INHIBITION OF ERYTHROCYTE BAND 3 TYROSINE PHOSPHORYLATION: CHARACTERIZATION OF A NOVEL THERAPY FOR SICKLE CELL DISEASE AND MALARIA

by

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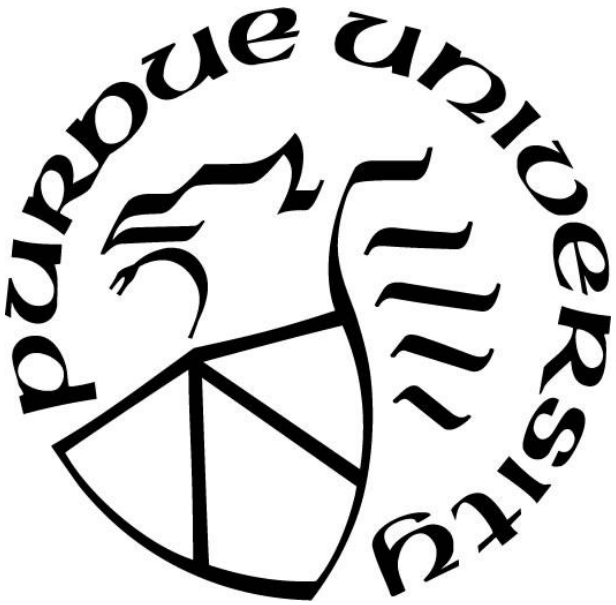

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ACT	Artemisinin combination therapy
CCH	Cincinnati Children's Hospital
CM	Complete medium
DHA	Dihydroartemisinin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	U.S. Food and Drug Administration
Hb	Hemoglobin
Hb A	Healthy Adult Hemoglobin
Hb F	Fetal Hemoglobin
Hb S	Sickle Hemoglobin
Hct	Hematocrit
HPMECs	Human pulmonary microvascular endothelial cells
HUVECs	Human umbilical vein endothelial cells
IC_{50}	Half maximal inhibitory concentration
Im	Imatinib
IRB	Institutional review board
iu	International units
MetHb	Methemoglobin
MFI	Mean fluorescent intensity
MP	Microparticle
MPs	Microparticles
OV	Sodium orthovanadate
PBS	Phosphate buffered saline
PBS-G	Phosphate buffered saline supplemented with 5 mM glucose
PPQ	Piperaquine
PTKs	Protein tyrosine kinases
PTPs	Protein tyrosine phosphatases
PTP1B	Protein tyrosine phosphatase 1 B
RBC	Red blood cell
RBCs	Red blood cells
iRBCs	*Plasmodium falciparum*-infected red blood cells
Rcf	Relative centrifugal force
ROS	Reactive oxygen species
Rpm	Revolutions per minute
SCD	Sickle cell disease
SCD-T	Transfused sickle cell disease sample
SEM	Standard error of the mean
SHP-1	Src homology region 2 (SH-2) domain-containing phosphatase 1
SHP-2	Src homology region 2 (SH-2) domain-containing phosphatase 2
SOC	Standard of care

SOD	Superoxide Dismutase
TBST	Tris buffered saline containing tween-20
U.S.	United States of America
W.H.O.	World Health Organization

ABSTRACT

While the molecular defect that cause sickle cell disease has well been established, the cause of vaso-occlusive crisis remains elusive and largely debated upon. Majority of studies have linked the painful episodes to polymerization of sickle hemoglobin following its deoxygenation. The variability of the disease symptoms among patients, compounds efforts for a holistic therapy. Hydroxyurea, a stimulator of Hb F induction and a widely used treatment, has ameliorated the complication of SCD but it is only effective in 50% of the patients. Expression of Hb F lowers the content of Hb S in blood and hence reduces oxidative stress caused by Hb S denaturation. Sickle cell disease severity depends on several factors. Most importantly, the ability of red cell to sickle dominates all other determinants. While deoxygenation of sickle hemoglobin may be inevitable, the duration with which the red cell remains in the deoxygenated state can be manipulated. Deoxygenation is a transient process that when compared to the time taken to develop the long filaments of deoxyhemoglobin to causes severe sickling, the red cell would have been cycled back to the lungs and re-oxygenated to restore the healthy conditions of the cell. In fact, if sickle cells would flow as fast as healthy erythrocytes, the detrimental impacts of sickling such as vaso-occlusive crisis, would not be a concern for this disease. Unfortunately, the unstable sickle hemoglobin undergoes denaturation through auto-oxidation, which imposes oxidative stress to the cells. The oxidative stress inhibits erythrocytes tyrosine phosphatases, a course which subsequently impair their constitutive action against the tyrosine kinases. In the end, a net tyrosine phosphorylation state in the red cell membrane proteins, most notably the transmembrane protein band 3, succeeds. Band 3 tyrosine phosphorylation abrogates the protein's interaction with ankyrin and spectrin-actin cytoskeleton, hence the cytoskeleton loses its major anchorage to the membrane thus engendering membrane destabilization. A destabilized erythrocyte sheds membrane fragments in form of microvesicles/microparticles and discharges free hemoglobin into the extra cellular matrix. In consequence, the microparticles power initiation of coagulation cascade through activation of thrombin, while free Hb inflicts inflammation, scavenges nitric oxide which is necessary for vasodilation and induces further oxidative stress within the microvasculature, and activates expression of adhesion receptors on the endothelium. Taken together, these events culminate in entrapment of red cells (not naming leucocytes and platelets) in the microvasculature, blockade of blood vessels and further damage of erythrocytes through prolonged deoxygenated

state thus terminating in tissue injury, strokes, and organ damage, amid vaso-occlusive episodes which always require hospitalization and extensive medical care for survival. Band 3 tyrosine phosphorylation and membrane weakening is not unique just to SCD, but also a druggable target for malaria. Malaria, a disease that is touted as the evolutionary cause of sickle cell disease, surprisingly thrives through the same mechanism. Briefly, malaria parasite consumes hemoglobin for its DNA synthesis, and in the process generate reactive oxygen species from denatured hemoglobin that feeds into the oxidative stress which triggers band 3 tyrosine phosphorylation. In this case however, a destabilized membrane offers perfect conditions for merozoites' (malaria daughter parasites) egress/exit out of the cell to begin infecting other red cells. Ultimately, the ensuing anemia and organ dysfunction leads to patient's death. Treatment of diseased cells with imatinib and other Syk inhibitors effectively reversed membrane weakening. A stabilized membrane not only survives longer in circulation to alleviate SCD symptoms but also traps and starves malaria parasite leading to termination of the parasitic infection. With band 3 tyrosine phosphorylation at center stage, this dissertation explores the above events in an effort to unveil a novel therapy for sickle cell and malaria diseases. First, the therapeutic strategy regarding SCD is discussed in detail beginning with non-transfused patients and ending in additional mechanistic study on inactivation of the principal erythrocyte's protein tyrosine phosphatase 1 B, PTP1B. The dissertation then provides an initial proof of concept on efficacy of imatinib in treatment of malaria as a monotherapy and its efficacy when used in a triple combination therapy with the standard of care treatment. Finally, I outline an alternative possible mechanism of action of quinine against malaria.

CHAPTER 1. A REVIEW OF SICKLE CELL DISEASE, MALARIA AND THEIR LINKAGE

1.1 Abstract

Sickle cell disease (SCD) is a hereditary monogenic hemoglobin disorder that affects millions of people worldwide, majority of which belong to the African ancestry. It is caused by a point mutation in the β-globin chain of adult hemoglobin whereby a glutamic acid in the 6th position is replaced with valine. Under deoxygenated state of erythrocytes, the non-polar amino acid, valine, introduces a hydrophobic interaction between beta globin chains of different hemoglobin molecules resulting in extensive polymerization of hemoglobin within red blood cells. The polymers change erythrocyte's morphology from the normal biconcave/discoid shape to various shapes predominated by a sickle/crescent morphology. Unlike healthy adult hemoglobin (Hb A), sickle hemoglobin (Hb S) is unstable thus prone to auto-oxidation reactions that impose oxidative stress to the cell and its milieu. Sickle cell disease is characterized by poor blood rheology, which is the major cause of pain and organ failure. Since its discovery, treatment efforts of SCD have largely relied on disease management/modifying regimens to alleviate pain and extend lifespan of patients. Of the many therapies at play, hydroxyurea stood out as the most impactful treatment option hitherto. In fact, the advent of hydroxyurea therapy, has significantly improved patients' lifespan in the last four decades to ~45 years in the United States of America where the drug is widely used. However, the patients' quality of life remains largely compromised due to relentless painful episodes and the inevitable progressive organ failures. While gene therapy and bone marrow transplant approaches are making good progress and touted as breakthroughs for a curative therapy for SCD, their costs and high expertise required to administer may limit the number of patients that can access this treatment.

1.2 Introduction

Sickle cell disease falls under a large umbrella of hemoglobinopathies (diseases that affect blood) that, in several ways, continue afflicting irreversible, and often-intolerable damages to patients throughout the world. In a general definition, hemoglobinopathies are characterized by defect(s) in the hemoglobin which in turn affect the normal function and/or viability of

erythrocytes; the hemoglobin carrying cells within the animal kingdom.[1] Besides sickle cell disease, thalassemia is the next major hemoglobinopathy. Thalassemia is caused by an imbalance of the alpha and beta globin chains of hemoglobin.[2] Other hemoglobinopathies include, but not limited to, vein thromboembolism, hemophilia, hereditary hemorrhagic telangiectasia and von Willebrand disease. In similarities, these disorders have a common functional miscue of insufficient supply of oxygen to the overall patient's body. Sequentially, the deficient oxygen delivery to tissues/organs opens an array of lifelong and often incapacitating episodes of pain that can suddenly turn fatal. In contrast to other hematologic disorders, malaria is caused by a parasite invasion and replication within erythrocytes leading to destruction of a significant number of red cells which also leads to insufficient oxygen delivery to tissues. This chapter will focus on a general review of sickle cell disease and malaria alongside principles bridging the two diseases.

Sickle cell disease (SCD) is a global problem that impacts the well-being of patients, their families, and local communities.[3–7] Approximately 300,000 infants are born each year with SCD, with ¾ of the births occurring in sub-Saharan Africa[6,8] and only a fraction of them are expected to survive past young adulthood.[8–11] This high mortality rate in Africa is attributed to multiple factors including a lack of universal newborn screening for early SCD detection, a dearth of therapeutic options to mitigate disease consequences,[12–14] and infections[15–17] such as pneumonia, meningitis, and malaria that may kill patients with SCD due to compromised splenic function.[6,16,18,19] While therapeutic interventions[10,12] are readily available in most developed countries, life expectancy is still only ~45 years[18] and the quality of life remains compromised due to frequent vaso-occlusive crises, progressive organ damage, recurring strokes, chronic pain, and addiction to narcotics that arises from efforts to relieve pain.[7,20–22] With ~100,000 people still suffering from SCD in the USA,[23,24] the need to expand and optimize therapeutic options remains critical.

On the other hand, malaria affects 228 million people with a fatal case load of 405,000 reported in 2018 alone.[25] Again, majority of the incidence and death cases arise from Africa where *Plasmodium falciparum* relentlessly continue with its seemingly uninterrupted existence despite availability of effective treatment alternatives. Regrettably, this disease is unproportionally deadly to the young with over 67% of total deaths coming from children under 5 years of age.[25]

While sickle cell disease may not be eliminated easily due to its hereditary/genetic nature and ethical concerns surrounding elimination attempts, malaria undoubtedly warrants eradication on the face of earth. Elimination efforts are ongoing, but their success is being hampered by rising

resistance to nearly all anti-malarials (including the once mighty artemisinins[26,27]) and emergence of insecticide resistant mosquitoes, notwithstanding logistical constraints in indigent endemic territories. Significant gains have been made so far but the present indicators of success are disheartening since the precipitous decline in malaria cases from 2010 to 2014 has plateaued, suggesting a necessity for a change of tact.[25] To further illustrate the complexity and perilous nature of malaria, the outbreak of 2019 novel coronavirus (COVID-19) pandemic has been projected to reverse ~20 years of gains on the fight against malaria parasite.[28,29]

Malaria is one of the oldest known human disease and it has been documented to be the major evolutional cause of sickle cell disease. Although it was meant to make humans less susceptible to malaria deaths, the genetic mutation gave rise to an even deadlier disease, sickle cell anemia. Nonetheless, the evolutional mutation resulted to an additional benign form, the sickle cell trait, which is resistant to malaria.[30–32]

This chapter will explore the pathogenesis of sickle cell disease, burden, and the current treatment options for the disease. It will also provide a succinct overview of malaria with regards to pathogenesis and therapeutic strategies. In the end, a link between the two diseases and an eventual similar treatment scheme would be presented.

1.3 Pathogenesis of sickle cell disease

1.3.1 Polymerization of sickle hemoglobin

Sickle cell disease is caused by a point mutation in the beta-globin chain of hemoglobin where glutamic acid (polar amino acid) is replaced with valine (a non-polar amino acid) to produce sickle hemoglobin. The hydrophobic effect of valine turned out to be the key element during deoxygenation state of the defective erythrocytes whereby it causes a hydrophobic interaction between two β-globin chains of different hemoglobin molecules.[33–35] The hydrophobic motif could eventually develop into long fibers/filaments in a series of steps (Figure 1.1: steps 1-2) which in turn further interacts with each other in a twisted conformation (Figure 1.1: step 3) thus imposing alterations on erythrocytes' membrane.[34] The polymerization of hemoglobin leads to a change in morphology of red cells (RBCs) from the normal discoid shape to crescent like (sickled) shape. This cell deformation triggers a concatenation of events whose feedback is detected clinically/symptomatically in form of vaso-occlusive crisis, acute chest syndrome, anemia,

jaundice, among other disease features.[36] Vaso-occlusive crisis, the hallmark of SCD, consist of incessant episodes of pain resulting from deficient oxygen delivery to tissues/organs and ischemia-reperfusion injury.[37,38]

The end product of polymerized Hb S can be reversed back to the healthy monomers of hemoglobin when the blood is reoxygenated.[38] However, repeated cycles of sickling yield irreversibly polymerized Hb S that permanently distort the cells rendering them rigid and fragile hence prone to membrane damage or hemolysis under any oxygen saturation state.[35,39,40] Hemolysis may result from physical trauma of the fragile red cells within its vascular cycling journey from and back to the lung or from oxidative stress conditions within the cells.[41] Hemolysis not only cause anemia due to loss of blood cells, but also produce excessive amount of free hemoglobin and heme. Free hemoglobin consumes nitric oxide, a vascular tone regulator, which further exacerbate overall blood rheology due to defective vasodilation, thus impairing oxygen delivery to tissues.[42,43]

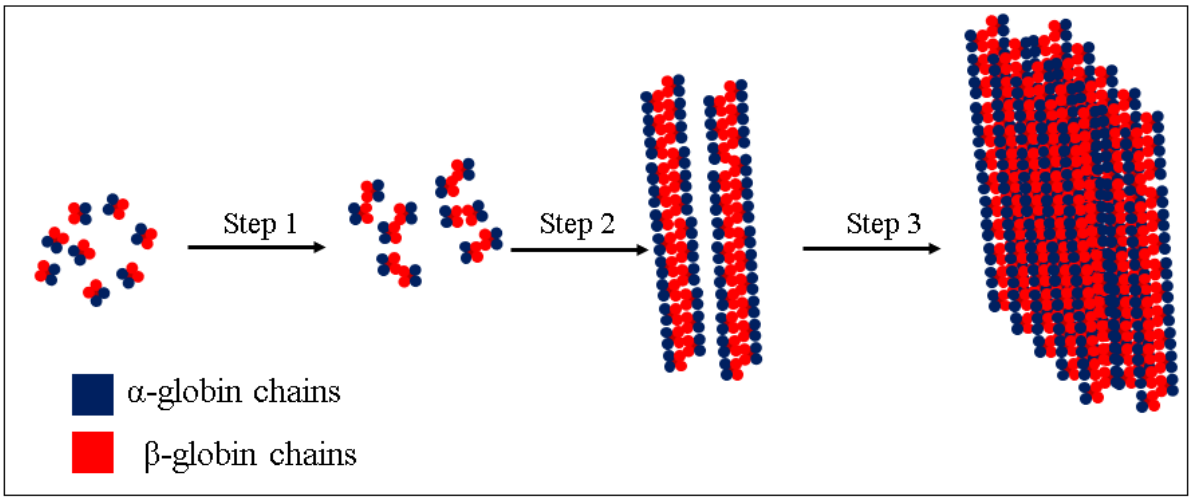


Figure 1.1 Molecular representation of sickle hemoglobin polymerization. Step 1: initiation of sickling, step 2: development of hemoglobin fibers and step 3: long sickle hemoglobin filaments interacting with each other.

1.3.2 Dehydration of sickled erythrocytes

Intracellular concentration of Hb S in red cells is a critical element with regards to Hb S polymerization and its downstream effects. During sickling or decreases in cellular pH, efflux of potassium ions (K^+) is initiated alongside a concomitant loss of water from the cell which

ultimately yield denser cells. In the long run, permanently sickled erythrocytes are generated. Since irreversibly sickled cells cannot assume their normal flexibility/shape even under fully oxygenated conditions, they are prone to entrapment within microvascular flow.[34] In fact, irreversibly sickled erythrocytes have been reported to be increased prior to, and reduced during/after vaso-occlusion events, suggesting initiation of occlusion, and entrapment/lysis of the cells respectively.[44]

Mean volume of erythrocytes is regulated by two prominent cation channels, viz; potassium chloride (K-Cl) cotransport[45,46] and calcium activated potassium channels (Gardos channel).[47,48] The K-Cl cotransport is activated by acidosis and cell swelling, while the Ca^{2+} activated K^+ channel is turned on by high intracellular Ca^{2+} concentration. In SCD, Ca^{2+} influx is aided by deoxygenation or sickling-induced permeation of the ions. To maintain cell's electrical neutrality, K^+ ions are concomitantly lost as more Ca^{2+} accumulate within the cell.[44] Another cation channel which contributes to dehydration but to a lesser extent than the preceding channels, is the cation sensitive PIEZO1 channel. The channel is activated by cation fluxes during deoxygenation condition as well as its sensitivity to cell mechanical alterations.[49,50] Table 1.1 below summarizes the cation channels that are responsible or partly activated for dehydration of sickle cells.

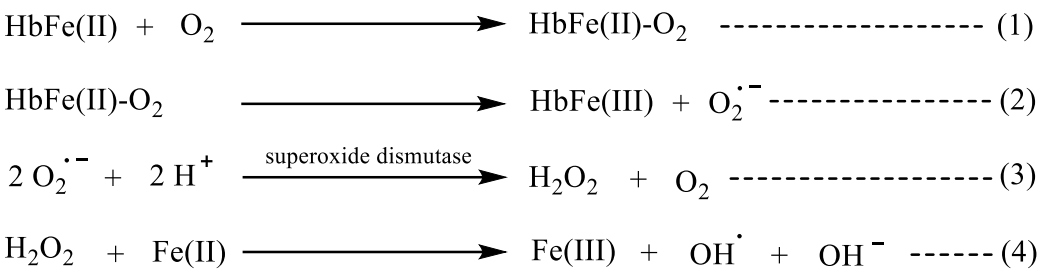
Table 1.1 Cation channels responsible for dehydration of sickle cells

Channel	Condition for activation	Permeant Cations
K-Cl Cotransport (KCC)	Acidosis and cell swelling	K^+, Cl^-
Ca^{2+}-activated K^+ channel (KCCN4)	Influx of Ca^{2+}/increased intracellular Ca^{2+} concentration, deoxygenation	K^+
Deoxygenation-induced (P_{sickle} or PIEZO1)	Sickling	K^+, Na^+,Rb^+, Ca^{2+}, Mg^{2+}

1.3.3 Oxidative stress

Reflecting the widely variable morbific pathways of SCD, oxidative stress is one other major pathogenic route of this hematologic disorder. Since Hb S is unstable, it undergoes accelerated auto-oxidation reactions that initiate oxidative stress within the red cells.[51] In this pathway, a series of reactive oxygen species (ROS) such as superoxide ions, hydroxyl radicals, hydrogen peroxide and high oxidation state iron Hb radicals are produced. These oxidative

reactions products cause lipid peroxidation and oxidation of proteins/enzymes. The reactions also produce free heme and iron which tend to associate with the membrane consequently imposing membrane defects through oxidation of lipids and proteins.[52] These oxidative reactions happening within the red cells can be simplified to reflect the most crucial products/steps as shown below.



After oxygen binding (equation 1), the resultant oxyhemoglobin undergoes auto-oxidation to produce methemoglobin (MetHb) and the superoxide radical (equation 2). As a defense mechanism against reactive oxygen species, the red cell detoxify itself of the superoxide with the aid of superoxide dismutase (SOD) in presence of hydrogen ions (equation 3). The resulting hydrogen peroxide can be eliminated by glutathione or catalase to yield water or water and oxygen, respectively.[53] However, the elimination process of the peroxide is slow, therefore the molecule lingers within the cell.[52] Unfortunately, hydrogen peroxide also undergoes further oxidative reactions involving hemoglobin and heme-free iron to yield radical complexes of hemoglobin with varying oxidation states of iron, and hydroxyl radicals, respectively (equation 4).[52] Hydroxyl radical is a potent oxidant that interacts with several cellular components.

