated vascular
endothelium. These observations show that ponatinib and nilotinib have the potential to influence the formation of platelet thrombus and the release of adhesive molecules into the blood. There may be other contributory factors triggered by ponatinib or nilotinib TKI therapy that may also lead to the development of the pathological thrombi. It is however evident that ponatinib or nilotinib stimulates an event which is very important for platelet and vascular endothelial cell activation. Thereby predisposing to a prothrombotic inflammatory phenotype.

In conclusion, investigative studies on the effects of TKIs in CML patients on modulating the formation of arterial thrombus are highly warranted. This study, despite having a limited sample size provides substantial evidence on the potential role of ponatinib or nilotinib in inducing the formation of platelet thrombus, which leads to increase of thrombotic events with CML patients treated with TKIs, and increased secretion of sP-selectin, proinflammatory cytokines and soluble adhesion molecules levels which may potentiate atherogenesis via the global effects on physiological function of platelets and endothelial cells.
Chapter 7: GENERAL DISCUSSION
Tyrosine kinase inhibitors are a relatively new treatment for Chronic Myeloid Leukemia (CML) and have improved the 10 years survival rate of CML patients in the chronic phase to 80 percent \[299\]. The first TKI that was introduced in 2000 for treating CML was Imatinib (Gleevec) as a frontline therapy which revolutionised CML chronic phase treatment thereby improving survival outcomes and quality of life significantly. Nilotinib and dasatinib were developed as second generation TKIs for treatment to address issues of imatinib resistance and/or intolerance \[300\]. Despite the increase in survival rate of the patients who were treated using the TKIs like imatinib, issues have emerged regarding the use of TKIs to treat CML \[301\]. One of the issues arising is the increase in resistance to dasatinib, nilotinib, and imatinib. Due to the side effects and challenges linked to dasatinib, imatinib, and nilotinib, ponatinib which is a new third generation TKI inhibitor multi-kinase drug has been developed to deal with mutational resistance for T315I. Ponatinib can inhibit T315I Bcr-Abl mutant kinases selectively, especially those that are not sensitive to new Bcr-Abl inhibitors obtainable at clinics \[302, 303\]. Being a third generation TKI, ponatinib uses a distinct mode of interaction in comparison to the other TKIs because it binds with superior affinity compared to other TKIs \[304\]. However, it was initially withdrawn due to serious vascular adverse effects \[304\].

The main intention of this study was to evaluate the effects of other TKIs and ponatinib in both mouse and human models in vitro, ex vivo and in vivo to establish its capability to provoke a prothrombotic condition. Thus, this study gives numerous novel insights on the results of other TKIs compared to ponatinib on platelet and endothelial activation together with thrombus growth under physiological conditions of arterial shear. The findings have broadened the
comprehension of the effect of ponatinib on the function of platelets and the endothelium and have exposed potential mechanism used by ponatinib to stimulate a prothrombotic situation. Differences in the modulation by ponatinib that involve the formation of platelet thrombus in vivo have also been established. Furthermore, the influence of dasatinib and imatinib on the thrombus growth over time where the two TKIs have inhibitory consequences on the platelet:ECM and platelet:platelet interactions involved in thrombus formation in mice and humans.

Nilotinib and ponatinib enhance arterial thrombotic occurrence in blood vessels and the introduction of PAOD over time according to clinical studies [285, 305]. On the other hand, dasatinib and imatinib do not produce this outcome. The study gives an insight into the consequence of ponatinib as an activator of platelets in vitro and indicates one of the mechanisms which increases the risk of vascular events. This study showed that a single acute dose of ponatinib treatment contributed to the potentiation of platelet activation.