Eventually, the ROS overrun the red cell's antioxidant capabilities and then the intrinsic cellular redox balance is abolished. In essence, all the antioxidants' molecules/enzymes are reported to be diminished or have reduced activity in sickle cell disease.[54] Conceivably, the ROS also catalyze oxidation of protein tyrosine phosphatases which are required to maintain low levels of protein tyrosine phosphorylation.[55] With respect to inhibition of protein tyrosine phosphatases (PTPs), the accompanying constitutive function of protein tyrosine kinases (PTK) is lost thus allowing the action of PTK to go on uninterrupted (discussed in the next and later chapters of this dissertation).

It should also be noted that oxyhemoglobin (HbFe(II)-O_2) can consumes nitric oxide and generate metHb. The former action leads to a dysfunctional endothelium while the latter powers more deleterious processes within the red cell as stated earlier.[56]

1.3.4 Adhesion to the microvasculature

While polymerization of sickle hemoglobin and dehydration present matters of the red cell alone, microvasculature offers an array of features that feeds into the pathogenesis of sickle cell disease in relation to other cell types. Although it is still evolving and extensive research ongoing globally, a few things remain clear. First, the release of heme or free plasma Hb is documented to trigger expression of adhesion receptors on the endothelium (cellular beds through which red cells and its diluent plasma flow through).[57] Activated adhesion receptors on the endothelium include P-selectin, E-selectin as well as cell adhesion molecules such as vascular adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule (ICAM).[57–61] Interaction of sickle cells with activated endothelial receptors is aided by adhesion molecules such as basal cell adhesion molecular (BCAM), α4β1 integrin and CD36 which are expressed on erythrocyte's plasma membrane.[62]

Arguably, Hb S polymerization is not the lone causative agent of vaso-occlusive crisis since erythrocyte's capillary transit time is shorter than time required for complete polymerization of Hb S (time required for initiation of Hb S polymerization is designated as delay time).[38,40] In essence, cells in circulation can easily evade imminent development of the long fibers of Hb S polymers (Hb S gelation) within transit time frame. Therefore, there must be other underlying factors that dictate blockage of micro vessels to initiate the painful episodes. As the red cell journeys through microvascular beds, the receptors on its membrane interact with those on the endothelium. As a result, the rate of blood flow is retarded and in worse cases, cells stick to the capillary walls causing clogging and blockade. Subsequently, the red cell transit time is increased offering prime conditions for extensive polymerization of Hb S due to prolonged deoxygenation state of the cells thus initiation of occlusion of the microvasculature.[62]

1.4 Global burden of SCD

Sickle cell disease is distributed across the globe, but majority of the cases populate the tropics, especially Africa. Outside the tropics, SCD cases arose from inter-continental population movements.[9] While better management of the disease is much advanced and has resulted in prolonged lifespan in developed countries, the burden in low resource settings where majority of the patients reside remain concerning.[11] In fact, children suffering from sickle cell disease barely make past young adulthood in such regions.[8,11,63] Lack of newborn screening and standard care for managing SCD is largely attributed to the low lifespan of SCD sufferers in developing countries.[8,63,64] Moreover, the remote nature of majority of the tropical residences make it barely accessible rendering provision of emergency support arduous.[10] Similarly, collection of data for up-to-date information, in these regions remains unreliable and has always been the reason why SCD population's estimates in such areas remains unreliable.[9,10,65,66]

Physiological complications arising from sickle cell disease are highly variable from patient to patient.[67] The most common SCD problems include anemia, stroke, dactylitis, nephropathy, retinopathy, acute chest syndrome, and osteonecrosis.[6,21,68,69] These complications can cause sudden death with acute chest syndrome and anemia being the early markers of imminent death.[68–70] Most of these complications can be corrected or altered following good care but in the long run, an eventual permanent organ damage ensues leading to death.[70] Unfortunately some SCD complications arise from treatment of the same disease. Alloimmunization, for example, results from continued blood transfusion in an effort to control anemia and slow down the rate of organ damage.[71–74] Patients undergoing blood transfusion are often prescribed to iron chelation therapies and phlebotomies to control the excess iron in the body.[75]

Economically, SCD burden remains wanting. To live with sickle cell disease means the patient is always under an emergency watch list. Any time there is a vaso-occlusive incident, the patient is bound to be subjected to costly hospitalization.[20,76] To reduce the cost of hospitalization, many patients resort to use of the lesser costly narcotics to relieve pain.[3,5] Narcotics cause addiction further exacerbating the quality of life of patients.[21] Regrettably, lost work times send the patient into unbreakable poverty traps which may limit majority of SCD sufferers from comprehensive medical coverage.[77]

Patients suffering from SCD are not spared of social and psychological anxieties, most of them arise from a presumed doom in life and hence regarded as inconsequential participants of the

society. Societal neglect highlights the worst part of social struggles.[78,79] Neglect exist also among medical personnel involved in giving guidance for treatment choices especially during transition from pediatric to adult care where patients take charge of their critical decisions.[80] As a measure of social burdens at the highest level, stigma is a common feature among SCD patients.[81] Better medicines or management healthcare would likely help reduces these psychological imbalances of SCD patients. While hydroxyurea undoubtedly provided hope since its first use following FDA approval, there has not been much progress or improvement in lifespan of SCD sufferers thereafter. Clearly, new therapeutic interventions are still needed.

1.5 Current and envisioned treatment regimens for SCD disease

Molecular diseases pose major challenges when it comes to their treatment tactics. Being the first molecular disease[82] to be discovered may suggest an improved treatment regimen for sickle cell disease. While major leaps have been made in improving patient's lifespan through non-genetic approaches, treatment of SCD is still basic since major therapies only seek ways to alleviate symptoms of the disease.[12] Moreover, there has been no curative treatment that has earned approval by global drug regulatory authorities such as the food and drug administration (FDA) in the US or any international drug approval entity. However, a breakthrough in gene therapy and bone marrow transplant offer a glimpse of hope for a curative treatment of the disease, notwithstanding the treatment cost and organ donor mismatch concerns. In this section, a summary of treatment or disease management approaches will be provided.

Treatment strategies for SCD may be categorized based on the pathogenic routes of the disease. Approaches for treatment have been focused on inhibiting any of the pathogenic processes enlisted in section **1.3**. Keeping in mind that the defective erythrocytes in SCD are continuously being produced and destroyed at higher rates than rates in the case of healthy red cells, the treatment methodologies are lifelong and often require switching from one treatment to another depending on the disease's state or complications. In the event that all treatment regimens become defective, the medical personnel turn to blood transfusion[83] as a last resort to save their patients. Additionally, blood transfusion is unequivocally needed whenever a patient experiences vaso-occlusive crisis or any sudden loss of blood.[13,83]

1.5.1 Targeting Hb S polymerization

Polymerization of Hb S is the most upstream effector of SCD[34] and therapeutic approaches impeding this process should unequivocally prevent extensive damage resulting from downstream sequelae of sickling.[84] Therapies targeting polymerization of hemoglobin have been explored. In this regard, the treatments either directly inhibit sickling such as the use of anti-sickling agents or indirectly retard polymerization of Hb S as it is the case for induction of fetal hemoglobin (Hb F) through hydroxyurea therapy.

Advancement in use of anti-sickling agents has gained traction in the recent past.[84,85] These agents are designed to bind sites of the Hb S that are required for polymer formation and/or increase oxygen affinity of Hb S. Of the many agents in trials, GBT440 is the golden standard and has recently earned FDA approval.[85,86] GBT440 (also known as voxelotor) acts by reversibly binding the N-terminal of α-globin chains which in turn allosterically stabilizes Hb S in the oxyhemoglobin state which in turn prevent sickling.[86,87] Benefits of the drug have been realized in resolving anemia, increasing the lifespan of erythrocytes, reducing the amount of reticulocytes, and mitigate vaso-occlusion.[86] Other anti-sickling agents with a similar mechanism of action to voxelotor include 5-Hydroxymethylfurfural and pyridyl derivatives of vanillin and triazole sulfide.[85]

Hydroxyurea is the first FDA approved drug for SCD to be on the market.[88] Hydroxyurea (also known as hydroxycarbamide), is the most successful drug available for treatment of sickle cell disease. The drug does not directly inhibit polymerization of sickle Hb but rather it modulates the immune system to make more fetal Hb (Hb F) and also increase total Hb cncentration.[89] In other words, hydroxyurea acts as a very potent, yet transient inhibitor of ribonucleotide reductase thus offering myelosuppressive effects leading to stimulation of erythroid progenitors with enhanced production of F cells (cells with increased concentration of Hb F).[90,91] Fetal hemoglobin is made up of two alpha and two gamma chains of globin($\alpha 2\gamma 2$), but among SCD patients, there is a possibility for production of a mixed tetramer with one gamma and one sickle beta globin ($\alpha 2\beta^S\gamma$) just like the case with Hb A having ($\alpha 2\beta^A\beta^S$). Irrespective of globin chain mixtures and unlike $\alpha 2\beta^A\beta^S$ which may interact with Hb S polymers, $\alpha 2\beta^S\gamma$ does not participate in polymer formation due to the hydrophilic amino acids glycine at $\gamma 87$ and aspartic acid at $\gamma 80$.[91]

Fetal Hb has a very high affinity for oxygen and has been shown to reduce incident rates of vaso-occlusive crisis. Erythrocytes that contain Hb F have been shown to have longer lifespan

compared to cells with Hb S only.[92] This aspect of fetal hemoglobin proves to reduce cases of anemia resulting from premature damage of red blood cells. Presence of Hb F has also been demonstrated to enhance production of nitric oxide.[93] Nitric oxide is an important signaling molecule required for regulation of vascular tonal dilation. This added advantage of Hb F improves overall rheology of blood thus lowering the rates of pain episodes, acute chest syndrome and other complications of SCD that would otherwise develop from effects of strained blood vessels.[93]

Other drugs with similar effects as hydroxyurea have also been discovered. These include pomalidomide, decitabine, metformin, etc.[12] These drugs influence biosynthesis of fetal hemoglobin through different mechanisms.[12]

1.5.2 Targeting the endothelial system

It has now become clear that polymerization alone cannot fully explain the pathophysiology of SCD. Considering that delay time is much longer than red cells' transit time, other aspects of blood rheology must contribute to the development of thorough sickling. As noted in section 1.3.4, the rate of blood flow in the body dictates the degree of gelation of Hb S.[33,38,94] Due to their sticky nature, sickle cells experience a retardation in flow rates. An increased vascular rate of flow could potentially slow down the development of Hb S fibers, an ingenious approach of treating upstream effectors of sickle cell disease by targeting related downstream sequelae. The microvascular rate of flow revolves around adhesion receptors present on the red cells and on the endothelium. With the elevated levels of free heme and Hb S, the endothelial cells are often activated to express adhesion receptors that initiate adsorption of the red cells to the vascular cell walls which concomitantly initiate recruitment of other cell types such as leucocytes and platelets.[48] The activated platelets and leucocytes not only adhere to the sickle cells forming cellular aggregates but also attach to the endothelium leading to a state of hypercoagulation. In the end, a reduced rate of overall blood flow ensues thus offering prime conditions for blockage of blood vessels and enhancement of Hb S polymerization due to increased delay time.[38]

Anti-adhesion therapies have emerged to attempt to address these problems. In this therapeutic category, anti-selectin drugs have been the most successful, perhaps due to expression of selectins by the endothelial cells' surfaces as well as in other blood cells (except red blood cells) which are involved in adhesion.[12] In fact, the FDA approved a first-in class drug, crizanlizumab-

tmca (ADAKVEO, developed by Novartis) in November, 2019.[95] Crizanlizumab, an anti-P-selectin monoclonal antibody, has been shown to reduce vaso-occlusive crises for ≥16 years SCD patients.[95] A year afterwards, another anti-adhesion molecule; rivipansel (GMI-1070) was granted a "rare pediatric disease designation" by FDA which highlight the need for this class of drugs for an improved treatment of SCD.[96] A pan-selectin inhibitor that inhibits both P-selectin and E-selectin, rivipansel has gone through clinical trials and it has been shown to arguably shorten time for resolution of vaso-occlusive thus reducing the need for narcotics.[97] Other anti-adhesive treatment options are in the horizon and curious readers may read a review by Telen *et al*[12] for more information.

The downside of this treatment strategy contestably rests upon the fact that the endothelium may remain exposed to its activators if free heme and Fe^{3+} from damaged red cells are being fed into the vascular system. While this treatment may help reduce damage of red cells and hence release of free heme and Hb S, other pathways such as increased band 3 tyrosine phosphorylation could contribute to generation of free heme/Hb S, as will be explained in the next chapter of this dissertation. Like other non-genetic approaches for SCD treatment, the use of anti-adhesive agents is suggestive of continual use of the drugs for any meaningful alleviation of SCD symptoms to be realized. It would be interesting to find out how combination therapies such as use of anti-adhesive agents alongside anti-sickling drugs impact outcomes of sickle cell disease.

1.5.3 Antioxidant therapies for SCD

The myriad reactive oxygen species produced following auto-oxidation of sickle hemoglobin are harmful to the red cell, endothelium, and other blood cells. Although these reactions also take place in healthy erythrocytes, their rates are indeed slower, and the redox balance remain unperturbed.[54] Erythrocytes have a pool of anti-oxidant enzymes that are designed to neutralize the oxidative reactions or oxidative stress products.[54,98,99] Though sickle erythrocytes also have these anti-oxidant molecules, they are depleted due to the excessive reactive oxygen species being produced at any given time. Baseline levels of antioxidant molecules/enzymes such as hemopexin, haptoglobin, and glutathione, or activity level of superoxide dismutase are all reduced in SCD patients.[54,100] Therefore, the sickle erythrocyte remains prone to toxic effects of oxidative stress.

To rescue cells from oxidative stress, attempts have been made to introduce antioxidant therapies for sickle cell disease management.[101] A plethora of such therapies exist ranging from supplementation of hemopexin and haptoglobin to use of molecules that directly neutralize the reactive oxygen species or enhance production of endogenous antioxidant enzymes or introduce amino acid precursors for production of the antioxidants. The most successful antioxidant therapies hitherto are the use of L-glutamine and N-Acetylcysteine.[101,102] Both drugs have been approved by the FDA, for treatment of sickle cell disease.[101] L-glutamine functions by increasing biosynthesis of nicotinamide adenine dinucleotide (NAD) which is required for restoration of balance of redox reactions, while N-acetylcysteine is required for synthesis of glutathione thus increasing the levels of antioxidants in sickled erythrocytes.[102]

1.5.4 Targeting cell hydration

Owing to their defective membrane deformability/inflexibility, irreversibly sickle cells are the culprits of initiating pain episodes in SCD. These cells are thought to be produced through cellular dehydration of sickle erythrocytes.[103] The activation of cation channels as explained in section 1.3 is associated with a concomitant loss of water from the cell. The resulting dense cells have increased cellular concentration of Hb S and are incapable of maneuvering the vascular flow, thus prone to sickling and lysis, respectively.[104]

Maintaining cellular hydration levels would lower Hb S concentration leading to decreasing chances of polymer formation. Dehydrated cells also lack membrane deformability and may clog the micro-vessels consequently triggering vaso-occlusion.[104] Therapies aiming at restoration of sickle red cell hydration to near normal levels have been tried but none of them have earned FDA approval hitherto, perhaps due to the complex nature of dehydration processes and cation loss/gain in erythrocytes and toxicities associated with these class of drugs as well as ubiquitous presence of similar cation channels in other cell types.[104] Gardos channel blockers, clotrimazole and senicapoc for example, have received extensive studies but none of these drugs have translated into the clinic due to hepatopathy side effects,[105] and inability to reduce vaso-occlusive crisis,[106] respectively.

1.5.5 Blood Transfusion

Blood transfusion is one of the mainstays rescue therapeutic option for people suffering from SCD. It involves transfusing donor matched blood from a healthy individual to a sickle cell patient. The strategy aims to lower percent of Hb S in blood and at the same time reduces the rate of generation of sickle erythrocytes. It is mostly applied when a rapid resolution of anemia is needed especially during or after vaso-occlusive events. The therapy also offers an archetypical approach for restoring abnormal transcranial doppler velocity and acute chest syndrome.[107,108] Additionally, the treatment is often preferred when patients experience frequently recurring vaso-occlusive crises, anemia, and multiple organ failures.[70,83]

With the transfused healthy cells being the main oxygen-carriers, the expected outcome of blood transfusion is efficient oxygen delivery, reduced rates of erythropoiesis, and hence reticulocytes, and an improved overall blood rheology. While these expected outcomes have been realized, the treatment may not be tolerated long term due to progressive iron build-up, risks of the often-lethal delayed hemolytic reactions resulting from undetected donor mismatches as well as difficulties in finding a match at any given time.[35,72,73,109]

1.6 Linkage between malaria and sickle cell disease

Besides the already explained evolutionary cause of sickle cell disease, i.e., malaria being the evolutional cause of SCD, the two diseases share one common element: oxidative reactions of hemoglobin which impose a disarray on the redox balance within the affected erythrocytes. The redox imbalance has far reaching consequences as explained in the preceding sections. An additional common element between malaria and sickle cell, is the elevated levels of band 3 tyrosine phosphorylation which results from the oxidative conditions within diseased red cells. Low et al, have extensively studied the impact of band 3 tyrosine phosphorylation with respect to red cell membrane integrity. One of the major outcomes of these studies is the observable disruption of membrane-cytoskeletal interactions leading to rigidity and loss of deformability of red cells wherever the degree of band 3 tyrosine phosphorylation is elevated.[110–112] As a major part of this dissertation which has already been published, we have demonstrated that band 3 tyrosine phosphorylation is elevated in sickle cell disease. Because malaria and sickle cell disease share

this common membrane weakening problem, we explored the possibility of a similar treatment for both diseases.

While sickling may be the most well agreed-upon feature causing change in RBC morphology in SCD, a new mechanism has emerged from this dissertation. The mechanism involves breaching the link between the membrane and cytoskeletal network of proteins that are responsible for maintaining red cell elasticity and flexibility. Sickle hemoglobin undergoes hastened auto-oxidation generating an avalanche of reactive oxygen species. The ensuing oxidative stress leads to inactivation of protein tyrosine phosphatases (PTPs).[113,114] Lost PTP activity causes band 3 tyrosine phosphorylation.[115] Studies on membrane chemistry by Low PS *et al*,[110,116] have revealed that band 3 tyrosine phosphorylation causes membrane destabilization leading to loss of free Hb and microparticles from the red cells. As the membrane is lost, dehydration sets in and may in turn activate volume sensitive tyrosine kinases that further cause band 3 tyrosine phosphorylation.[117]

Band 3 is a prominent red cell membrane protein that constitutes ~25% of membrane proteins. The protein offers anchorage to the cytoskeleton network of flexibility-maintaining proteins such as actin and spectrin.[118–120] Specifically, band 3 forms a bridge with ankyrin and the junctional complex.[110] Such an interaction is relevant to flexibility/deformability of erythrocytes. During phosphorylation, the interaction of band 3 with the cytosolic network of proteins is abrogated. The ensuing membrane destabilization causes shedding of microparticles and release of free Hb. Both MPs. and free Hb would go on to contribute to a further oxidative stress and inflammation of the endothelium. An activated endothelium expresses adhesion molecules which traps RBCs within circulation thus triggering vaso-occlusive crisis in sickle cell.

With regards to malaria, the parasite consumes hemoglobin and throughout the course of Hb digestion, ROS are produced which trigger similar events stated above. In this case though, the destabilized membrane resulting from band 3 tyrosine phosphorylation offers exit pathways for merozoites (young malaria parasites) from its host cell to begin a new series of invasion of RBCs.[112,121] In the end, the red cells are destroyed leading to anemia and associated complications which ultimately robe the patients of their vitals and hence death.

While the role of band 3 tyrosine phosphorylation with regards to proliferation of malaria parasite has already been exhaustively investigated, this dissertation unveils novel pathways in which this modification of membrane proteins catalyzes the pathogenesis of sickle cell disease. A

novel therapy for use of inhibitors of band 3 tyrosine phosphorylation[55] in treatment of sickle cell disease will be explored. Additionally, a discovery that quinine, and possibly other quinolines, mediates parasite death through inhibition of band 3 tyrosine phosphorylation will also be presented.

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CHAPTER 2. INHIBITION OF BAND 3 TYROSINE PHOSPHORYLATION: A NEW MECHANISM FOR TREATMENT OF SICKLE CELL DISEASE

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2.1 Abstract

Many hypotheses have been proposed to explain how a glutamate to valine substitution in sickle hemoglobin (HbS) can cause sickle cell disease (SCD). We propose and document a new mechanism in which elevated tyrosine phosphorylation of Band 3 initiates sequelae that cause vaso-occlusion and the symptoms of SCD. In this mechanism, denaturation of HbS and release of heme generate intracellular oxidants that cause inhibition of erythrocyte tyrosine phosphatases, thus permitting constitutive tyrosine phosphorylation of Band 3. This phosphorylation in turn induces dissociation of the spectrin-actin cytoskeleton from the membrane, leading to membrane weakening, discharge of membrane-derived microparticles (that initiate the coagulation cascade) and release of cell-free HbS (that consumes NO and activates the endothelium to express adhesion receptors). These processes promote vaso-occlusive events that cause SCD. We further show that inhibitors of Syk tyrosine kinase block Band 3 tyrosine phosphorylation, prevent release of cell free Hb, inhibit discharge of membrane-derived microparticles, increase sickle cell deformability, reduce sickle cell adhesion to human endothelial cells, and enhance sickle cell flow through microcapillaries. In view of reports that imatinib (a Syk inhibitor) successfully treats symptoms of sickle cell disease, we suggest that Syk tyrosine kinase inhibitors warrant repurposing as potential treatments for SCD.

2.2 Introduction

Although most symptoms of SCD are thought to be caused by occlusion of the microvasculature,[1–4] the mechanistic steps underpinning these vaso-occlusive events are still debated. Included among the most cited mechanisms are: 1) loss of erythrocyte viscoelastic properties deriving from sickle hemoglobin (HbS) polymerization, red blood cell (RBC) dehydration, and membrane rigidification,[1,3,5–9] 2) activation of adhesion receptors on the vascular

endothelium and/or erythrocyte membrane,[10–13] and 3) initiation of thrombosis by RBC-derived microparticles (MPs) and the subsequent activation of platelets by thrombin and other coagulation factors.[12–17] Vaso-occlusive processes may result in tissue hypoxia, ischemia-reperfusion injury, organ damage and associated morbidities, and debilitating pain that results in significant suffering and may require medical treatment/hospitalization.[18,19] Sickle cell hemolysis, reduced cell lifespan, and anemia can further aggravate clinical symptoms.[20,21]

While RBC dehydration,[3,5,6,22] loss of membrane deformability,[3,23] and increased RBC/endothelial cell adhesion[10–12] undoubtedly contribute to SCD, an increasing number of researchers now propose that additional pathologic sequelae may arise from weakening of erythrocyte membrane leading to discharge of MPs[12,14,15,17] and free hemoglobin (Hb).[15,24] In this hypothesis, accelerated denaturation of HbS[25] hemichrome formation[26,27] and release of heme may collectively induce oxidative stress within the RBC.[26,28,29] Increased oxidative stress can then cause inhibition of RBC tyrosine phosphatases that normally prevent constitutive Band 3 tyrosine phosphorylation.[30–33] Upon inhibition of these phosphatases, over-phosphorylation of Band 3 then induces global destabilization of the erythrocyte membrane,[34,35] accelerating intravascular hemolysis and MP release. The increased plasma hemoglobin and heme can 'activate' the vascular endothelium, causing expression of adhesion receptors (e.g., p-selectin, E-selectin and von Willebrand factor)[10–12] and sequestration of the vasodilator, nitric oxide (NO),[11,36–38] while the release of MPs can trigger intravascular thrombosis via activation of prothrombin.[12,14,15,17] When initiated concomitantly with loss of RBC deformability and enhanced vaso-adhesion, the sequelae associated with membrane weakening can aggravate an already compromised blood flow, leading to micro-emboli and progressive tissue damage.

In this paper, we explore the role of Band 3 tyrosine phosphorylation and consequent membrane weakening in development of the symptoms in SCD. We first show that the extent of tyrosine phosphorylation of Band 3 correlates with the percent of HbS in sickle erythrocytes, the concentration of cell free Hb in the patient's plasma, and the number of RBC-derived MPs in a patients' peripheral blood. We next document that blockade of tyrosine phosphorylation of Band 3 with tyrosine kinase inhibitors prevents release of cell-free Hb and discharge of RBC-derived MPs, while concomitantly enhancing sickle cell deformability, reducing sickle cell sickling at lower O_2 pressures, and enhancing sickle cell flow through microcapillaries. Finally, we also establish that imatinib treatment suppresses adhesion of erythrocytes to heme-activated

endothelium. Based on evidence from many labs that MPs,[12,14–17] cell-free Hb,[36,39,40] reduced sickle cell deformability, and enhanced sickle cell adhesion to the endothelium[10–12,23,41] contribute to the pathology of SCD, we argue that Band 3 tyrosine phosphorylation inhibitors could constitute a potent therapy for treatment for SCD.

2.3 Methods

2.3.1 Processing of SCD Blood Samples

All sickle cell blood samples were obtained following informed consent using procedures approved by the local IRBs. Venous blood was collected from patients (genotype SS or $S\beta^0$) and healthy volunteers into EDTA-containing vacutainer tubes and maintained at 4°C until use. Samples were centrifuged at 800 rcf for ten minutes and plasma was removed for analysis of MPs and cell-free Hb. RBC pellets were washed 3x with phosphate buffered saline, pH 7.4, containing 5 mM glucose (PBS-G) and the buffy coat was aspirated after each wash cycle.

2.3.2 Quantitation of Band 3 Tyrosine Phosphorylation

Washed RBCs were suspended at 30% hematocrit (Hct.) in PBS-G and treated with either 5 μM drug (imatinib, PRT062607 or R406) or vehicle (control) for 4 hours at 37°C under 50 rpm shaking. The 5 μM drug concentration was determined from the minimum concentration of imatinib required to completely reverse band 3 tyrosine phosphorylation within 4 h. RBC membranes were prepared and processed for western blotting as described in supplemental information. Band 3 tyrosine phosphorylation intensity was quantitated using image J software. To induce Band 3 tyrosine phosphorylation and membrane fragmentation in healthy erythrocytes, blood from healthy donors was washed and cells were suspended at 30% Hct in PBS-G containing either 5μM imatinib or vehicle (DMSO; ≤0.5% v/v to minimize impact of the vehicle on cells, Supplemental Fig. 1) at 37 °C for 1 h. After the 1h incubation, 2 mM sodium orthovanadate (OV) was added to both imatinib-treated and untreated cells and cells were incubated for 4 h at 37 °C while shaking at 1400 rpm.

2.3.3 Quantitation of Erythrocyte-Derived Microparticles and Free Plasma Hemoglobin

Plasma from sickle cell samples was centrifuged 2x at 2500 rcf for 15 minutes to remove platelets, and 100 µL supernatant containing MPs was incubated for 20 minutes on ice with 0.5 µL mouse-anti-human glycophorin A antibody (BD Biosciences #562938). To test if the MPs. are CD71 positive 1.0 µL of CD71 antibody (BD biosciences #12-0711-82) was added alongside the glycophorin A antibody. Samples were diluted with 1 mL stain buffer (BD Biosciences #554656), transferred to BD Trucount™ tubes (BD Biosciences #340334) and analyzed on Attune NxT Flow Cytometer utilizing violet fluorescent trigger channel[42]. The absolute number of MPs was calculated as follows,

$$Absolute\ microparticles\ count = ((Glycophorin\ A\ positive\ events)/(bead\ events)) \times ((Number\ of\ trucount\ beads)/Volume)$$

For evaluation of the effect of tyrosine kinase inhibitors on release of MPs, 500 µL sickle cells, suspended at 30% Hct in PBS-G, were treated for 1 hour with desired tyrosine kinase inhibitor or vehicle control, and shaken at 1400 rpm for 4 h. Newly released microparticles were separated from residual RBCs by centrifuging at 800 rcf for 10 minutes, collecting the supernatant, and quantitating the MPs as described above.

Cayman's hemoglobin colorimetric assay was used to quantitate cell-free Hb according to manufacturer's instructions.

2.3.4 Measurement of Red Blood Cell Deformability

Erythrocyte deformability was measured using Technicon™ Ektacytometer and plotted as elongation index versus shear stress. Data were acquired while accelerating the ektacytometer from 0 to 250 rpm and shear stress was calculated:

$$Shears\ stress = (2\pi \times viscoscity\ (poise) \times rpm \times radius\ of\ cylinder\ (cm))/(60 \times gap\ between\ the\ two\ cylinders\ (cm))$$

2.3.5 Measurements of RBC flow through microcapillaries

The effect of imatinib on the rate of sickle cell flow through 5µm x 6.5µm microcapillaries at different oxygen pressures was measured using a high speed camera mounted onto an inverted

microscope focused on a microfluidic device through which sickle blood was flowed at constant pressure (1.6 psi), as described in Supplement Information and reference[43].

2.3.6 Oxygen Gradient Ektacytometry (Oxygenscan)

To determine the point of sickling (PoS) upon deoxygenation, oxygenscans were performed using a Laser Optical Rotational Red Cell Analyzer as described[44]. Patient samples were washed and resuspended at 20% hematocrit in HBSS modified with 10 mM HEPES and 10 mM $MgCl_2$ and incubated with 5 µM imatinib or DMSO for 4 h at 37 °C. 300×10^6 RBCs were added to 5 ml OxyIso solution (RR Mechatronics, Zwaag, The Netherlands) and loaded into the Lorrca where they were subjected to constant shear stress (30 Pa). Deformability was measured while pO_2 was gradually decreased from 150 mmHg to <20 mmHg before reoxygenation with ambient air. The PoS was determined as the pO_2 at which samples reached 95% of their initial deformability and began to sickle.

2.3.7 Measurements of RBC adhesion to endothelial cell functionalized microchannels

Microfluidic channels were fabricated and incubated with fibronectin prior to coating with a monolayer of human umbilical vein endothelial cells (HUVEC) and human pulmonary microvascular endothelial cells (HPMECs) as described earlier[16,45]. Two hours prior to analysis of RBC adhesion, adherent HUVECs and HPMECs were activated with 40µM heme to induce expression of adhesion receptors [16]. Freshly isolated sickle cells were simultaneously incubated in basal medium for 4 hours and perfused through the microchannels at a physiological shear stress level of 1 dyne/cm^2 in precisely controlled physiological hypoxia (SpO_2 of 83%)[16]. The SpO_2 level was chosen to be pathologically relevant to SCD based on clinical studies [46]. Non-adherent RBCs were washed away, and adherent cells were counted (see details in Supplemental Information).

2.3.8 Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) and F-test on linear regression. Statistical significance was set at 95% confidence level for all tests (p<0.05).

2.4 Results

2.4.1 Hematologic parameters of SCD patients

To test the hypothesis that sickle cells are distinguished by increased tyrosine phosphorylation of Band 3, membrane weakening, and release of both cell-free Hb and MPs, we focused studies on RBCs from non-transfused children (age range 3-20 years, mean 9.3 years; n=48) with SCD; all undergoing hydroxyurea treatment. As shown in Fig. 1A, the concentration of cell-free Hb in patient plasma was more than twice that of healthy volunteers, i.e. in agreement with previous studies[12,15,24,47]. Moreover, the number of MPs, identified as glycophorin A positive particles of 0.1-1.0 μm diameter (Supplemental Fig.2), were also more than twice as abundant in patients than healthy volunteers (panel B), i.e. also consistent with previous observations[12,14,15,48]. It is worth noting that the Glycophorin A positive microparticles identified herein are CD71 negative (Supplemental Figure 3) which indicates that the MPs. observed are mainly derived from mature erythrocytes. Because release of MPs would be expected to render the membrane-depleted erythrocytes less deformable (due to their smaller surface to volume ratios and higher Hb concentrations[2,3,5,23,41]), the reduced deformability of SCD blood versus normal controls (panel C) was expected. Importantly, the nearly linear correlation between MP count and cell-free Hb in each patient's blood sample (panel D) suggested a possible relationship between cell-free Hb and RBC-derived MPs. The weakening of the membrane by tyrosine phosphorylation of Band 3 could account for this correlation.

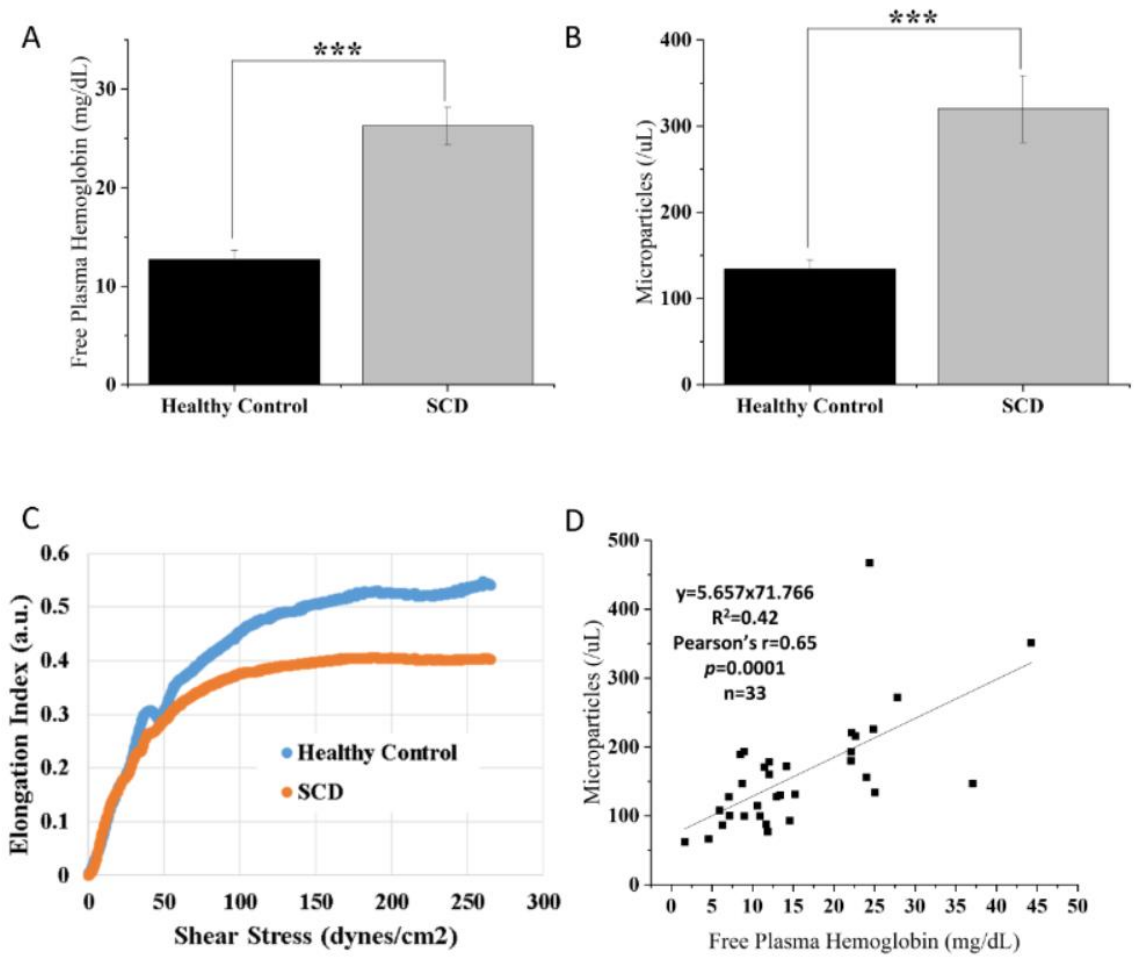

Figure 2.1 Quantitation of cell-free Hb and erythrocyte membrane-derived MPs in blood from patients with SCD. The concentration of cell-free Hb in the plasma (**A**) and numbers of erythrocyte membrane-derived MPs (**B**) were evaluated in both patients with SCD and healthy controls (n = 48). Erythrocyte elongation index as a function of increasing shear stress (i.e. deformability) was also compared for SCD and control blood samples (**C**), representative sample of three different healthy controls and five different SCD patients). A plot of free plasma Hb versus MP count was constructed to explore a possible common mechanism leading to their production (**D**). (Error bars are expressed as SEM, *** denotes $P \leq 0.005$ computed from one-way ANOVA or student t-test; Pearson's r = 0.65, and P = 0.0001 in panel **D**). (Hb, haemoglobin; MP, microparticle; SCD, sickle cell disease; SEM, standard error of the mean).

2.4.2 Characterization of Band 3 tyrosine phosphorylation in SCD erythrocytes

It has been frequently reported that oxidative stress leads to inactivation of erythrocyte tyrosine phosphatases[30,31,49] that in turn allow unimpeded tyrosine phosphorylation of Band 3 by constitutively active tyrosine kinases[30,35,50]. Because this tyrosine phosphorylation induces an intramolecular interaction in Band 3 that causes dissociation of the spectrin-actin cortical

cytoskeleton from the membrane[34,35], we hypothesized that oxidative stress deriving from premature HbS denaturation[25,28,29] might initiate a phosphorylation cascade that would lead to dissociation of the spectrin-based cytoskeleton from the membrane, causing membrane destabilization and fragmentation. To test this hypothesis, we compared tyrosine phosphorylation of Band 3 in sickle cells and healthy controls. As shown in Fig. 2A, tyrosine phosphorylation of Band 3 in healthy cells was almost undetectable, whereas phosphorylation in sickle cells was prominent. Evidence that phosphorylation in sickle cells was dependent on their levels of HbS is provided in panel B, where a positive correlation (Pearson's r=0.70) and a significant linear relationship (p=0.008) was observed between Band 3 tyrosine phosphorylation and percent HbS in each patient's sample.

Documentation that tyrosine phosphorylation of Band 3 was likely also related to release of Hb into plasma was demonstrated by a significant correlation (p=0.02, Pearson's r=0.63) between these two parameters (panel C). Furthermore, an indication that Band 3 tyrosine phosphorylation was related to release of MPs is shown in panel D (Pearson's r=0.72, p=0.01). These data suggest that elevated tyrosine phosphorylation of Band 3 in sickle cells is related to the membrane destabilization that causes release of MPs and free Hb.

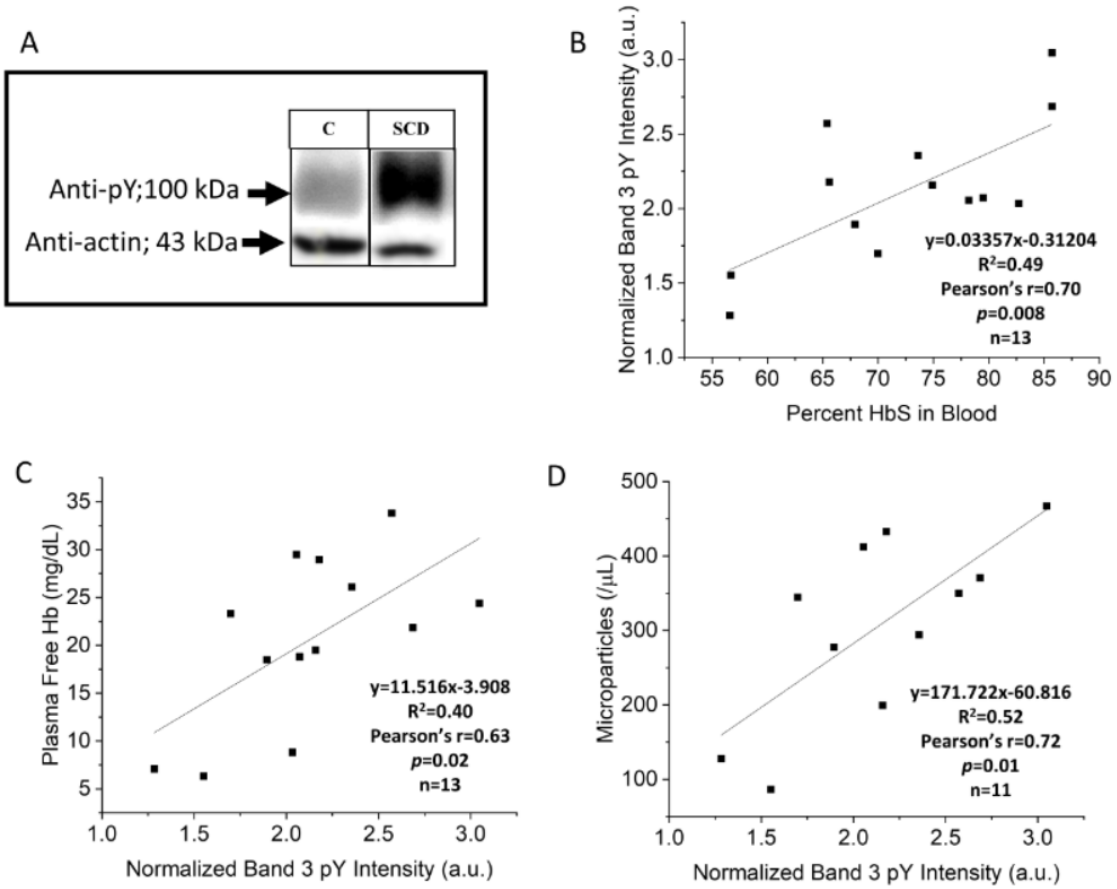

Figure 2.2 Analysis of the relationship between Band 3 tyrosine phosphorylation, cell-free Hb and MP count in sickle cell blood samples. A) Representative anti-phosphotyrosine immunoblot of Band 3 in erythrocyte membranes from a healthy control (C) and a sickle cell patient (SCD) treated with hydroxycarbamide. The correlation between Band 3 tyrosine phosphorylation and percentage of HbS in the blood sample (B), cell-free Hb in the plasma (C), and RBC membrane-derived MPs (D), is also plotted. (Statistical data from F-tests are as indicated on the respective figures). (Hb, haemoglobin; MP, microparticle; HbS, sickle haemoglobin; RBC, red blood cell).

2.4.3 Inhibition of Band 3 tyrosine phosphorylation

To further test the hypothesis that Band 3 tyrosine phosphorylation might promote release of Hb and MPs from sickle erythrocytes, we explored the effect of imatinib on tyrosine phosphorylation of Band 3. As shown in Fig. 3A, healthy erythrocytes displayed low levels of Band 3 phosphorylation, whereas sickle erythrocytes exhibited higher levels of phosphorylation. Moreover, treatment of sickle erythrocytes with 5 µM imatinib lowered their Band 3 tyrosine phosphorylation to levels similar to control cells, demonstrating that imatinib can inhibit the natural tyrosine phosphorylation of Band 3 in sickle cells. As documented in panels B and C,

imatinib treatment also reduces release of cell-free Hb and MPs, suggesting that Band 3 phosphorylation is directly related to both characteristics of sickle blood. Importantly, although the tyrosine phosphorylation of Band 3 and accompanying Band 3 conformational changes are readily reversible, Hb and MP release are not reversible (Puchulu-Campanella et al, 2016; Ferru et al, 2011; Hierso et al, 2017).