Compared to CRP and collagen, ADP is a known weak agonist in the development of platelet aggregation. In chapter three, ponatinib was shown to potentiate the ADP-mediated response of platelet aggregation. In addition, activation of platelets takes place in reaction to vessel injury which is caused by various receptors like thrombin, PAR-4, and thrombin receptors PAR-1. PAR-1 has been discovered to be the central thrombin receptor and has a high affinity on human platelets whereby it links to a separate sets of heterotrimeric G-proteins to control signaling pathway [306]. The PAR-1 mediated α-granule release in normal healthy platelets of donors is potentiated by nilotinib and ponatinib. Despite the mechanism being unclear, there is a possibility that it has off target effects that act on PAR-1 mediated α-granule pool secretion.
of P-selectin [307]. Therefore, this study demonstrates ponatinib as promoting aggregation of platelets in response to adenosine diphosphate (ADP) which was not seen with dasatinib, imatinib, and nilotinib. Besides, ponatinib and nilotinib were both shown to enhance exposure of platelet activation marker, P-selectin (CD62) from the alpha particle (α-granule) following PAR-1 stimulation, although there was no effect after stimulation of CRP and thrombin.

Once platelets have been activated, α-granules secrete their contents where P-selectin is exposed and quickly translocated to the surface of platelets [308]. It takes part in causing inflammation by mediating the interaction. Ponatinib is connected to the reactions to endothelial injury or stimulating vascular tissue and also enhances exposure of P-selectin which implies the activation of platelets. However, the release of α-granules was not enhanced in PAR-4 mediated activation of platelets. Previous studies have shown that human platelets have different α-granule pools that are; PAR-1 mediated α-granule which releases fibrinogen comprising pro-angiogenic proteins and vascular endothelial growth factor (VEGF) while PAR-4 mediated α-granule on the other hand, releases vWF-entailing anti-angiogenic proteins and Endostatin [309]. This explains the differences in the releases of platelets in α-granule protein pools in the two autonomous groups. It is well recognised that thrombin is the most potent physiological stimuli and has a prominent pro-inflammatory nature [310].

Nonetheless, ponatinib did not demonstrate any enhancing effect on the release of α-granule by thrombin-mediated exposure of activating platelets and this can be made clear by the fact that thrombin is associated with many receptors like PAR-4, platelet glycoprotein (GP)1b-IX-V receptor and PAR-1. It is important to note that GP1b binding spot for thrombin is abundant on platelet membrane; thus, full activation of platelets by thrombin is necessary.
In chapter three, the effect of TKI’s to modulate platelet:ECM and platelet: platelet interactions under arterial flow conditions was examined using both human and mouse model. Ponatinib's effect on the formation of thrombus under in vitro conditions of arterial shear flow had not been studied entirely unlike imatinib, dasatinib, and nilotinib in previous studies [238]. The study tested the ability of other TKIs versus ponatinib to modulate the growth of thrombus under in vitro arterial flow conditions by intravital microscopy which permitted the capture of real time images from C57BL/6 mouse and human samples. The experiments showed that nilotinib and ponatinib TKIs facilitated the growth of thrombus under in vitro arterial flow conditions on immobilised type I collagen over time tested at all doses. In contrast, dasatinib and imatinib reduced platelet adhesion and thrombus formation on immobilised type I collagen with the thrombus height, area and volume thrombus was significantly reduced at 4 and 6 minutes of perfusion when compared against vehicle control of whole blood.

The work done previously by Loren et al., appears to disagree with my results of in vitro thrombus formation that was stimulated by ponatinib [222]. The cause of this difference is not completely clear but it likely due to variation in test systems. These workers used a shear flow rate of 2200s⁻¹ on type I collagen and blocked the collagen-coated surface with denatured BSA while this study used arterial shear flow rate of 1800s⁻¹ on type 1 collagen without coating blocked by BSA. Another factor relates to solubilisation of ponatinib [222]. In this study, 25 mM citrate buffer pH 2.75 achieved maximal solubilisation of ponatinib. For this solubilisation it provides optimal bioactive ponatinib for stimulation of platelet activation and thrombus formation under arterial flow conditions.
A study by Gratacap et al., showed that CML patients treated with imatinib reduced thrombus formation significantly on immobilised type I collagen under physiologic arterial shear condition whereas patients treated with dasatinib formed smaller thrombi than imatinib. The similar group of research also accounted that blood from mice treated with dasatinib formed smaller thrombi on type 1 collagen under arterial shear flow conditions [47].