Because imatinib inhibits several kinases besides Syk [51], the question arose whether other more Syk-specific inhibitors might similarly suppress Band 3 tyrosine phosphorylation in sickle cells. As shown in Fig. 3, incubation of sickle cells with either 5 μM PRT062607 (panel D) or R406 (panel E) resulted in an analogous diminution of Band 3 tyrosine phosphorylation. Moreover, the same Syk-specific inhibitors also suppressed MP and Hb release from both sickle cells (Supplemental Fig. 4) and o-vanadate treated healthy cells (Supplemental Fig. 5). These data demonstrate that Syk-specific inhibitors also suppress the tyrosine kinase that phosphorylates Band 3 in SCD, suggesting that at least one of the kinases that phosphorylates Band 3 in SCD is Syk.

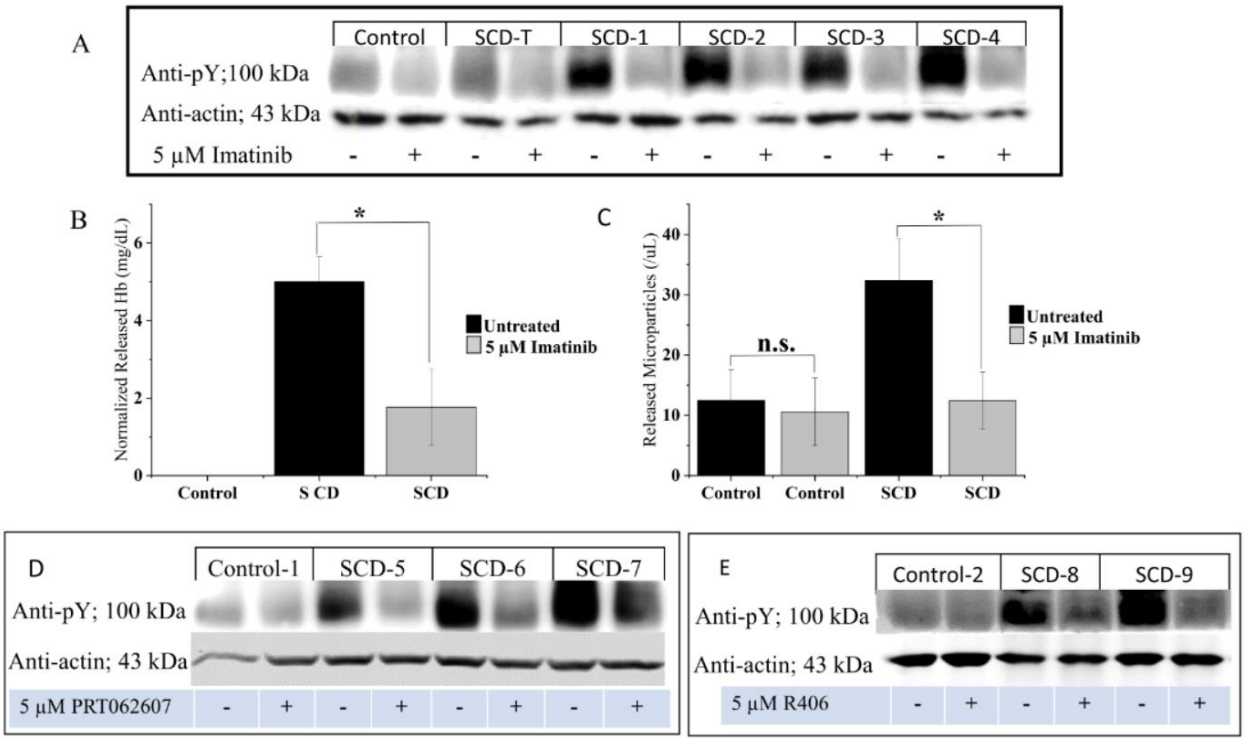

Figure 2.3 Effect of treatment of sickle cells with imatinib or other tyrosine kinase inhibitors on: A) tyrosine phosphorylation of Band 3 in healthy erythrocytes (control), erythrocytes from a SCD-T patient, and erythrocytes from non-SCD-T patients treated with hydroxycarbamide, **B**) release of cell-free Hb (n = 5), and C) discharge of RBC membrane-derived MPs from non-transfused sickle cell blood samples in vitro (n = 6). Panels **D** and **E** illustrate the effect of two Syk-specific inhibitors on tyrosine phosphorylation of Band 3 in erythrocytes from healthy controls, and non-transfused patients on hydroxycarbamide. Whole blood samples from healthy controls or SCD patients were washed three times in PBS-G and incubated for 4 h at 37°C in PBS-G, containing or lacking 5 lM imatinib, or 5 lM PRT062607, or 5 lM R406 while shaking at 50 (A), or 1400 (BE) rpm prior to analysis. Note that the sickle cell patient receiving transfusions (SCD-T) exhibits no increase in Band 3 tyrosine phosphorylation. (Error bars are expressed as SEM, *denotes P ≤ 005, computed from one-way ANOVA). (SCD-T, transfused sickle cell disease; Hb, haemoglobin; RBC, red blood cell; MP, microparticle; PBS-G, phosphate-buffered saline with glucose; SEM, standard error of the mean).

2.4.4 Erythrocyte membrane stability

Next, because defects in molecular bridges connecting the erythrocyte membrane to its cortical spectrin-actin cytoskeleton have been shown to compromise erythrocyte deformability[34,35], we examined whether inhibition of Band 3 tyrosine phosphorylation might restore the disrupted bridges and thereby improve sickle cell deformability. First, sickle blood samples were incubated for 4 hours in the presence or absence of 5 µM imatinib and then examined by ektacytometry for changes in cell deformability. As shown in Fig. 4A, although the deformability of sickle cells was

lower than that of healthy controls, incubation with imatinib improved their deformability. Second, the deformability of sickle erythrocytes under constant shear stress was examined during de-oxygenation and re-oxygenation of the sickle cells[44]. As shown in Fig. 4B, sickle RBCs pre-incubated with imatinib exhibited higher baseline deformability, initiated sickling only when exposed to lower pO_2 (point of sickling 5%), and displayed improved minimal deformability (EImin, panel B) compared to untreated cells from the same patient. Third, because pO_2-regulated RBC capillary flow velocity relates mechanistically to cell deformability[43], we studied the flow of sickle erythrocytes through microcapillaries at controlled pO_2 (panel C). Relative to untreated cells, imatinib-treated cells were found to experience a significant increase in capillary velocity that improved as the concentration of imatinib was increased (panel C). Since similar observations were obtained at all other O_2 pressures examined, we conclude that imatinib improves the flow of sickle cells through microcapillaries.

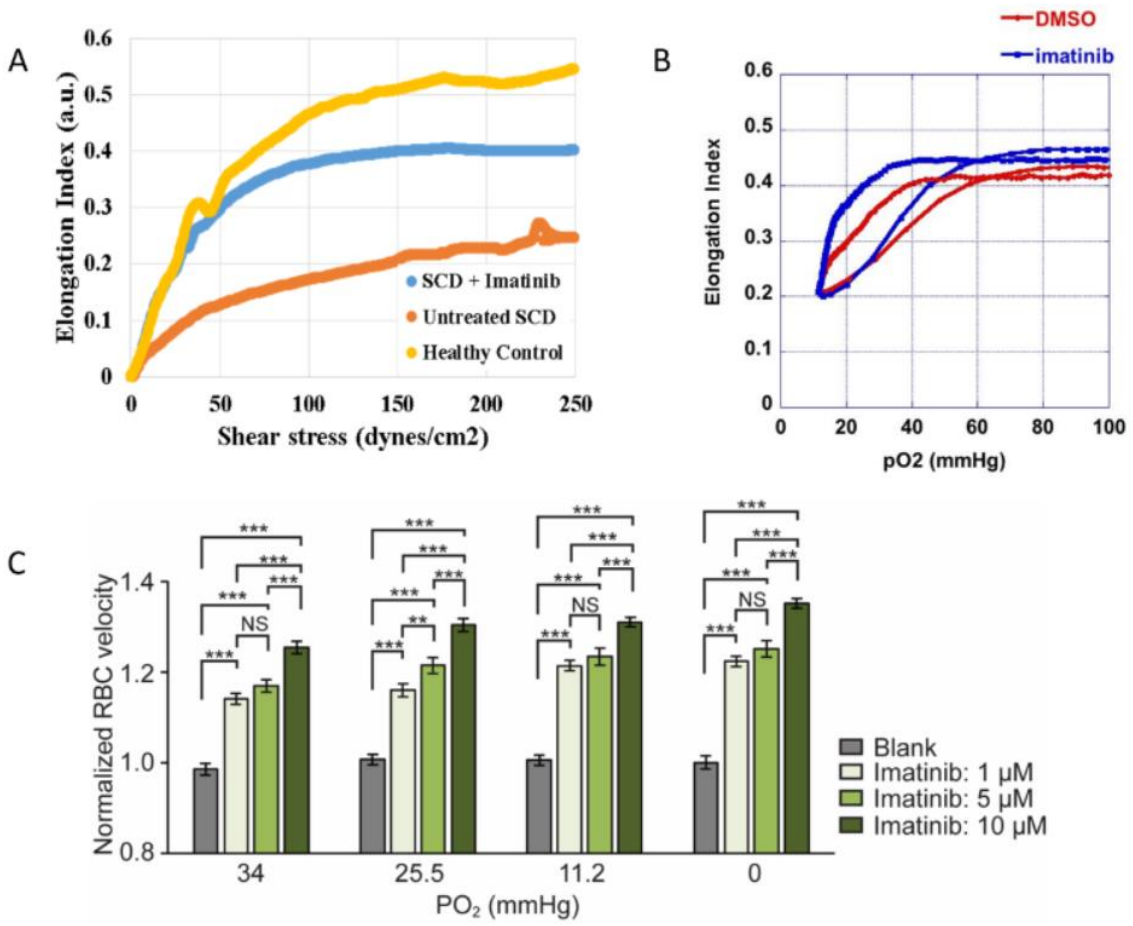

Figure 2.4 Effect of imatinib on the deformability and rheology of sickle cells at different oxygen pressures. A) The deformability of healthy erythrocytes and washed sickle cells is determined by ektacytometry in the presence and/or absence of 5 lM imatinib. **B**). A representative scan of inhibition of sickling after treatment of blood from SCD patients with imatinib or DMSO (vehicle control) as detected by Oxygenscan. Elmax, point of sickling (PoS5%) and Elmin are recorded as the sample is deoxygenated and then reoxygenated after 4 h incubation with 5 μM imatinib or an equal volume of DMSO carrier. Note both the left-shift in PoS5% and higher Elmin in sickle RBCs following incubation with imatinib, which are indicative of improved deformability of the cells. **C**) Washed sickle or healthy cells were incubated for 4 h at 37°C in the indicated concentrations of imatinib and then equilibrated at the indicated partial pressures of oxygen, prior to analysis of flow rates. [***denotes $P \leq 0.05$, figures are representative of three (panel **A**) and five (panels **B** and **C**) different patient samples]. (SCD, sickle cell disease; DMSO, dimethyl sulfoxide; Elmax or Elmin, Eadie-Hofstee linearisation maximum or minimum deformability).

2.4.5 In vitro induced membrane weakening

To directly demonstrate that tyrosine phosphorylation of Band 3 promotes membrane weakening and release of cell-free Hb and RBC membrane-derived MPs, we induced tyrosine phosphorylation of Band 3 in healthy erythrocytes by treatment with the tyrosine phosphatase

inhibitor, orthovanadate, and then examined release of cell-free Hb and MPs in the presence and absence of imatinib. As shown in Fig. 5, treatment of control RBCs with orthovanadate induced tyrosine phosphorylation of Band 3 (panel A), release of cell-free Hb (panel B) and discharge of MPs (panel C) in a manner that could be inhibited by imatinib. These data demonstrate that tyrosine phosphorylation of Band 3 constitutes the cause of Hb and MP release and that imatinib prevents these pro-embolic processes by inhibiting Band 3 tyrosine phosphorylation.

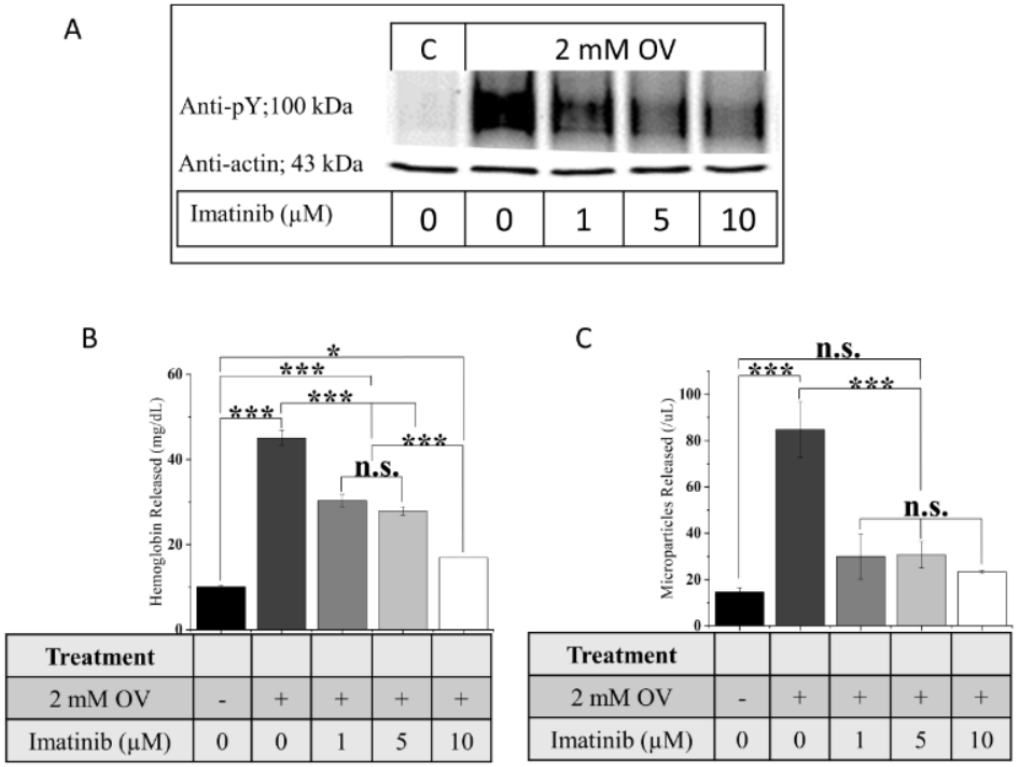

Figure 2.5 Effect of imatinib on release of cell-free Hb and membrane-derived MPs from healthy RBCs following treatment with the tyrosine phosphatase inhibitor OV. Effect of sodium OV and imatinib on (**A**) Band 3 tyrosine phosphorylation, (**B**) release of cell-free Hb, and (**C**) discharge of membrane-derived MPs from healthy HbAA RBCs. Cells were incubated with the indicated concentrations of imatinib and OV for 4 h with shaking at 1400 rpm prior to analysis. (Error bars are expressed as SEM, * denotes $P \leq 0.05$, **denotes $P \leq 0.025$, ***denotes $P \leq 0.01$ computed from one-way ANOVA) (OV, orthovanadate; Hb, haemoglobin; MPs, microparticles; RBC, red blood cell; SEM, standard error of the mean).

2.4.6 Activation of the endothelium

Finally, we examined the effect of imatinib on adhesion of flowing sickle cells to heme-activated endothelial cells. As seen in representative images of adherent RBCs (Fig. 6), untreated

sickle cells (panels A and B) are more adherent to heme-activated HUVECs and HPMECs under hypoxia than imatinib-treated sickle cells (panels C and D). The mean adhesion of naïve sickle cells was 383 ± 57 (control) compared to 171 ± 30 for imatinib-exposed sickle cells (panel E; n=13 patients; p<0.001, paired t test). These data suggest that imatinib can further reduce vaso-occlusive events by suppressing adhesion of sickle cells to activated endothelial cells.

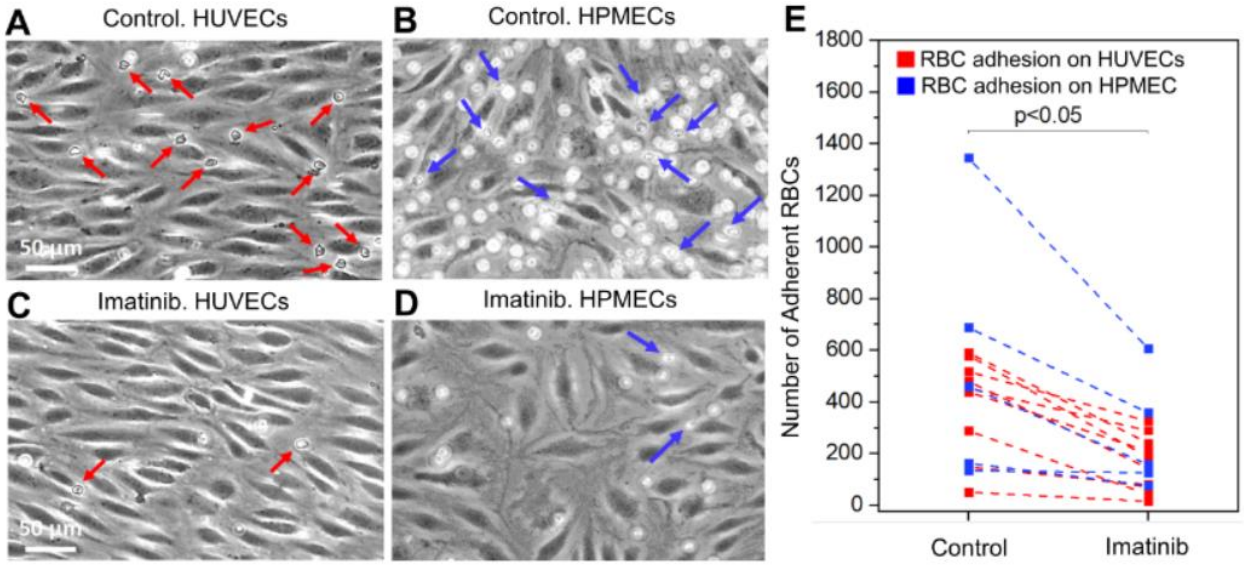

Figure 2.6 Sickle RBC adhesion to heme-activated endothelial cells under physiologic hypoxia in microfluidic channels in vitro. Representative images of adherent RBCs to heme-activated endothelial cells are also shown in the control group (**A**, **B** on HUVECs and HPMECs) and in the imatinibtreated group (**C**, **D** on HUVECs and HPMECs). Arrows indicate RBCs adherent to endothelium. (**E**) Sickle RBC adhesion to heme-activated endothelial cells is significantly reduced by imatinib (5 lM) treatment, compared with control (vehicle, DMSO) treatment (N = 13 subjects, mean adhesion of untreated vs. imatinib-treated sickle cells ±SEM = 383±57 vs. 171±30, P < 0.001, paired t-test).) (RBC, red blood cell; HUVEC, human umbilical vein endothelial cells; HPMEC, human pulmonary microvascular endothelial cells).

2.5 Discussion

Multiple publications have reported that oxidative stress is elevated in sickle cells[11,28,29], that this oxidative stress inhibits erythrocyte tyrosine phosphatases[26,30,31,50], and that inhibition of erythrocyte tyrosine phosphatases leads to elevated tyrosine phosphorylation of Band 3[34,35,50]. We document here that elevated tyrosine phosphorylation of Band 3 causes destabilization of the membrane, promoting the release of both MPs and cell-free Hb[34,35,52]. Recognizing that erythrocyte-derived MPs[12,14,15,48] and cell-free Hb[12,15,47] are pro-embolic, we formulated the

hypothesis that elevated oxidative stress in sickle cells should sequentially induce: 1) heightened tyrosine phosphorylation of Band 3[30–32,50,53,54], 2) destabilization of the sickle cell membrane[34,35], 3) release of MPs and cell-free Hb[4,12,14,15,47], 4) activation of adhesion receptors on the vascular endothelium by the released cell-free Hb and heme[10,11,14,16], 5) stimulation of micro-embolisms by the prothrombotic MPs[4,12,14,15], 6) enhancement of adhesive properties of sickle cells[10–13,39], and 7) induction of vaso-occlusive events due to concurrent activation of the above processes. The data presented here provide strong evidence that these sequelae do in fact occur in SCD and that Band 3 tyrosine phosphorylation is critical for their occurrence.

In addition to the effects of inhibitors of Band 3 tyrosine phosphorylation on SCD symptoms outlined above, we also envision that these inhibitors may exert other unanticipated positive effects on SCD. Although inhibition of MP and HbS/heme release can be predicted to reduce micro-embolic events, the concomitant reduced blebbing/loss of erythrocyte membrane area should also improve sickle cell deformability by maintaining a higher cell surface to volume ratio, thereby improving the flow of sickle erythrocytes (Fig. 4A, C). This maintenance of sickle cell volume should also suppress the cell's tendency to sickle, since the delay in sickling is related to the 30th power of HbS concentration (i.e. a change in only 8% in RBC volume will cause a 10-fold change in the lag time before sickling)[8,9] and prevention of membrane loss will prevent the associated increase in cytoplasmic HbS concentration (Fig. 4B). The observed decline in sickle cell adhesiveness upon treatment with imatinib (which must derive from an effect on the erythrocyte membrane since the endothelial cells were not exposed to imatinib) should also improve sickle cell flow through the vasculature, and this improved flow should reduce the time each sickle cell remains deoxygenated, thereby further decreasing the tendency to sickle (Fig. 6)[8,9]. And while our studies did not examine sickle cell lifespan, it's predictable that inhibition of Band 3 phosphorylation should also improve sickle cell survival, since prevention of membrane loss should prolong maintenance of RBC flexibility and thereby reduce its susceptibility to phagocytosis by macrophages[55,56] and hence improve SCD-associated anemia.

Erythrocyte deformability is thought to depend on three parameters: i) the cell's surface to volume ratio, ii) the viscosity of the cell's cytoplasm (which is determined by the concentration of Hb), and iii) the intrinsic deformability of the RBC plasma membrane[3,5]. As shown in Fig. 4, both sickle cell deformability and sickle blood rheology are improved within 4 hours of exposure to imatinib. Because significant changes in either RBC volume or surface to volume ratio did not

occur over this short time span, the rapid improvement in RBC rheology must have derived from an enhancement in membrane deformability. These data therefore suggest that restoration of the disrupted bridges between Band 3 and the spectrin-actin cytoskeleton by imatinib can improve membrane deformability. The fact that exposure of the isolated sickle cells to imatinib also reduced their tendency to bind heme-activated human endothelial cells (Fig. 6) also suggests that imatinib has a positive effect on sickle cell membrane properties.

With more potent kinase inhibitors readily available[34], the question naturally arises why we selected imatinib to test involvement of Band 3 phosphorylation in SCD. Following initial observations that inhibition of Band 3 phosphorylation by tyrosine kinase inhibitors suppressed release of cell-free Hb and MPs, it seemed logical to explore whether any SCD patients might have fortuitously been treated for another disease with such inhibitors. Upon screening FDA-approved tyrosine kinase inhibitors for inhibition of Band 3 tyrosine phosphorylation, we found that imatinib was an effective inhibitor at clinically relevant concentrations. We then looked for reports in the literature where chronic myelogenous leukemia (CML) patients who coincidentally suffered from SCD might have been treated with imatinib. We found two anecdotal publications that essentially reported the same observation, namely that administration of imatinib successfully treated the symptoms of SCD in their CML patient [57,58]. Although neither author linked his/her findings to any RBC property, their results nevertheless suggest that an inhibitor of Band 3 tyrosine phosphorylation could constitute a therapy for SCD. While chronic use of imatinib should not be considered for treatment of SCD in children because it can stunt a child's growth,[59,60] a well-designed short term clinical evaluation of imatinib in a more mature population could provide a proof-of-concept test that would inform whether a search for a more selective inhibitor of band 3 tyrosine phosphorylation might be worthwhile.

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CHAPTER 3. IMPACT OF TREATMENT ON SICKLE CELL DISEASE CLINICAL BIOMARKERS AND THEIR INDICATION OF DISEASE STATUS

3.1 Abstract

Blood transfusion is one of the most crucial live saving options for people suffering from anemia, especially in disorders such as sickle cell disease where sudden blood loss can happen any time. Whilst hydroxyurea remains the first line of defense for sickle cell disease, blood transfusion is regarded as the chief therapy to deliver rapid resolution of anemia, prevent primary and secondary stroke, and provide increased oxygen affinity of red cells, thus offers an immediate alleviation of sickle cell complications. In this study, an assessment of blood samples from sickle cell disease patients undergoing either blood transfusion or hydroxyurea treatment revealed similar clinical outcomes with respect to baseline anemia and reticulocytosis. The extent of band 3 tyrosine phosphorylation in both treatment groups is above observable healthy levels, although recently transfused patients' phosphorylation levels can sometimes be the same as that of healthy controls depending on the content of adult sickle hemoglobin present in blood. Because transfused patients and patients treated with hydroxyurea in this study have similar steady state levels of reticulocytes, it is conceivable that depletion of erythrocytes within circulation is occurring at equal rates between the two treatment groups. Regardless of patients' treatment regimen, inhibiting residual band 3 tyrosine phosphorylation using imatinib or Syk inhibitors would minimize hemolysis resulting from weakened red cell membranes. Restoring red cells' membrane integrity would fundamentally increase the lifespan of erythrocytes within circulation and hence relieve patients of stress erythropoiesis and the accompanying progressive organ damage common to SCD sufferers.

3.2 Introduction

Blood transfusion is one of the key treatment options for people suffering from sickle cell disease (SCD). Although hydroxyurea remain the long term standard therapy,[1,2] only 50% of patients respond to the treatment.[3,4] Non-responsive patients resort to blood transfusion as the next major alternative therapeutic intervention.[5] At the molecular level, blood transfusion is

administered purposely for two main reasons: to reduce the percent sickle hemoglobin (%Hb S) and, to increase blood's oxygen carrying capacity in lieu of normal erythrocyte being the principal oxygen carriers.[5,6] Basically, sickle red cells have diminished oxygen binding affinity.[7–10] With regards to clinical outcomes, the major goal of long-term blood transfusion in sickle cell disease is to prevent primary and secondary stroke in patients with abnormal transcranial doppler velocities (TCD).[6,11,12]

Blood transfusion can be performed through three major approaches, viz: simple, manual, and automated exchange. Simple transfusion primarily involves transfusing healthy blood into a patient without removal of any blood components from the patient, thus increasing the cumulative total red cell content, i.e., to correct anemia or to raise hematocrit levels.[5] On the other hand, manual and automated exchange involves removal of blood from the patient and subsequently replacing it with healthy blood.[5] The choice of transfusion methodology depends on the reason for transfusion and the available expertise. Simple transfusion requires little expertise relative to manual exchange. Automated transfusion (also known as red cell exchange) requires sophisticated machinery and high level of expertise to administer.[5] Although sophisticated and expensive, automated transfusion appears to be the safest option due to minimized progressive iron overload.[5]

Like any other treatment for SCD, blood transfusion does not come without demerits.[13] Among the most reported undesirable effects include alloimmunization, gradual iron build-up, frequent and extended hospitalization, and the associated costs.[6,13–16] Although automated red cell exchange approach stated above offers safer and reduced accumulation of iron, it is often expensive and sparingly available in low-resource settings where majority of SCD patients reside.[17] Bearing in mind the general socio-economic aspects of the affected population, there is a need for an affordable, safe and easy to administer treatment for sickle cell patients.

This chapter explores the general hematologic parameters of sickle cell patients regarding the two core treatments: blood transfusion and hydroxyurea. First, a general visual perspective of the degree of hemolysis in SCD is provided alongside the magnitude of sickle hemoglobin (%Hb S) in the same patients' blood. The next section outlines disease's modifying impact of blood transfusion relative to hydroxyurea treatment vis-à-vis the extent of free plasma hemoglobin (Hb) and erythrocyte-derived microparticles (MPs.), and red cells' membrane deformability. Impact of transfusion on band 3 tyrosine phosphorylation is also explored. Finally, the core biomarkers of anemia (total hemoglobin Hgb and reticulocytes), and their correlation with blood levels of Hb S

was investigated to provide an overview of the influence of treatment on erythropoiesis and a role imatinib or other Syk kinase inhibitors could play under observed circumstances.

3.3 Methods

3.3.1 Quantitation of general hematologic parameters of SCD patients

Except for the case of free plasma hemoglobin, all other kinds of hemoglobin were quantified by Cincinnati Children's Hospital (CCH) hematologists. Hemoglobin electrophoresis was used to determine the content of sickle hemoglobin in patients' blood samples. Additionally, numbers of reticulocytes were also determined in CCH with the aid of flow cytometry.

3.3.2 Quantitation of free plasma Hemoglobin and erythrocyte-derived microparticles

Protocols used to determine amount of free plasma Hb, MPs. and erythrocyte membrane's deformability are as described in Chapter 2, as well as in one of our previous publication.[18]

3.3.3 Quantitation of band 3 tyrosine phosphorylation

Blood samples from healthy donor and SCD patients was centrifuged at 800 rcf for 10 minutes. Using a 1 mL pipette or aspiration, plasma and the white buffy coat were removed leaving behind a pellet of packed erythrocytes. The packed RBCs were washed three times with phosphate buffered saline that is supplemented with 5 mM glucose (PBS-G). After the final wash, packed RBCs were diluted with PBS-G at 1:1 v/v ratio. To prepare ghost membranes for Western blot analysis, 100 µL of the RBC suspension was added to 1.5 mL tubes and immediately placed on ice. This step is followed by addition of 1400 µL of ice-cold ghost buffer (5 mM Na_2HPO_4, 1 mM EDTA) that contains protease and phosphatase inhibitors. Samples were incubated on ice for an additional 30 minutes after which they were centrifuged at 13000 rpm and 4 °C for 15 minutes. The ensuing ghost pellets were washed two times or until the all the pellets are devoid of the hemoglobin's red coloration. After the last wash, 100 µL of 4x SDS loading buffer was added to each tube, boiled at 95 °C for 5 minutes then immediately stored in -20 °C until use. The gel electrophoresis was performed by loading 20 µL of dissolved samples into 10% polyacrylamide gel and run at 100 V for 2 hours (h). The resolved proteins were then transferred onto a

nitrocellulose membrane at 100 V for 2 hours. Immunostaining was performed as described in chapter 2.[18]

3.3.4 Statistical analysis

Setting the confidence level at 95% ($p \leq 0.05$), statistical significance was computed using one-way ANOVA. Unless stated elsewhere within the chapter, error bars are expressed as standard error of the mean, SEM. The *p* value in linear regression graphs is computed based on how significantly different the slope is from zero, with a $p \leq 0.05$ regarded as statistically significant as well.

3.4 Results

3.4.1 Visual perspective of the degree of hemolysis in SCD

Anemia is one of the major hallmarks of SCD. A visual observation of plasma from sickle cell patients and a healthy donor control, show a more intense red coloration (hemoglobin color) among the patients' samples than that visually discernible from the control, **Figure 3.1**, A. Further scrutiny of the percent of sickle hemoglobin in each of the patient's erythrocytes reveals that patients with a high percentage of Hb S in their red cells contain elevated degrees of hemolysis, **Figure 3.1**, B. That the hemolysis levels among the patients below (all undergoing hydroxyurea treatment) varies widely, with some indistinguishable from healthy control, the photographic standpoint provide an appraisal of clinical variability[4,19–23] of the disease.

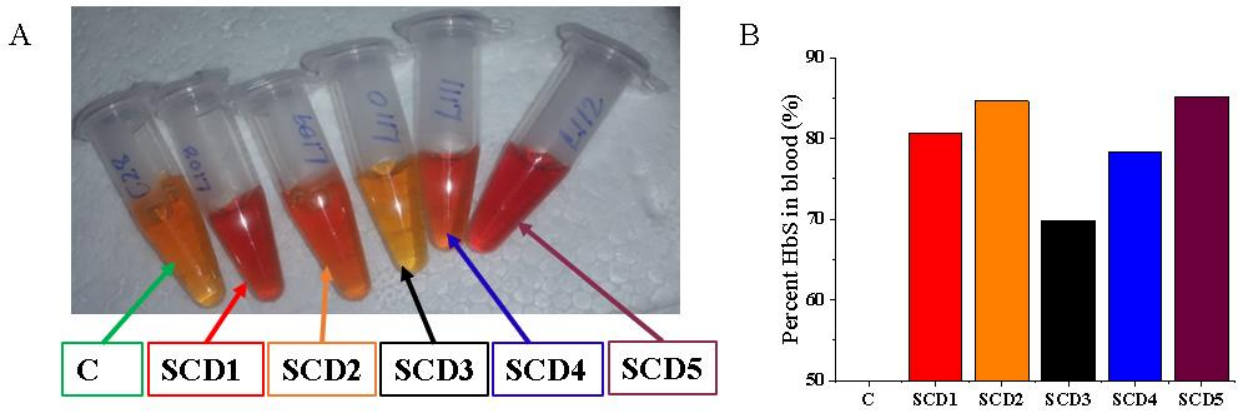

Figure 3.1 Visual perspective of sickle cell disease patients' plasma. Image of plasma from a healthy control (C) and sickle cell disease samples (SCD1 to SCD5), **A**, and sickle hemoglobin content (%Hb S) among SCD patients, **B**.

3.4.2 General hematologic parameters of sickle cell disease

Although sickle blood samples have been repeatedly shown to contain elevated concentrations of both cell-free Hb[24–30] and RBC-derived MPs,[31–38] variabilities in these parameters[4,19–23] have precluded correlating their magnitudes with any of the proposed molecular mechanisms of SCD. To determine whether this variability might have arisen from differences in the therapy received by the patients, comparison of the same parameters in SCD patients undergoing treatment with hydroxyurea versus those receiving chronic blood transfusions was explored. As expected, the percent Hb S in any patient's blood sample depended strongly on the therapy received, with Hb S content being more than twice as high in non-transfused patients as transfused patients (**Figure 3.2**, A). Reflecting real-life conditions, where compliance to hydroxyurea-treatment is not always perfect, the concentration of free Hb in the plasma was higher in hydroxyurea-treated than transfused patients (**Figure 3.2**, B), and RBC-derived membrane microparticles were similarly found more abundant in hydroxyurea-treated than in transfused samples (**Figure 3.2**, C). However, the observed difference is not statistically significant, an aspect which could be linked to wide variability in disease symptoms among SCD patients. Due to dilution of sickle blood with healthy erythrocytes during transfusion, the observed intermediate degree of red cell membrane deformability of transfused patients relative to healthy control and hydroxyurea-treated patients is not surprising (**Figure 3.2**, D).

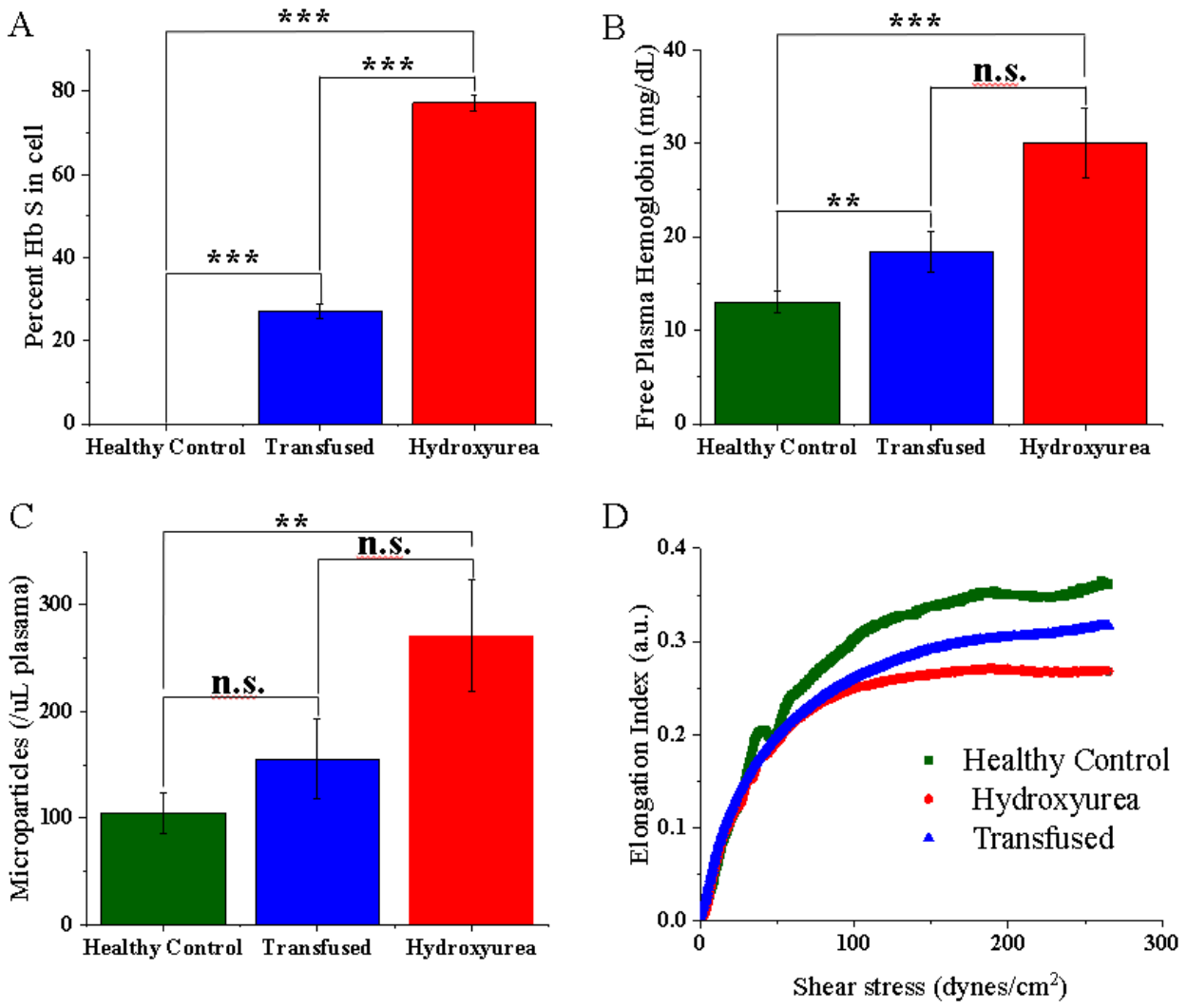

Figure 3.2 General profile of sickle cell patients' hematologic parameters. Average percent of sickle hemoglobin in blood samples from SCD patients undergoing treatment with either hydroxyurea (~77%) or blood transfusion (~27%), **A**. Measurement of free plasma Hb in the indicated patient populations, **B**. Measurement of RBC-derived microparticles in plasma from the indicated patient populations, **C**. Analysis of the deformability of erythrocyte membranes derived from the indicated patient populations, **D**. Error bars are expressed as standard error of the mean. (**A**: n=15 and 53 for transfused and hydroxyurea; **B:** n=24, 9 and 48 for healthy, transfused and hydroxyurea; **C:** n=18, 9 and 47 for healthy, transfused and hydroxyurea; respectively)

3.4.3 Quantitation of band 3 tyrosine phosphorylation

Because healthy erythrocytes will overpopulate sickle cells following blood transfusion, the degree of band 3 tyrosine phosphorylation in erythrocytes from this population of patients would be expected to be intermediate of that observed in healthy donors' and non-transfused patients' samples. As reported elsewhere[18] and as shown in **Error! Reference source not found.**,

A and B, this contention is reinforced. Of note, is the statistically significant higher degree of phosphorylation in transfused patients than healthy controls which points out that the effects of phosphorylation-induced membrane weakening are also present among sickle cell patients receiving blood transfusion.

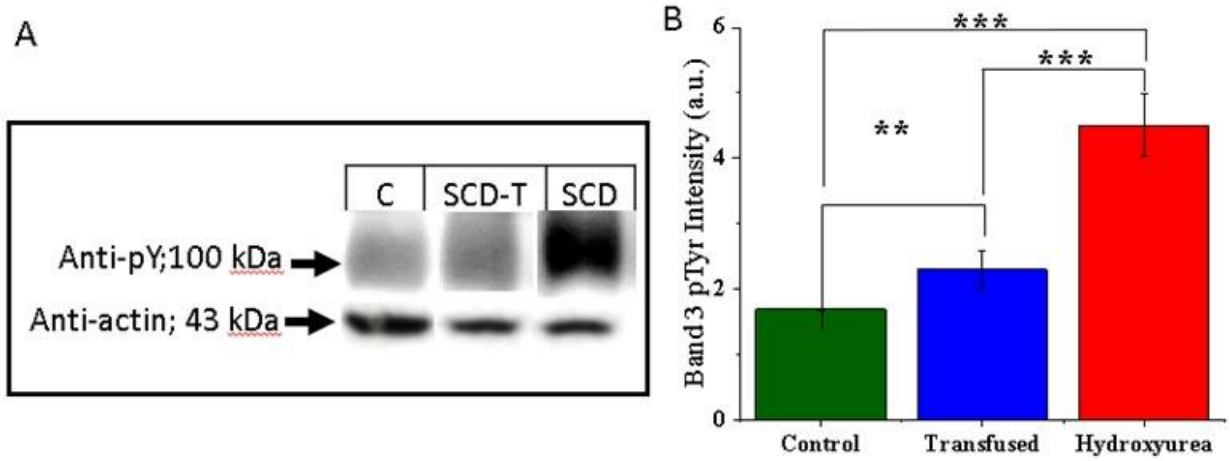

Figure 3.3 Band 3 tyrosine phosphorylation intensity in erythrocyte membranes from healthy donor (C), transfused (SCD-T) and hydroxyurea-treated (SCD) sickle cell patients. Immunostaining of RBCs' actin is shown as a loading control. The 100 kDa band is band 3, panel **A**. Quantified band 3 tyrosine phosphorylation intensity from Image J densitometry (n=7, 5 and 16 for control, transfused and hydroxyurea, respectively), panel **B**. Error bars are expressed as standard error of the mean.

3.4.4 Anemic biomarkers of transfused and hydroxyurea-treated patients are indistinguishable

After characterization of hematologic parameters and extent of band 3 tyrosine phosphorylation, the next question was to examine whether the two different treatments have considerably different effects on anemia biomarkers. **Figure 3.4** illustrates a similar baseline level of total hemoglobin (Hgb) and reticulocytes in blood from transfused and hydroxyurea-treated patients. For reference, the healthy Hgb and reticulocytes ranges are 10-15 g/dL[39] and 0.8-2.5% [40] respectively. That the average number of reticulocytes is the same between these two core therapies, suggest absence of a substantial difference in erythropoiesis. A different research group reported a similar observation between transfused and untreated patients.[41] Given that the severity

of sickle cell can be assessed based upon reticulocytosis, these data imply that the patients in subject may have identical disease status.