TKIs have a different impact on the thrombus platelet formation with emerging evidence of a direct effect of ponatinib producing vascular arterial occlusive events [311]. Furthermore, for CML patients, ponatinib and nilotinib appear to trigger PAOD over time in a dose-dependent manner [312]. In mice treated with ponatinib a single acute dose of 3, 10 or 30 mg/kg or 25 mg/kg of nilotinib, various methods were used to evaluate the development of thrombi in vivo in C57BL/6 mice, and they comprised evaluation after FeCl₃ vascular induced injury of both the carotid artery and mesenteric arterioles.

In chapter four, the potentiation of ponatinib versus the other TKIs was examined under ex vivo arterial shear flow rates to induce thrombus growth, was of interest in this chapter. Both ponatinib and nilotinib TKIs had potentiated a significant increase in thrombus growth over time as compared to vehicle control. In contrast, thrombus growth type 1 collagen was limited by dasatinib and imatinib treatment at 4 and 6 minutes of perfusion which was similar to vehicle control. Our results showed that a single acute dose of ponatinib of 3, 10 and 30 mg/kg or nilotinib 25 mg/kg enhanced thrombus ex vivo growth on mobilised type 1 collagen in contrast to vehicle control over time.
GPIb-IX-V, integrin α2β1, integrin αIIbβ3, CD9 and GPVI are the main glycoprotein receptors which exist on the surface of the platelets, and they play an important role in platelet regulation [313]. Dasatinib, however, does impair the platelet association with collagen and leads to downregulation of collagen GPVI/FcR γ chain ITAM coupled signaling pathway giving rise to bleeding events and limiting the growth of thrombus [314].

Since ponatinib treatment of C57BL/6 mice resulted in a prothrombotic phenotype, it was necessary to check if ponatinib modulated expression of platelet glycoproteins or the conformational change of integrin αIIbβ3 to an activated state in vivo. The studies showed that 3 mg/kg ponatinib did not affect platelet glycoprotein expression or modulate the agonist induced conformational change of integrin αIIbβ3 in vivo. The study demonstrated similar capability of binding to JON/A mAb from both treated and untreated C57BL/6 mice.

In chapter four either intravital microscopy or laser doppler flow probe to examine the effect of TKIs on FeCl₃ induced vascular injury of carotid artery or mesenteric arterioles in vivo. The findings indicated that mice that underwent nilotinib and ponatinib treatment increased the rate at which the thrombus growth occurred in vivo at six, eight and ten minutes. This study and others proved that mice treated with dasatinib or imatinib reduced thrombus growth [221]. Thus, this study emphasises that dasatinib and imatinib TKIs have the capacity to limit platelet thrombus growth in vivo.

Haemoglobin oxidation was induced by FeCl₃, resulting to haemolysis of erythrocytes producing endothelium denudation and as a consequence exposure of the ECM molecules
including type I collagen [315]. It is recognised that dysfunctioning of the endothelial cells plays a major role in arterial thrombus growth, linked with disfunctioning of platelet, the bond of engaged leukocytes, and promoting the proinflammatory cytokines release [316]. After platelet stimulation, adhesive proteins like CD40L and P-selectin are produced to support the platelets activation and enhance the arterial thrombus formation in vivo [290]. This study showed a reduction in thrombus growth after FeCl$_3$ induced vascular injury in C57BL/6 mice treated with dasatinib as compared to the sham control indicating a major variance in comparison to nilotinib, ponatinib or vehicle control.

In chapter four, my study showed that mice treated with dasatinib and imatinib, led to a delay in achieving 95% occlusion of the carotid artery when compared to vehicle control following induction of FeCl$_3$ injury of the carotid artery. The induction of FeCl$_3$ thrombosis involves adhesion and activation of platelets at site of vascular damage via contact with sub-endothelial adhesive proteins including type 1 collagen and vWF connected to their respective receptors. Nilotinib and ponatinib TKIs induced a prothrombic state in in vitro, ex vivo and in vivo after induction of FeCl$_3$ injury as shown by preceding studies whereas both dasatinib and imatinib TKIs limited thrombus development. In contrast, ponatinib and nilotinib potentiated thrombus growth over time.