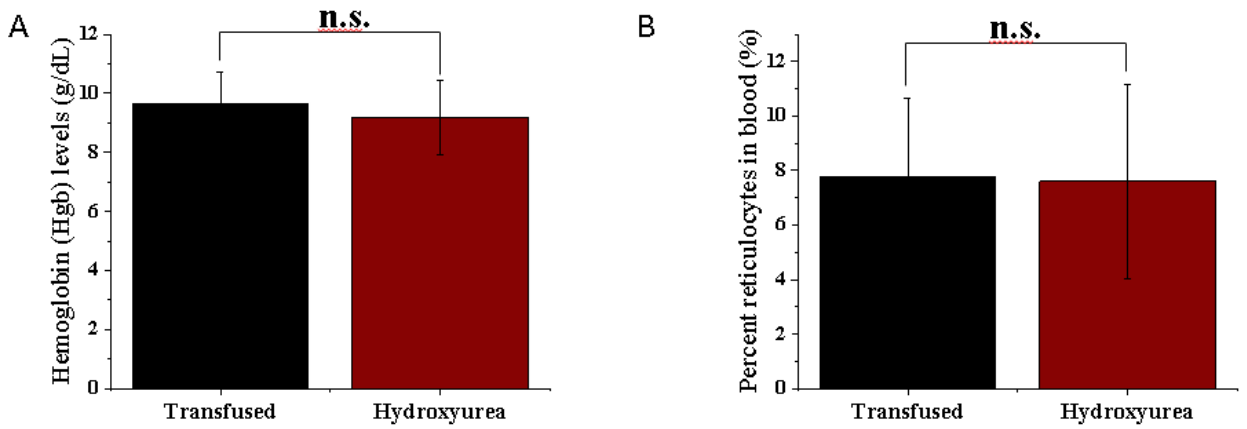

Figure 3.4 Comparison of anemia biomarkers of transfused (n=15) and hydroxyurea-treated (n=34) SCD patients: A. Quantity of total hemoglobin, and **B.** reticulocytes among sickle cell patients. Error bars are expressed as standard deviation.

3.4.5 Correlation of total hemoglobin, Hgb with reticulocytes

Linear regression plots were employed to test whether transfusion and hydroxyurea treatment modify correlation of total hemoglobin with reticulocytes. As illustrated in **Figure 3.5**, both patients' groups display a moderate negative correlation between Hgb and percent reticulocytes (Pearson's r=-0.4 to -0.5). However, the correlation of the two parameters is weaker in transfused patients (panel A) than it is among hydroxyurea-treated patients (panel B).

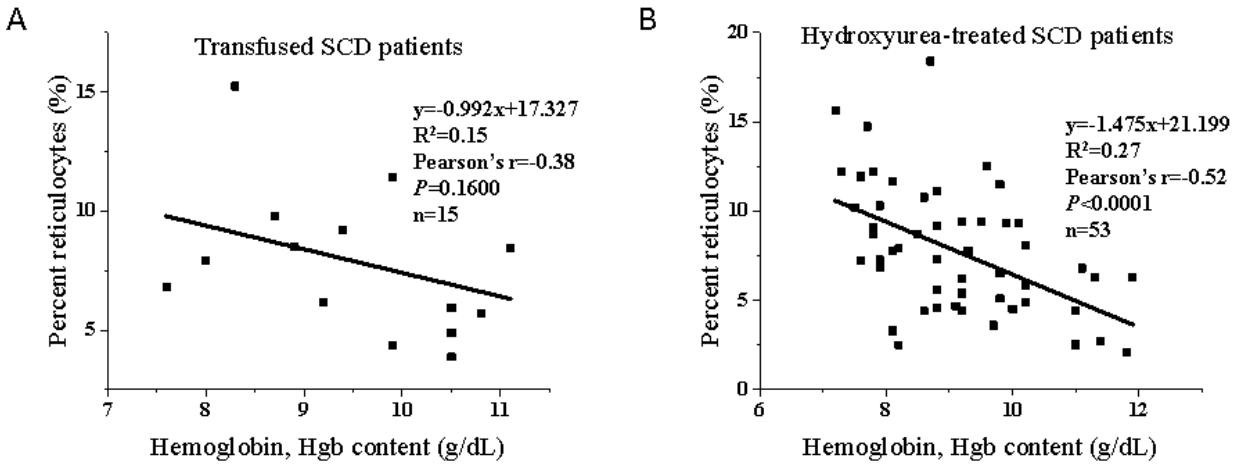

Figure 3.5 Correlation of total hemoglobin with reticulocyte numbers in SCD patients treated with transfusion **(A)** or hydroxyurea **(B).**

3.4.6 Correlation of sickle hemoglobin, Hb S with reticulocytes

High content of percent sickle hemoglobin, Hb S among sickle cell patients is known to associate positively with vaso-occlusive episodes[42–45] and this in turn is expected to cause destruction of red cells within circulation. The ensuing anemia is unequivocally envisioned to trigger explosive erythropoiesis. High levels of circulating reticulocytes indicate heightened erythropoiesis. The observed positive correlation of %Hb S with reticulocytes further confirm this long-held tenet. As is the case with total hemoglobin, correlation of %Hb S with reticulocytes in transfused patients (**Figure 3.6**, A) is weaker than that observed in non-transfused SCD patients (**Figure 3.6**, B). In this case however, the correlation in hydroxyurea-treated patients is very strong (Pearson' r =0.74)

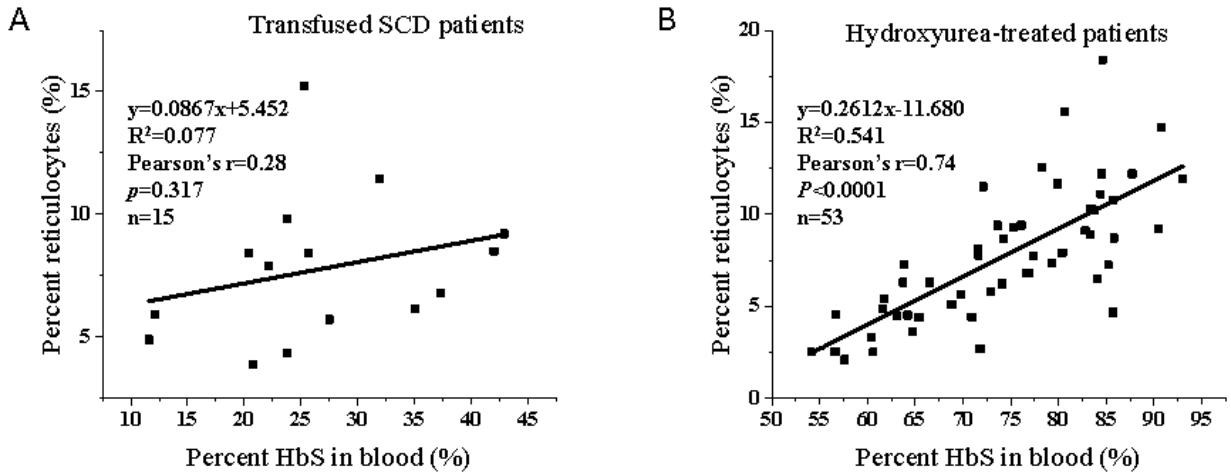

Figure 3.6 Correlation of sickle hemoglobin with reticulocyte numbers in SCD patients treated with transfusion **(A)** or hydroxyurea **(B).**

3.5 Discussion

Theoretically, one can imagine that replacing sickle erythrocytes with healthy blood would completely solve the problems of sickle cell disease since healthy red cells would dominate erythrocytes involved in oxygen delivery throughout the body. However, continuous replenishment of defective erythrocytes through an above normal rate of erythropoiesis, washes out the transfused healthy blood leading to interminable cycles of blood transfusions.[46] In time, an inevitable iron build-up in the patients supervenes. To correct the iron overload, patients under chronic blood transfusion are often prescribed to iron chelation therapies[47,48] and therapeutic phlebotomies,[49,50] notwithstanding the associated costs.[51,52] Additionally, unforeseen immune response in the form of delayed hemolytic transfusion reactions,[16,53–55] poses major risks for chronically transfused SCD patients. While blood transfusion unequivocally saves lives; principally in the short term, its extended use plainly causes untenable burden to the recipients.

Despite the >70% of healthy blood in transfused patients (**Figure 3.2**, A), the elevated levels of free plasma hemoglobin and microparticles among the patients relative to healthy control, is suggestive of an incessant systemic process of damage of RBCs. The release of Hb and MPs. can be attributed to the elevated degree of band 3 tyrosine phosphorylation. Although in-depth examination of band 3 tyrosine phosphorylation among SCD patients' blood samples revealed a substantially reduced tyrosine phosphorylation among transfused patients relative to non-transfused patients, the moderately elevated tyrosine phosphorylation can still be reversed by

imatinib.[18] As a result, imatinib may slow down hemolysis and fragmentation of the sickle cell population and would possibly increase the timespan between transfusions, which, in essence, may reduce rates of iron overload. However, due to overpopulation of sickle erythrocytes with healthy donor blood, transfused patients may not be well suited for an initial proof of concept clinical trial of imatinib stated in chapter 2.

That the average number of reticulocytes of the patients under investigation is more than twice the normal range[40] indicates an ongoing disease status, with little if any, disease modification. Because both blood transfusion and hydroxyurea treatment display similar baseline levels of reticulocytes, neither treatment appear to alleviate the systemic impact of sickle hemoglobin. It is therefore with the best interest of sickle cell patients that new studies ought to put more emphasis on reducing premature destruction of red cells. Presciently, the advent of imatinib may offer novel avenues directed at increasing the lifespan of erythrocytes which would sequentially solve the lifelong anemia, organ damage and the inevitable hospitalizations holistic to SCD patients.

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CHAPTER 4. IMPACT OF AGE ON SEVERITY OF SICKLE CELL DISEASE; AN OVERVIEW OF PATIENTS YOUNGER THAN 2 YEARS

4.1 Abstract

Complications of sickle cell disease start to arise when expression of fetal hemoglobin (Hb F) switches to sickle hemoglobin (Hb S). Under healthy conditions, the switch is hasty and often reaches baseline levels at 6-10 months of age while children suffering from sickle cell disease experience persistently elevated levels of Hb F up to 24 months after birth. Since high Hb F content ameliorates impacts of SCD due to its high oxygen binding affinity and anti-sickling properties, clinicians have turned to hydroxyurea to induce production of Hb F containing erythrocytes. The therapy has experienced more success than any other there is. However, response to the therapy has remained highly variable and therefore undermines an all-inclusive and affordable remedy for SCD sufferers. With the non-responsive patients constituting nearly 50% of the SCD population under hydroxyurea treatment, more therapy is clearly needed for the other defenseless patients. Additionally, hydroxyurea treatment does not fully address impacts of the disease even in the responsive patients. In this chapter, an illustration of the impacts of Hb F more so in younger patients on hematologic parameters will be discussed. Although high levels of hydroxyurea are associated with less indicators of membrane weakening, suggesting better blood rheology, the fact that reticulocytes levels remain more than double the levels of healthy individuals, accounts for the need to rescue the population of erythrocytes that contain high content of Hb S to minimize the burdensome erythropoietic process among the patients. As already pointed out that band 3 tyrosine phosphorylation is an active process in sickled erythrocytes, tyrosine kinase inhibitors present promising treatment for all sickle cell patients and may conceivably delay the onset of SCD complications especially when used alongside hydroxyurea.

4.2 Introduction

Sickle cell disease (SCD) is a genetic disorder that manifest early in life and remains a problem throughout a patient's life. The disease affects mostly people of African descent and over 75% of the global patients reside in Africa. In Africa as well as other indigent regions, children barely survive past young adulthood.[1] Not only do the children loose life from sickle cell disease

complications but also suffer victims from co-morbidities such as meningitis, pneumonia, malaria and HIV/AIDs.[2,3] The advent of newborn screening for early disease detection, vaccination against meningitis and prophylactic use of penicillin to ward off bacterial infections, has enabled young children to live past young adulthood.[2] Nonetheless, these preventative care stratagems do not address vaso-occlusion, the main causal event of SCD suffering, at any point in life.

The extent of SCD's severity increases with patients age and as a matter of fact, infants younger than 2 years show a paucity of symptoms due to high content of fetal hemoglobin (Hb F).[4] As infant's fetal hemoglobin continues with its natural decline and its consequential replacement with sickle hemoglobin (Hb S), complications and lethality of the disease beget lifelong episodic suffering.[4] To prevent the toxic events triggered by Hb S, clinicians have turned into use of hydroxyurea therapy to enhance production of erythrocytes that contain fetal hemoglobin. This treatment tactic has contributed immensely to lengthened lifespan of the patients wherever it is practiced. However, induction of Hb F using hydroxyurea modulation has not been effective for all patients[5–8] and, given the lifelong nature of SCD, the effectiveness of the therapy may not be sustainable especially later in life when chronic organ dysfunction takes centerstage.[9] Additionally, responsive patients are still prone to vaso-occlusive crisis[8,10] thus the battle to improve quality of the prolonged lifespan remains a crucial aspect that needs new therapies to minimize such episodic events.

Sickling of erythrocytes following polymerization of sickle hemoglobin (HbS) has been the basis of vaso-occlusive crisis.[11–13] However, recent developments have proved that adherence of cells to the microvasculature,[10,14–16] activation of coagulation cascades,[14,17,18] depletion of nitric oxide (NO)[19–21] and a general poor blood rheology[10,22] substantially contribute to initiation of vaso-occlusive crisis. As explained in chapter 2, free plasma hemoglobin and erythrocyte-derived microparticles (MPs.) contribute to the upstream causal events of the above stated findings. Briefly, cell free Hb scavenges NO,[23,24] and its associated heme activate the endothelium to express adhesion receptors[15] and induce oxidative stress within the cells and in the extravascular milieu.[25–27] Microparticles on the other hand are largely responsible for initiation of coagulation through activation of thrombin.[28,29] Additionally, production of free plasma Hb and MPs. reduces the cells' volume which heighten intracellular concentration of Hb S (thus more Hb S polymerization) and make the cells more rigid culminating in poor blood flow.[30,31] Inhibiting the release of these upstream stimulants (Hb and Mps.) would likely prevent or minimize initiation of vaso-occlusive

crisis and reduce rates of erythropoiesis among the young. Since children suffering from SCD still have well-functioning organs, reducing the toxicities associated with free Hb/heme and MPs. may likely help minimize overworking of several organs such as the spleen and liver.

Studies on erythrocytes derived from SCD patients (Chapters 2 and 3), established an extensive tyrosine phosphorylation of band 3.[32] Since this phosphorylation is present in all sickle cells, and that it is the critical element triggering membrane destabilization through discharge of free plasma Hb and MPs., its inhibition could constitute an effective treatment for every SCD patient. This chapter ushers in new information on the role of age, and present grounds with which imatinib or Syk inhibitor's therapy offers a viable treatment for younger patients. While band 3 tyrosine phosphorylation largely depends on the %Hb S in blood, and that the intensity of this phosphorylation is not dependent on age, this chapter demonstrates that younger patients have lower levels of free plasma hemoglobin (Hb) and erythrocyte-derived microparticles (MPs).

4.3 Methods

The methods employed in this chapter for measurement of hematologic parameters, enumeration of erythrocytes-derived microparticles, quantitation of free plasma Hb, and determination of band 3 tyrosine phosphorylation are as detailed in chapter 2 and elsewhere.[32]

4.4 Results

4.4.1 Hemoglobin profile of age grouped SCD patients

To understand sickle cell disease's severity in patients younger or older than 2 years, an analysis of baseline hemoglobin content will be provided, and comparative conclusions made. On average, %Hb S in blood among patients younger than 2 years is lower than that observed in patients over 2 years of age; **Figure 4.1** A. To gauge the impact of age on responsiveness to hydroxyurea, a comparison of the extent of Hb F expression was also examined. As expected, and in agreement with **Figure 4.1** B, %Hb F was found to be higher in the younger patient population. Whereas the differences observed herein are not statistically significant, the data convey a better response to hydroxyurea among patients younger than two years, which agrees with previous reports.[9]

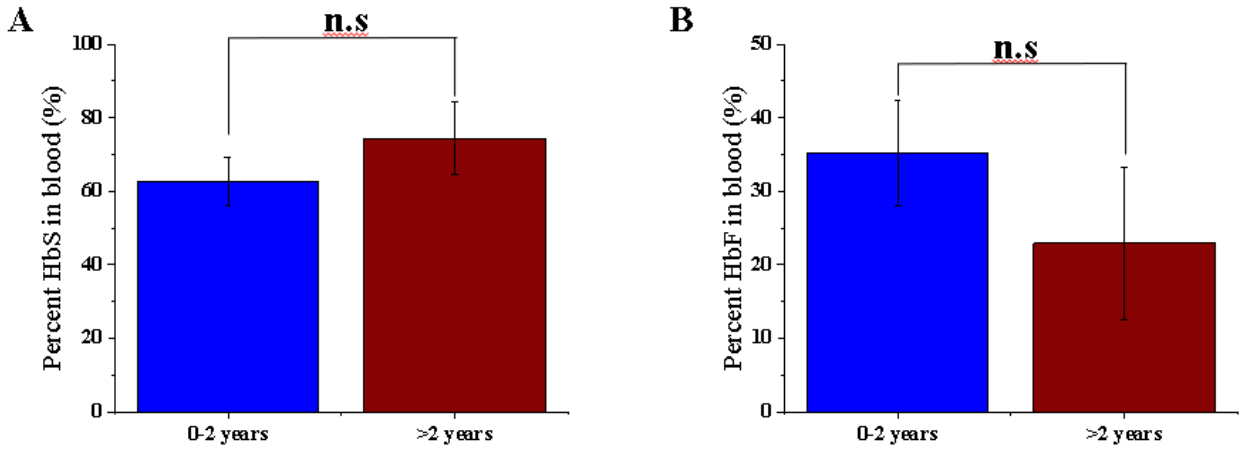

Figure 4.1 Hemoglobin content in blood from hydroxyurea-treated SCD patients above and below 2 years of age: A, Percent sickle hemoglobin (%Hb S), and **B** percent fetal hemoglobin (%Hb F). Error bars are expressed as standard deviation. (n= 6 for 0-2 years and n= 53 for >2 years populations)

4.4.2 Hematalogic markers of membrane stability

Patients under 2 years display significantly lower amounts of free plasma Hb ($p<0.001$) and erythrocyte-derived microparticles ($p<0.001$) in circulation relative to older patients (**Figure 4.2** A and B). There is no statistically significant difference on the amount of microparticles of patients under 2 years and healthy controls. However, free plasma Hb in healthy control is substantially lower than that observed in patients under 2 years ($p=0.0495$). On the other hand, patients older than 2 years have significantly elevated levels of free plasma Hb and MPs. relative to healthy control.

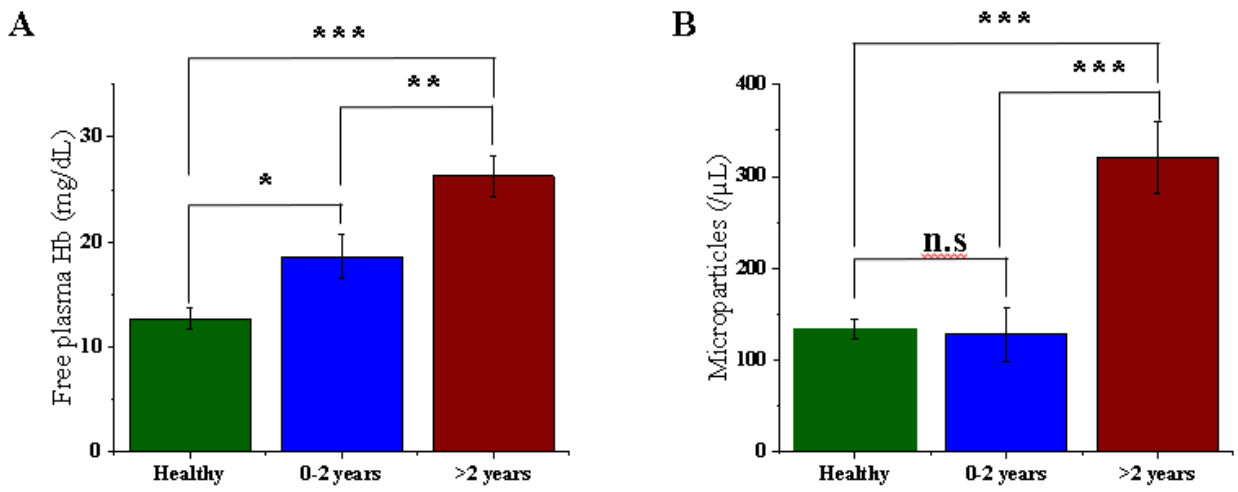

Figure 4.2 Free plasma Hb (A) **and red cell-derived microparticle** (B) **content in the plasma of hydroxyurea-treated SCD patients above and below 2 years of age. (**n=6, 15 and 30 for healthy, 0-2 year, and >2 years populations, respectively).