Apart from the vasodilators such as calcium channel blockers regulating and making sure that there is enough blood flow, it is evident that the endothelium also plays a significant role [317]. The primary function of the vasodilators is to prevent the aggregation of the platelets [318]. When the vasodilator secretion increases, the anti-thrombotic and anti-aggregation of the platelets is achieved which in turn raises the activities of adenylate cyclase and guanylate

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cycloise which increase the levels of c-AMP and c-GMP. Apart from thromboxane A2, angiotensin II, ROS, and endothelin-1 are also vasoconstrictors that are secreted by the endothelium. Through the cell surface expression of thrombomodulin, the endothelial cells regulate coagulation by interaction with thrombin that leads to binding and activation of protein C to downregulate coagulation through inactivation of FVa and FVIIIa. [247].

The endothelial cells are indeed delicate, and this makes them susceptible to injury or disease which may affect wound repair processes. Detaching of the endothelial microparticles into the blood circulation system results from endothelial cell injury after a vascular inflammation [319]. The rise in the fragments of the endothelial cells, as well as endothelial dysfunction, are what is used to quantify the shedding of the endothelial microparticles [320]. Remarkably, hypertension is a core cause of dysfunctioning of the endothelium [321]. The blood vessels lose their capacity to vasodilate which is a way to identify if a person has hypertension [322]. Endothelial dysfunction leads to the lower accessibility of NO, and therefore the oxidative pressure escalates within the cells. Cardiovascular risk is linked with any disturbance experienced in the endothelium [323]. According to previous studies, the dysfunctioning of the endothelium can also be influenced by chronic inflammation [324].

Specific possible mechanisms of how ponatinib stimulates arterial occlusive events are being proposed. These include fibroblast growth factors receptor kinases (FGFR), the vascular endothelial growth factor receptor (VEGFR) and angiopoietin receptor (Tie-2) are potential targets of ponatinib TKI on multiple receptor pathways that may be capable of predisposing to vasospastic or hypertensive conditions. There is also the apparent association between thrombotic and proteinuria microangiopathy with the ponatinib TKI drug [325].
Ponatinib inhibition of VEGFR is vital because it reduces migration and the functioning of the HUVEC [312]. Moreover, to exert some anti-antigenic activity in HUVEC and zebrafish, ponatinib blocks VEGFR in a AKT and P13 kinase-dependent manner [326]. From these studies, there arose a question on the function of VEGFR pathway in preventing unfavourable vascular occurrences.

The use of either nilotinib or ponatinib to block the Ang/Tie-2 pathway could block the pro-inflammatory effects due to the up-regulation of the cytokine levels. Cell-to-cell interactions or cell-to-matrix can also result from the blockade, and they activate the platelets while at the same time progressing the vascular complications with time. A retrospective CML's patient study at MD Anderson showed that 2.1% vascular events of patients treated with nilotinib were linked with vasospasm and thus lacking macro evidence of atherosclerotic lesions [327]. Higher doses of nilotinib to 400 mg QD as compared to the former 300 mg QD heightened the vasospasm influence [230]. According to the early acute vascular effects, the cause of vasospasm is seen to be nilotinib, ponatinib or possibly dasatinib therapy [327]. However, research has shown the chronic effects of TKIs over time of amplified atherosclerosis, which primarily affects patients with cardiovascular risk factors [328]. Interestingly, ponatinib TKI has been shown to have the capacity to attenuate vasorelaxation and vasoconstriction of mouse aortic rings in vitro [231]. COX-2 inhibitor, diclofenac, eNOS synthase inhibitor, L-NNA as well as CCB, nifedipine were able to reverse this process and these observations suggested that vasoconstriction prostanoids are associated with ponatinib treatment [231].

While COX-1 and COX-2 share about 65 percent of homology, the two COXs are quite diverse in function [329]. For COX-1, fatty acids and arachidonic acid are the chosen substrates while
2-arachidonoyl glycerol and fatty acid are the preference for COX-2 [330]. Even though COX-2 requires secondary absorptions of hydrogen peroxide than its counterpart, both need lipid peroxides for activation [331]. Therefore, in the presence of COX-1, COX-2 can be used to treat the conditions that cause inflammation without being stimulated.

It is through the smooth vascular muscle cells and the endothelial cells, that the blood vessel usually operates by expression of COX-1 and COX-2 with the latter being the major isoform. COX-1 contributes to the production of PGs while the COX-2 is attributed majorly with the generation of prostacyclin in human beings [332]. Within the cardiovascular system, thromboxane A$_2$ purposes as an accumulating factor which helps in the vasoconstriction in its production from COX-1 in platelets as well as the endothelial cells [333]. Although the inducible synthesis is never affected, the interruption of COX-1 can lead to the reduction of the integration of PG and in the blood vessels, prostacyclin is considered to be a potent vasodilator and an anti-aggregating cause [334]. It is arguable that COX-2 deletion phenotype is further severe because of its close association with an impaired inducible synthesis of PG [335]. Also, in the endothelium, prostacyclin is the main arachidonic acid metabolite [336].

Before the exposure to TKI, the interesting part of this study was to test if the prothrombotic state could be ameliorated by pretreatment with the use of either L-type calcium channel blocker, diltiazem, COX-2 inhibitor, diclofenac or eNos synthase inhibitor, L-NNA. The pro-inflammatory and prothrombotic phenotype could be reversed by pretreatment with the calcium channel inhibitor, diltiazem prior to nilotinib or ponatinib treatment as shown in this study. According to the evidence produced, a vasospastic effect may be produced by nilotinib and
ponatinib as this could trigger dysregulation or aid towards endothelial activation. However, platelet and endothelium are activated as a result of the impact on the blood vessel wall.

Diltiazem belongs to the benzothiazepine class of calcium channel blockers. It has the capacity to enhance vasodilatory tone by inhibiting calcium influx into vascular smooth muscle cells and increasing by endothelial eNOS expression. Diltiazem also increases the production of endothelial derived NO which would explain the potent inhibition of thrombus growth potentiated by either ponatinib or nilotinib TKIs. NO is a known potent bioactive inhibitor of platelet activation released from activated endothelial cells.

Consistent with this prothrombotic finding of ponatinib dose, any dose of ponatinib even as little as 15 mg QD can result in some severe vascular consequences [281]. Ponatinib treatment in ex vivo and in vivo mouse models are examined in this study over a single acute dose range between 3, 10 and 30 mg/kg. The conclusion of the study showed that prothrombotic phenotype could be produced by 3 mg/kg of ponatinib which equated to 45 mg QD applied to CML human patients. In 2014, there was introduction of protective actions to reduce the adverse vascular effects of ponatinib treatment. This included a reduction in prothrombotic risks related to the dose, lowering the dose intake from 45 to 30 to 15 mg QD. Patients with CML or Ph \(^+\) refractory leukemia use drugs that may expose them to a thrombo-inflammatory phenotype over time. Therefore, to lower the risks associated with these medicines and to lower vascular occurrences with nilotinib and ponatinib, investigating the use of anti-inflammatory or calcium channel anti-hypersensitive or anti-platelet measures should be considered.
The adverse events (AEs) of the arterial thrombotic status are said to be increased by ponatinib TKI as shown by numerous clinical studies [282]. Additionally, incidents of PAOD with the use of nilotinib overtime as a direct consequences on vasospasm of the blood vessels [295]. The effect was not observed by imatinib treatment but vasospasm has been reported with dasatinib use [337]. The reason above explains why young patients with low cardiovascular risk end up getting a form of PAOD that progresses quickly over time.

Therefore, this study intends to bring to light how treating patients with ponatinib influences the way platelets and endothelial cells function. Using intravital microscopy real-time imaging according to the first findings of the study, it can be noted that using nilotinib and ponatinib for treatment increased of ex vivo thrombus formation on type I collagen, unlike dasatinib and imatinib. Furthermore, the results according to this study proved that using nilotinib or ponatinib treatment increased soluble cytokines levels in chronically treated CML patients which are different from other TKIs. It was clear that common cardiovascular risk factors significantly increased incidence of vascular events. However, arterial vascular events were reported on CML patients who exhibited low-risk factors indicated the direct effect of ponatinib and nilotinib treatment [338].