4.4.3 Band 3 tyrosine phosphorylation

Despite the erratic nature of sickle cell disease, children under two years consistently display low levels of free plasma Hb and MPs. Since the extent of band 3 tyrosine phosphorylation is determined by the content of sickle hemoglobin, and that all non-transfused patients display elevated levels of band 3 tyrosine phosphorylation, an increase in phosphorylation in this age group is expected. When compared to healthy controls, the degree of band 3 is significantly elevated, **Figure 4.3**. Although band 3 tyrosine phosphorylation is elevated, the high amount of %Hb F may play a major role in contributing to the low amounts of free Hb and MPs. while the %Hb S concurrently give rise to a high level of band 3 tyrosine phosphorylation.

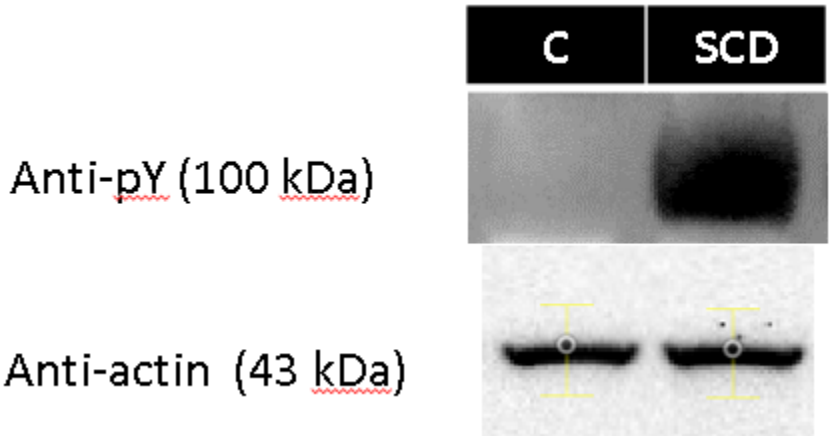

Figure 4.3 Band 3 tyrosine phosphorylation of: a healthy control (C), and a non-transfused 2-year-old child suffering from sickle cell disease (SCD).

4.4.4 Analysis of biomarkers of anemia in patients under and over 2 years of age

The principal biomarkers of anemia; a SCD's severity indicator, suggest a better health status for children under two years. With both age populations under steady state with respect to total hemoglobin content, the lower levels of reticulocytes suggest a slower rate of hemolysis on patients under 2 years of age, **Figure 4.4** A and B. This observation is attributed to the lower % Hb S and higher % Hb F present in the younger patients relative to patients older than 2 years.

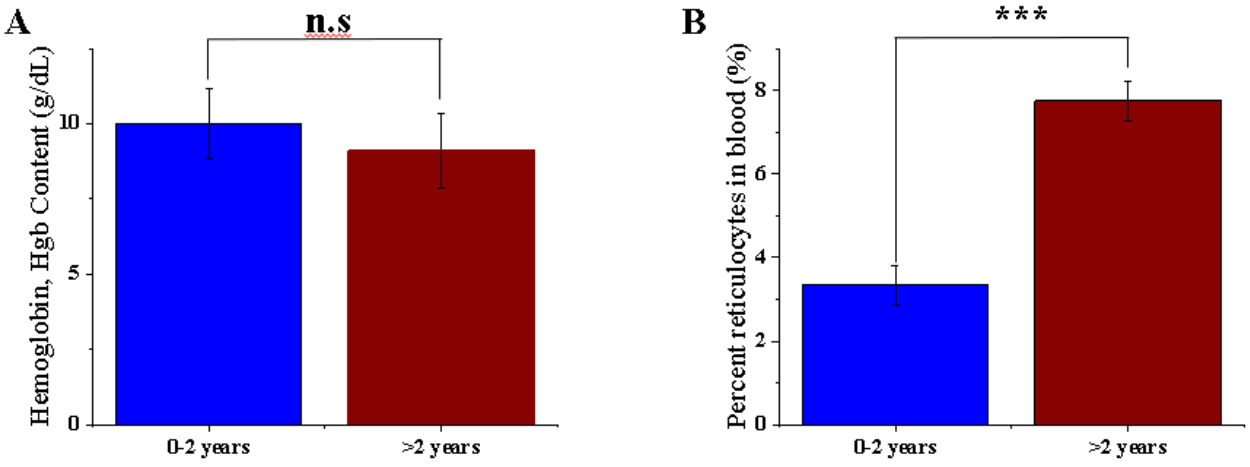

Figure 4.4 Analysis of biomarkers of anemia: A. total hemoglobin, and **B.** percent of reticulocytes in patients' blood. **(**n=6 for 0-2 years, and n=53 for >2 years populations, respectively).

An investigation on linear correlation of age to total hemoglobin and reticulocytes contents among SCD patients shows a weak negative correlation (Pearson's r=-0.27) of age with total hemoglobin which is not statistically significant (p=0.127), **Figure 4.5**, A. With respect to reticulocytes, however, a statistically significant (p=0.00275) and moderate positive (Pearson's r =0.5) correlation of age and reticulocyte content was observed, **Figure 4.5**, B. Based on the reticulocytes data herein, the positive correlation with age illustrates the increase in severity of the disease as patients age which is also an indication of a decline of efficacy of hydroxyurea treatment.

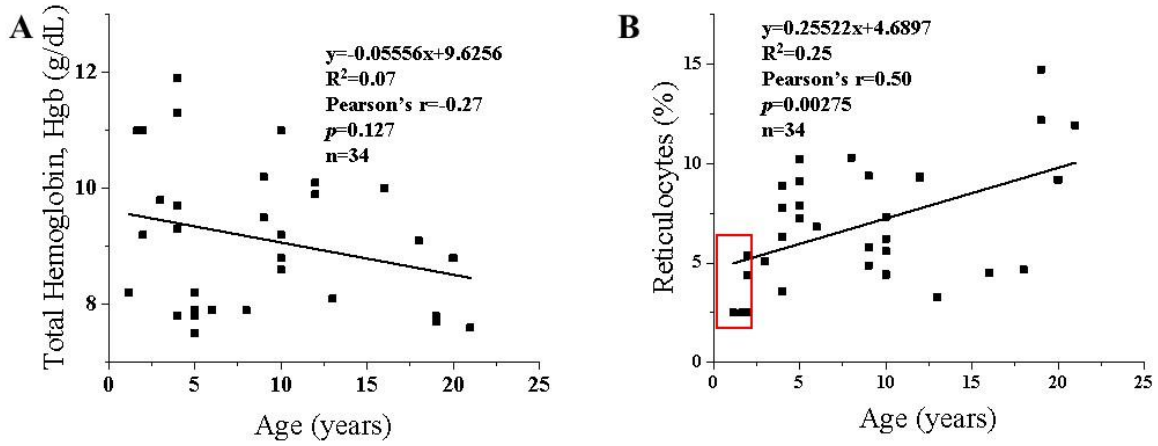

Figure 4.5 Effect of age on biomarkers of anemia. Correlation of age with total hemoglobin, **A,** and reticulocytes content among SCD patients, **B**. The red box in panel **B** highlights the region in which patients aged ≤2 years fall in on the scatter plot.

4.4.5 Effect of fetal hemoglobin on anemia

To firmly establish that the high content of fetal Hb is also a major contributing factor to a better health status in SCD patients under and over 2 years, linear regression analysis was performed to explore correlation of fetal Hb with total hemoglobin and reticulocytes' content in the patients under investigation. As expected, fetal Hb varies linearly with percent reticulocytes in blood and total hemoglobin with statistically significant (p=0.00003 and 0.0003) strong negative and positive correlations (Pearson's r=-0.61 and 0.54), respectively (**Figure 4.6**, A and B). Others have observed that hydroxyurea induced Hb F ameliorates the impacts of SCD on patients.[33–35]

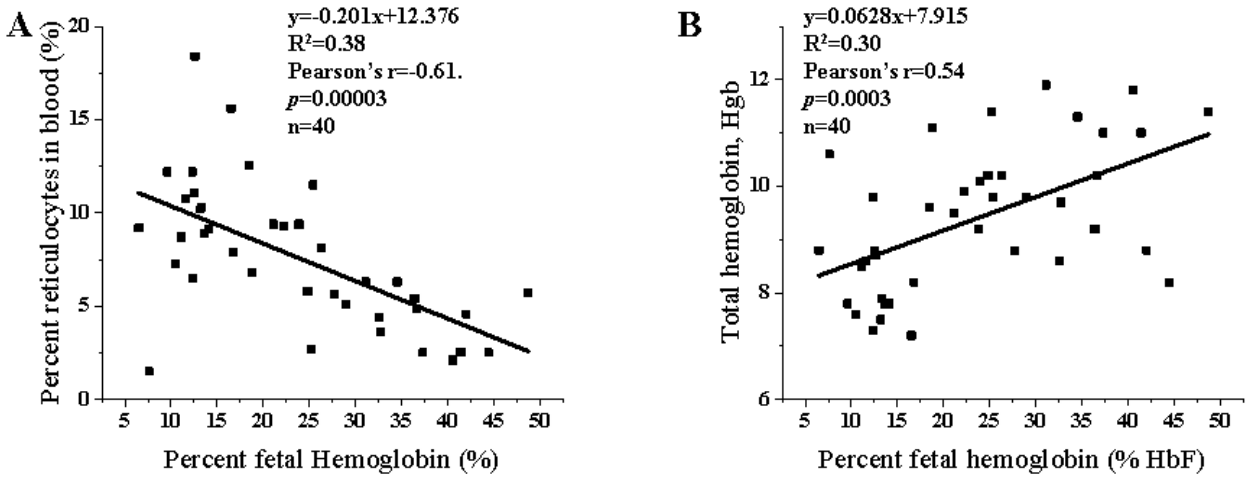

Figure 4.6 Correlation fetal hemoglobin on biomarkers of anemia: A. correlation of % Hb F with percent reticulocytes, and **B.** correlation of % Hb F with total hemoglobin in blood.

4.4.6 Representative view of band 3 tyrosine phosphorylation and hematologic parameters

For further clarity, a representation of the aspects already discussed above are presented herein. In **Figure 4.7**, an immunoblot featuring extent of band 3 tyrosine phosphorylation is provided alongside blood's content of Hb S, Hb F, and reticulocytes from the same representative patient samples. As the main therapies, patients SCD-1 and SCD-2 are on hydroxyurea while patient SDC-T1 is under chronic blood transfusion. Patient SCD-1 has the highest Hb S content and band 3 tyrosine phosphorylation intensity. Moreover, the same patient displays highest reticulocytes content which reflects the highest rate of destruction of red cells. On the other hand, patient SCD-2 contains topmost Hb F and intermediate degree of band 3 tyrosine phosphorylation intensity. In this patient and similarly to patients under 2 years of age, the high Hb F content alongside lowest reticulocytes suggest that the 37% Hb F content is responsible for slowed down anemic level. Lastly, patient SCD-T1 has the lowest band 3 tyrosine phosphorylation signal yet high reticulocyte count. This transfused patient is a testament that there is an elevated and ongoing destruction of red cells suggesting that the transfused blood is not enough to slow down the systemic harmful impact of the disease.

Taken together, these events and observations point out at the importance of maintaining membrane stability/elasticity using imatinib or Syk kinase inhibitor in treatment of sickle cell disease. Inhibiting band 3 tyrosine phosphorylation will slow down the hemolysis rates of the

population of affected cells and hence reduce abnormally high rates of erythropoiesis that would otherwise execute a systemic burden on the patients.[36–38]

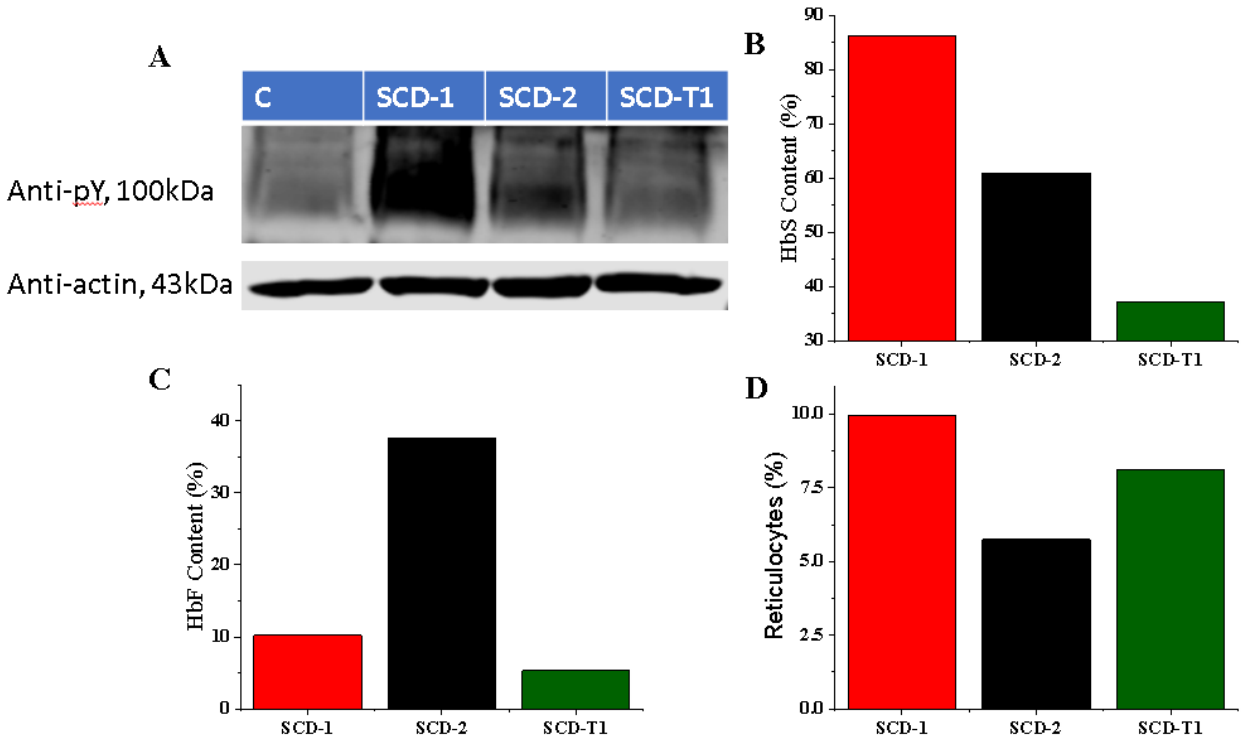

Figure 4.7 Representative of patients' samples illustrating band 3 tyrosine phosphorylation (**A**) and corresponding hematologic parameters of the same patients (**B-D**). [C stands for healthy control, SCD-1 and SCD-2 represents patients under hydroxyurea therapy, SCD-T1 is a sickle cell disease patient under chronic blood transfusion].

4.5 Discussion

Combination of low levels of Hb S and high content of Hb F is associated with reduced free plasma Hb and MPs. suggesting more stable erythrocytes' membranes (**Figure 4.1** and **Figure 4.2**). Because the average Hb F of patients ≤2 years old is more than a third of total Hb, the improved indicators of membrane stability (i.e., low levels of free Hb and MPs.) are unsurprising given that Hb S content influences the extent of band 3 tyrosine phosphorylation and the concomitant release of free hemoglobin and microparticles.[32] The population of cells containing high levels of fetal hemoglobin not only interferes with Hb S polymerization[39] but also provide a healthy alternative that is more efficient in oxygen carrying capacity.[4,40] Moreover, this group of cells has an improved lifespan[41] which in turn may prevent acceleration of anemia.

Although organ's functions is optimal for majority of SCD patients at earlier ages of life,[22,23,37] and that better organ function would quickly remove free Hb and MPs. from circulation,[19] the diminished reticulocytes among patients under 2 years of age strongly suggest improved survival of red cells. Whereas long lifespan of F-cells (Hb F containing cells) is unequivocal, survival of sickled erythrocytes (non-F cells) remains elusive.[41] However, the low levels of free plasma Hb and MPs. somewhat imply enhanced lifespan for the sickle RBCs population possibly due to improved blood rheology resulting from abundance of the vasodilator, nitric oxide, and reduced coagulation that would otherwise stem from excess free plasma Hb consumption of NO[15,19,20,42,43] and triggering of coagulation by MPs.[18,24,28]

Inhibiting the residual band 3 tyrosine phosphorylation will target the sickle cell population, improve their lifespan and could consequently lower reticulocyte count close to healthy baseline levels. Whether imatinib or a relevant Syk kinase inhibitor is used alone or in combination with hydroxyurea/blood transfusion, the treatment regimen constitutes a viable and all-inclusive approach for managing sickle cell disease. Concerns that imatinib and majority of kinase inhibitors may not be useful for children owing to their dwarfism side effects can be addressed with more selective Syk inhibitors. With young patients[44,45] and the common ischemia-reperfusion[46,47] in mind, synthetic research aiming to make selective Syk inhibitors should attempt to design compounds with little effect on growth factor receptors. More selective Syk inhibitors are already on the horizon,[48–50] offering a glimpse of hope that a better kinase inhibitor could soon be unveiled.

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CHAPTER 5. INVOLVEMENT OF PROTEIN TYROSINE PHOSPHATASE 1B IN ELEVATION OF BAND 3 TYROSINE PHOSPHORYLATION IN SICKLE CELL DISEASE

5.1 Abstract

Protein tyrosine phosphatases and protein tyrosine kinases constitutively function in mediating innumerable physiologic processes within several cell types. In human erythrocytes, the constitutive action of tyrosine kinases and tyrosine phosphatases maintains a low profile of membrane proteins' tyrosine phosphorylation. Down-regulation or overactivation of one enzyme without commensurate reduction or increase in activity of the other, respectively, would interfere with normal cell function or survival. In particular, the pro-oxidant state in sickle cell disease induces oxidative inhibition of protein tyrosine phosphatases. Additionally, spleen tyrosine kinase (Syk) has been shown to be overexpressed in sickle cell disease relative to healthy controls. Taken together, these events culminate in an increase in phosphorylation state of membrane proteins, most notably membrane protein band 3. Further exploration of the phosphatases responsible for band 3 dephosphorylation in sickle cell disease revealed that protein tyrosine phosphatase 1 B (PTP1B) is cleaved to generate a lower molecular weight fraction alongside the full-length enzyme. While this is a process that has been documented in several cell types such as platelets, there is no published evidence of this type of cleavage in sickle cell disease. Conceivably, the increased calcium influx in sickle cells may be responsible for catalyzing this type of PTP1B cleavage. The PTP1B is the major enzyme responsible for dephosphorylation of band 3 and its inhibition would, or cleavage might, be responsible for the observed elevated band 3 tyrosine phosphorylation in sickle cell disease as demonstrated in this chapter.

5.2 Introduction

Protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) work constitutively in regulation of several cells' physiologic processes.[1,2] Due to the complimentary action of the PTPs and PTKs, a dysregulation of the role of either would dramatically impact the cell's function and/or survival resulting in myriad diseases.[3–6] One of the most widely studied and known effect of phosphatases on health is the negative regulation of insulin by protein tyrosine

phosphatase 1B (PTP1B).[7] Protein tyrosine phosphatase 1B is ubiquitously expressed in several cell lines and it is also implicated in development of different cancers such as colon and breast cancers.[8]

In vitro, PTPs can be inhibited by oxidants, such as diamide and hydrogen peroxide/pervanadate,[9–11] thiol-alkylating agents e.g. N-ethylmaleimide,[9,12] and phosphatase inhibitors (principally sodium orthovanadate, OV).[13,14] When healthy erythrocytes are exposed to any of these pharmacological agents, a sudden increase in protein tyrosine phosphorylation, most notably on protein band 3, is observed.[15,16] Band 3 is a prominent red cell membrane protein which constitutes >25% of cell's membrane dry matter content.[17–19] Upon its tyrosine phosphorylation, a global membrane destabilization ensues leading to membrane fragmentation and hemolysis.[16,20,21] Vesiculation and fragmentation (discharge of microvesicles/microparticles; MPs) has been documented to happen as a result erythrocyte senescence.[22–25] In its own entirety, erythrocyte senescence is accompanied by gradual cell shrinkage and a concomitant increase in band 3 tyrosine phosphorylation as a result of membrane loss resulting from activation of tyrosine kinases and a gradual loss of protein tyrosine phosphatase activity.[22,25,26] It's also considered that red cells (RBCs) get rid of compromised membrane fragments through this process.[27,28]

As stated in the preceding chapters, sickle cell disease is characterized by heightened oxidative stress arising from denaturation of the unstable sickle hemoglobin (Hb S)[29–31] and a collateral depletion of anti-oxidant molecules/enzymes such as glutathione, and free heme- and hemoglobin-detoxifying agents – hemopexin and haptoglobin respectively – which ultimately renders the cell defenseless against oxidant damage and the accompanying inflammation.[30,32,33] This oxidative stress is linked to several complications of SCD such as inflammation, acute chest syndrome and pulmonary hypertension.[33–35] In fact anti-oxidants have been used to ease SCD's suffering as reviewed in chapter 1, suggesting the deleterious impacts of oxidative stress in sickle cell disease.[29,36]

Following the oxidative stress and the elevated Band 3 tyrosine phosphorylation in SCD,[5,37] it is conceivable that erythrocyte's PTPs could be inhibited in SCD and that the observed band 3 tyrosine phosphorylation could also arise from this phenomenon. Protein tyrosine phosphatases identified in human erythrocytes include the SH2-domain containing phosphatases, SHP-1[38] and SHP-2,[39] and protein tyrosine phosphatase 1B (PTP1B).[26] All the three PTPs have been reported

to dephosphorylate band 3 of healthy erythrocytes, however, their specific functions in SCD have not been explored.