The ex vivo thrombus formation as discussed in this study was examined blood samples from healthy normal donors, compared to chronically TKI-treated CML patients receiving dasatinib, imatinib, nilotinib, or ponatinib therapy. This approach was performed to compare results of chronically treated CML patients using TKIs or with a single acute dose of TKI treatment in a mouse C57BL/6 model. Under ex vivo flow conditions, the interaction between platelets with
type I collagen was enhanced in thrombus height, surface coverage (area) and thrombus volume in CML patients who had undergone nilotinib or ponatinib treatment.

On the other hand, in the ex vivo interaction of the platelets and type I collagen, there was inhibition in the thrombus formation over time on patients who have are treated with either dasatinib or imatinib TKIs compared to healthy human controls. Similar observations were made in chapter four of this study where nilotinib or ponatinib TKIs treated C57BL/6 mice, displayed great potential for thrombus formation unlike those being treated with dasatinib and imatinib, which acted as inhibitory modulators of thrombus formation and growth.

Importantly this study highlighted that single acute dose of ponatinib or nilotinib in C57BL/6 correlated with a prothrombotic phenotype demonstrated in chronically treated CML patients. In contrast, dasatinib and imatinib treatment reduced thrombus formation. The two findings showed a close correlation between the outcomes of TKIs on human beings and wild-type mice where the two subjects indicated that TKIs affected platelet: ECM and platelet: platelet interactions to modulate and thrombus formation over time under arterial flow conditions.

The unrestrained buildup of the activated platelets in circumstances of injured endothelium or ruptured atherosclerosis lesions in blood vessels where increased levels of type 1 collagen occur, can induce pathological thrombosis and which may result in myocardial infarction or cerebral ischaemic stroke. During this process, leukocytes interact with the endothelium, as a consequence of leukocyte rolling mediated by P-selectin which increases the inflammatory response and may predispose to atherothrombosis [339].
A variety of subclasses of cardiovascular diseases have showed increased levels of soluble P-selectin in plasma as a consequence of endothelial and platelet activation. In this study, plasma was obtained from chronically TKI treated CML patients on either dasatinib, imatinib, ponatinib, and nilotinib. My study showed that higher soluble plasma P-selectin levels were present in CML patients chronically exposed to nilotinib and ponatinib treatment. Nonetheless, there were no significant changes in patients on dasatinib or imatinib treatment compared to healthy controls.

The role of GPIbα of tethering to capture P-selectin on endothelium that is activated is shown in the interaction between the endothelium and platelets [340]. These results match up with the ones from chapter four which indicated that P-selectin cleavage with subsequent rise in soluble P-selectin plasma levels in wild-type mouse treated with either nilotinib or ponatinib highlighted platelet and endothelial activation in vivo. Based upon on these results, the rise in sP-selectin plasma levels in TKI treated mice or human CML patients, the activation of platelets and endothelium in vivo is influenced by nilotinib and ponatinib TKI therapy. This study highlighted that nilotinib and ponatinib TKIs increased secretion and production of cytokines known to be pro-inflammatory. This study demonstrated that nilotinib and ponatinib have great potential to influence platelet thrombus formation and secretion of soluble adhesion molecules, sVCAM-1 and sICAM-1 and pro-inflammatory cytokines (IL-6, TNF-alpha and IFN-gamma).

7.1.1 CONCLUSION

To conclude, chronically treated CML patients on ponatinib therapy is linked with augmented rates of atherothrombotic occurrences as evidenced by clinical data. Various effects and
insights of different TKIs on the role of platelet profiles resulting from human blood and murine samples have shown by this study. Imatinib and dasatinib treatments have inhibitory effects on platelet aggregation as proved by experiments in my study and from studies in other laboratories on platelet granule exocytosis and in vitro thrombus formation [237]. Ponatinib and nilotinib potentiated platelet alpha-granule release (P-selectin exposure) selectively as depicted by the studies in chapter three in reaction to PAR-1 in the human platelets and this implies that nilotinib and ponatinib have the ability to potentiate agonist induced platelet activation.

What is confirmed in the study is the possible association of ponatinib treatment for both human and murine platelets on platelet type I collagen in the enhancement of in vitro thrombus growth. Both nilotinib and ponatinib TKIs potentiated the growth of arteriolar thrombus growth over time. These TKIs modulated the platelet activation, adhesion and aggregation steps of thrombus formation. This may explain why ponatinib or nilotinib TKIs have the capacity to increase thrombus formation in settings of atherosclerosis and vascular disease.

Novel insights on how the formation of an arteriolar thrombus is influenced by TKIs via various models in vivo have also been highlighted. After a single acute dose of ponatinib or nilotinib selectively produced a prothrombotic state in vivo in the context of different vascular beds. Analysis of formation of platelet thrombus included FeCl₃ induced vascular injury of either mesenteric arterioles (80-100 µm in diameter) or of the carotid artery.
The study concluded that ponatinib 3 mg/kg was sufficient to reduce a prothrombotic phenotype which is equivalent to CML patients receiving 45 mg QD. In order to counter the prothrombotic effects of ponatinib treatment, altered measures were introduced in 2014. Recommendations were made together with safety actions to lower the amount of dose from 45 mg QD to 15 mg QD to reduce the dose-related prothrombotic effect for CML treated patients. Typically, these patients with CML and Ph+ refractory leukemia and receiving drug treatment with ponatinib developed a thrombo-inflammatory phenotype over time. To minimise these adverse effects of ponatinib TKI or nilotinib and lower the vascular events on patients treated with these TKIs, there is a possibility to implement the use of anti-inflammatory or anti-platelet drugs, or calcium channel blockers or direct oral anti-coagulant drugs.

Reducing the side effects of arterial thrombus development in ponatinib or nilotinib treated CML patients is warranted. This study provided substantial evidence on modulatory function of ponatinib or nilotinib to enhance the development of thrombus formation. It indicates a rise in the occurrence of thrombosis in patients with CML and who are treated with TKIs could potentially occur because of the increasing of levels of sP-selectin and cytokine secretion from endothelial cells and platelets. Based on these results, ponatinib and nilotinib TKIs have a prothrombotic effect that leads to thrombotic complications which can be reversed by pretreatment with L-type calcium channel blocker, diltiazem and by COX-2 inhibitor, diclofenac and to a lesser extent eNOS synthase inhibitor, L-NNA.

7.1.2 FUTURE DIRECTIONS

Data obtained from this study was from a small group of chronically treated CML patients treated with either ponatinib, nilotinib, imatinib or dasatinib therapy and thus a significant
constraint is the patient number. Further studied should explore the relationship between raised blood glucose and levels of xLDL and the thrombogenic potent in patients undergoing through the TKI treatment over time.

The ponatinib dose of 15 mg QD is currently administered in haematology clinics to CML patients or 300 mg QD nilotinib or 400 mg QD imatinib or 100 mg QD dasatinib. However, in research experiments involving animals, an acute single dose of TKIs was given orally to C57BL/6 mice to examine thrombus growth equated with the same dose given to CML patients. Thus, the requirement of advanced studies to analyse the TKIs effects on various mechanisms of thrombus growth and stability in a mouse model with bcr-abl Ph+ treated using TKI therapies is warranted.

The fact that the CCB diltiazem was able to reverse the prothrombotic and pro-inflammatory phenotype in a C57BL/6 mouse model it would be valuable to apply the CCB in human clinical trials. A clinical trial in nilotinib-treated or ponatinib treated CML patients with treatment arms of nilotinib or ponatinib alone versus treatment with calcium channel blocker, diltiazem in conjunction with TKI therapy. In addition, nilotinib or ponatinib-treated CML patients who develop vascular complications of coronary artery vasospasm or cerebrovascular accident could be treated in conjunction with L-type calcium channel blocker to reduce arterial occlusive events.

Further studies should examine the effect of other anti-platelet agents including ticagrelor or clopidogrel (P2Y₁₂ antagonists) or new anti-coagulants such as apixaban and rivaroxaban (FXa
inhibitors) to see whether the acute effects of ponatinib or nilotinib could be prevented. In addition, examine the effect of statins which seem to ameliorate a proinflammatory state.
Chapter 8: REFERENCES
References


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