This chapter documents the involvement of erythrocyte's PTP1B in band 3 tyrosine phosphorylation. First, an overall phosphorylation profile of SCD red cell membrane proteins will be provided followed by evidence of reduced phosphatase activity in sickle cells. Next, the chapter reveals that inhibitors of PTP1B are capable of inducing band 3 tyrosine phosphorylation. Although PTP1B is the major phosphatase responsible for dephosphorylating band 3,[22,26] SHP-2 and SHP-1 phosphatases were also investigated and since specific inhibitors of SHP-1 and SHP-2 did not induced band 3 tyrosine phosphorylation in healthy cells, they are not investigated any further in this study. An illustration of PTP1B cleavage follows as a plausible inhibitory mechanism. Overall, these information provide one of the mechanistic processes responsible for band 3 tyrosine phosphorylation in sickle cell disease.

5.3 Methods

5.3.1 Phosphorylation of erythrocyte membrane proteins from sickle cells

Blood samples from sickle cells patients were obtained with informed written consent and in accordance with Cincinnati Children's Medical Hospital IRB. After blood withdrawal, the samples were shipped overnight to Purdue University. The patient samples were subjected to centrifugation at 800 rcf for 10 minutes at room temperature. After separating plasma from packed red blood cells, the erythrocytes were washed three more times with phosphate buffered saline containing 5 mM glucose (PBS-G). Erythrocyte ghost membranes were immediately prepared by mixing 1:15 volume of packed RBCs with ghost buffer (5 mM NaH_2PO_4, 1mM EDTA) supplemented with phosphatase (sigma #P5726 and P0044) and protease (Bimake #B14001) inhibitor cocktails. Resulting suspension was incubated on ice for 30 minutes followed by centrifugation at 13000 rpm and 4 °C for 15 minutes. Membrane ghosts were washed 3 times or until they become white (indicative of complete loss of hemoglobin from the ghosts).

With minor modifications of the cold harbor springs recipe,[40] samples were prepared for Western blotting by solubilizing the ghosts in 4x Laemmli buffer containing 0.5M dithiothreitol (DTT), protease and phosphatase cocktail inhibitors. Samples were heated up to 90 °C for 5 minutes after which they were stored in -20 °C until use (used within one week of storage).

Electrophoresis was performed by loading 20-30 µL of samples onto 10% SDS-PAGE gels, run at 90, 120 and 150 volts for 30, 45 and 20 minutes, respectively. Proteins were immediately transferred onto a nitrocellulose membrane at 100 V for 2 hours. After transfer, the membranes were incubated with mouse anti-phosphotyrosine and rabbit anti-actin antibodies dissolved in TBST supplemented with 5% bovine serum albumin. Incubation was done overnight after which the buffer containing the primary antibodies was poured off and membranes washed three times with TBST. The membranes were then incubated with respective secondary antibodies conjugated with infrared dyes detectable at 800 nm and 700 nm wavelength. After washing with TBST (2 times) and phosphate buffered saline (PBS, 2 times), image scanning was performed using a LI-COR Odyssey infrared imager. Protein quantification/densitometry was conducted using image J.

To test activation state of Syk by evaluating the extent of its recruitment to erythrocytes' membranes, the proteins transferred to nitrocellulose membrane after SDS-PAGE protein separation were immunostained with rabbit anti-Syk antibody (1:2000) and visualized with the aid of LI-COR scanner as stated above.

5.3.2 Phosphatase activity of supernatants from sickle cells and healthy erythrocytes

Erythrocytes' supernatants were obtained by incubating sickle/healthy red cells with ghost's buffer (5 mM NaH_2PO_4, 1mM EDTA plus protease cocktail inhibitor) and spinning down the membrane fraction at 13000 rpm for 15 minutes at 4 °C. Supernatants were transferred to clean 1.5 mL tubes while the membrane pellets were discarded.

With minor amendments, the level of phosphatase activity in red cells derived from sickle cell patients' and healthy control samples was assessed as described previously.[41] Briefly, erythrocyte's band 3 was phosphorylated by incubating washed healthy red cells (suspended at 30% hematocrit) with 2 mM diamide for 30 minutes. Membranes were stripped with potassium iodide to generate potassium iodide-inside out vesicles (KI-IOVs) and then incubated with supernatants from healthy and SCD's red cells for 15 minutes. This method would expose the phosphorylated band 3 in KI-IOV membranes to phosphatases present in the supernatants. The level of phosphatase activity in the supernatants was assessed by gauging the level at which the supernatants dephosphorylate band 3 substrates in the KI-IOVs following a ten-minute incubation of 10 µL of the KI-IOVs with 50 µL of supernatants. To confirm that equal amounts of supernatants were used, 10 µL of each supernatant (solubilized in 4x Laemmli buffer) was run in

the same gel and their actin contents used as loading controls for that matter. Samples for Western blotting were prepared as described in section 5.3.1 above.

5.3.3 Induction of band 3 tyrosine phosphorylation by PTP1B inhibitors

Washed healthy erythrocytes suspended in PBS-G at 20% hematocrit were incubated with PTP1B inhibitor(s) [PTPV, PTPXVIII and Cayman PTP1B inhibitor #15782] at 37 °C for 30 minutes under 35 rpm shaking. Erythrocytes' ghosts were prepared as stated in section 5.3.1 and band 3 tyrosine phosphorylation detection conducted as explained in the same section.

5.3.4 Analysis of protein tyrosine phosphatase 1 B cleavage

To investigate the degradation status of PTP1B in sickle cells, samples were processed as explained in section 5.3.1, except that the transferred proteins in the nitrocellulose membrane were probe with anti-PTP1B antibody.

5.3.5 Comparison of full and short length PTP1B activity against phosphorylated band 3 and p-nitrophenyl phosphate

Erythrocyte's protein band 3 was phosphorylated by incubating 30% hematocrit red cells with 1 mM OV for 30 minutes at 37 °C. Control samples were incubated with vehicle lacking OV. Ghost membranes (prepared from the OV-treated cells as outlined above, section 5.3.1) were used to obtain inside out vesicles (IOVs) which were then stripped with potassium iodide to generate potassium iodide-inside out vesicles (KI-IOVs) based on protocols established previously.[20] To test the ability to dephosphorylate band 3 by the PTP1B enzymes (Sigma: Full length PTP1B #SRP0215-20µg; Lot. #3000920322, short chain PTP1B #SRP0212-20µg; Lot. #3001031203), 10 µL of the phosphorylated KI-IOVs were mixed with 50 µL of phosphatase buffer (25 mM HEPES, pH 7.4, 5 mM DTT, 10 µg/mL bovine serum albumin) and incubated for 1 h. The phosphatase buffer contained either 0.5µL of full length PTP1B or 4 µL of the short chain PTP1B enzyme. Unphosphorylated KI-IOVs were similarly incubated with the enzymes under the same conditions. The reaction was stopped by solubilizing the samples in 60 µL of 4x Laemmli buffer containing phosphatase and protease cocktail inhibitors and heated up to 90 °C for 5 minutes. Extent of band 3 dephosphorylation was assessed using immunoblots acquired as stipulated in section 5.3.1

To test the activity of equimolar concentrations of both short and long chain PTP1B, p-nitrophenyl phosphate phosphatase assay (ScienCell #8108) was used, and in accordance with manufacturer's instructions. Relative phosphatase activity of the short form of PTP1B to the long chain form was established.

5.3.6 Co-immunoprecipitation of band 3 with PTP1B

Healthy and sickle cell samples were washed with PBS-G and 100 µL of each sample was dissolved in 1.5 mL Pierce^{TM} lysis buffer (Thermofisher #87788) containing phosphatase and protease cocktail inhibitors. Lysed samples incubated on ice for 30 minutes, then centrifuged at 12000 rcf for 10 minutes. Supernatants were transferred into new 1.5 mL tubes and 10 µL of mouse anti-PTP1B (N-terminal, Sigma #MABS197) or anti-PTP1B (C-terminal; ECM Biosciences #PP2341) added then transferred onto a revolver and antibody binding allowed to progress overnight at 4 °C. On the next day, 25 µL of protein A/G agarose magnetic beads (Bimake #B23201) were added to each sample and incubated for an additional 4 h, after which samples were washed 5 times with PBS supplemented with protease cocktail inhibitor. After the last wash, 50 µL of 4x Laemmli buffer was added to each sample, heated to 90 °C. After SDS-PAGE and transfer of proteins to nitrocellulose membrane, the membrane was cut into two portions along the 80 kDa line. The higher molecular weight portion was incubated with mouse anti-band 3 while the lower molecular weight segment was stained with rabbit anti-PTP1B, antibodies. After washing with TBST and further incubation with respective secondary antibodies, the membranes were washed and then scanned with Li-COR infrared scanner.

5.3.7 Induction of PTP1B cleavage using Ca^{2+}/A23187

Healthy erythrocytes washed with PBS-G and suspended at 30% hematocrit were incubated with 1 mM $CaCl_2$ and 5 µM calcium ionophore A23187 for 1 hour at 37 °C under 35 rpm shaking conditions. Vehicle (dimethyl sulfoxide, DMSO) was added to control samples in place of 1 mM $CaCl_2$ and/or calcium ionophore A23187. Samples for Western blotting were prepared as explained in section 5.3.1 and probed with anti-phosphotyrosine and anti-PTP1B antibodies.

5.4 Results

5.4.1 Global tyrosine phosphorylation of SCD erythrocyte membrane proteins

A general tyrosine phosphorylation of erythrocyte membrane proteins was examined to assess the impact of oxidative stress on several proteins. As shown in **Figure 5.1** there is an above basal tyrosine phosphorylation of many membrane proteins. This is not surprising given the reported over-expression of Syk and Src kinases in sickle cells. Additionally, the data in **Figure 5.1** B, indicates that Syk (a major kinase responsible for band 3 tyrosine phosphorylation[14,41–43]) is recruited to the membranes of sickled erythrocytes which suggest its activation as reported by others.[42,44,45]

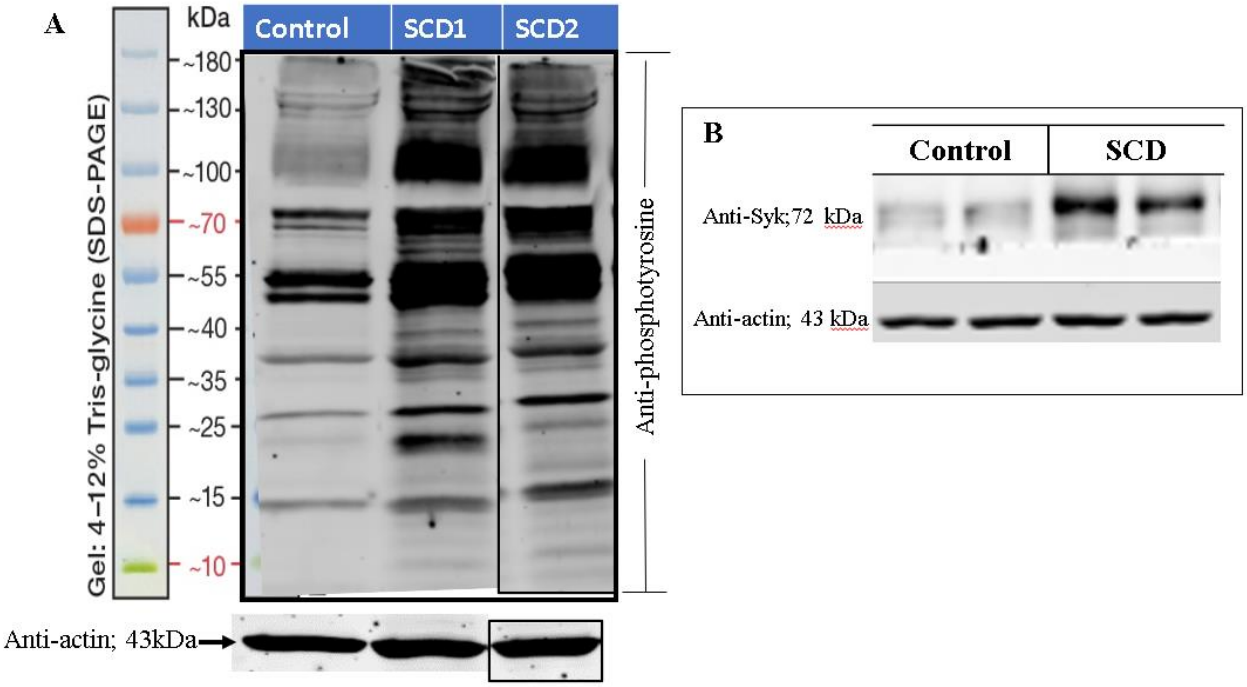


Figure 5.1 Global phosphorylation of membrane proteins and recruitment of Syk to the membrane. Erythrocytes' membrane proteins from healthy controls and sickle cell patients were resolved with SDS-PAGE and probed with anti-phosphotyrosine (**A**), or anti-Syk (**B**) antibodies. Anti-actin immunostaining was used as a loading control.

5.4.2 Phosphatase activity of supernatants from sickle cells and healthy erythrocytes

With the establishment that majority of membrane proteins of sickle erythrocytes are tyrosine phosphorylated, the question arose about the level of tyrosine phosphatase activity in these patients' samples. A crude estimation of phosphatase activity was examined by testing the extent

with which supernatants derived from SCD or healthy control RBCs dephosphorylate band 3 in diamide-phosphorylated KI-OVs. **Figure 5.2** illustrates that, KI-IOVs incubated with sickle cell supernatants display less reduction in band 3 phosphorylation intensity (i.e., lower phosphatase activity) as compared to KI-IOVs incubated with healthy control's supernatant which nearly abrogated the intensity of band 3 tyrosine phosphorylation (i.e., higher phosphatase activity). This observation in addition with the expected effects of oxidative stress on red cells,[46] is in agreement with previously published reports that indicates a reduction in phosphatase activity in sickled erythrocytes.[31]

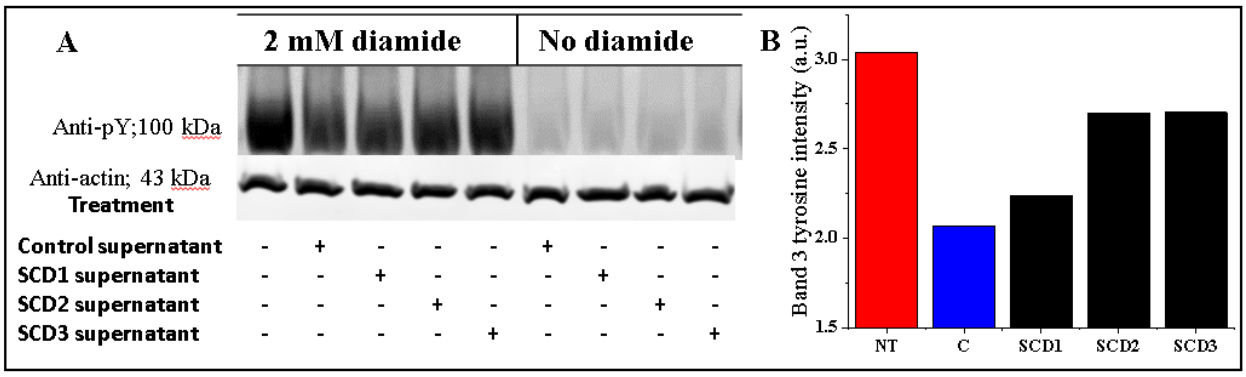


Figure 5.2 Assessment of tyrosine phosphatase activity in healthy and sickle cell patients' supernatants derived from red cells. Membranes were dephosphorylated with cytosolic contents of erythrocytes from a healthy control and 3 different sickle cell patients. Proteins were resolved by Western blotting and probed with anti-phosphotyrosine antibody (**A**) and intensity of band 3 computed using image J (**B**). Panel **B** represents band 3 phosphorylation intensity of the diamide treated samples (first 5 lanes of panel **A**).

5.4.3 Inhibitors of PTP1B induce band 3 tyrosine phosphorylation in healthy red cells

Because protein tyrosine phosphatase 1B is known to be very sensitive to orthovanadate (OV) concentrations as low as 1 µM,[13] the reported extensive band 3 tyrosine phosphorylation induced by this phosphatase inhibitor[5,16,20] may be an indication of complete abrogation of PTP1B activity despite inhibitory aspects of OV on other tyrosine phosphatases in red cells. To test whether specific inhibitors of PTP1B could initiate band 3 tyrosine phosphorylation, healthy erythrocytes were treated with 3 different specific inhibitors of PTP1B. As shown in **Figure 5.3**, the three PTP1B-specific inhibitors markedly stimulated band 3 tyrosine phosphorylation. The

data demonstrate importance of PTP1B in maintaining a low profile of band 3 tyrosine phosphorylation.

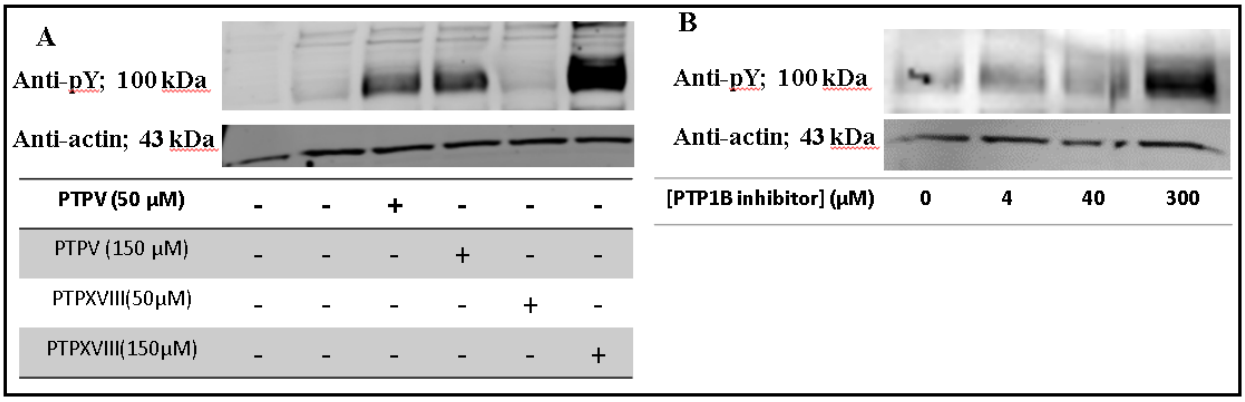


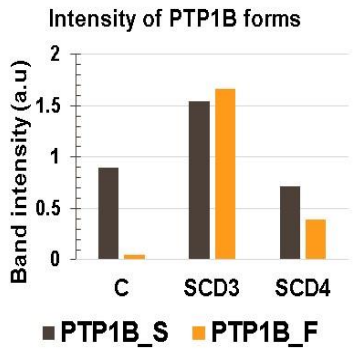
Figure 5.3 Initiation of band 3 tyrosine phosphorylation with specific inhibitors of PTP1B. Erythrocytes from healthy donors were treated with PTP1B inhibitors and extent of band 3 tyrosine phosphorylation determined via Western blotting. Initiation of band 3 tyrosine phosphorylation by different concentrations of PTPV and PTPXVIII (**A**) and Cayman PTP1B inhibitor (**B**).

5.4.4 Assessment of PTP1B cleavage in sickle cell erythrocytes

Protein tyrosine phosphatase 1 B is a prominent PTP in dephosphorylating band 3. While reactive oxygen species may promiscuously catalyze oxidative inhibition of several red cell PTPs, another mechanism that involve displacement of PTP1B from band 3 was proposed more than a decade ago.[26] With the elevated band 3 tyrosine phosphorylation in sickle cells, prescience of PTP1B cleavage was undertaken. To pursue the hypothesis, SDS-PAGE resolved membrane proteins were probed with anti-phosphotyrosine and anti-PTP1B antibody, clone FG6 (sigma #MABS197). The use of anti-phosphotyrosine herein, **Figure 5.5**, A (top panel), would illustrate how the extent of band 3 tyrosine phosphorylation correlates with PTP1B cleavage, **Figure 5.5**, A (bottom panel). Amazingly, PTP1B antibody recognized only one band at ~50 kDa in healthy control but detected two separate bands at ~50 kDa and ~42 kDa in sickle cell samples. Moreover, the observed cleavage is more pronounced in sickle cell patient SCD4 which had a 90.7% Hb S content relative to 64.2% Hb S of patient SCD5. It seemed the more cleavage of PTP1B there is, the more band 3 tyrosine phosphorylation.

To assess whether the degree of PTP1B cleavage correlates with the extent of band 3 tyrosine phosphorylation, densitometry computation, using Image J, was performed on several

immunoblots. The degree of cleavage (suggestive of degradation of PTP1B enzyme) was defined as the percent of the intensity of short chain PTP1B band of the total intensity of both full and short chain forms present. A sample calculation is provided in **Figure 5.4** below.



	Intensity of endogenous PTP1B bands from Image J estimations			
	Full chain	short chain	Total	%short chain
C	0.895786	0.052878	0.948664	5.573932
SCD3	1.547435	1.668915	3.21635	51.88849
SCD4	0.712079	0.392864	1.104942	35.55512

Figure 5.4 Sample calculation for the degree of PTP1B cleavage. The band intensity given on the table on the right are given in arbitrary units; (PTP1B_S and PTP1B_F stands for short (AA 1-321) and full chain (AA 1-435) PTP1B enzyme, respectively)

The data illustrate a statistically significant (p= 0.0021) positive correlation between the extent of PTP1B cleavage the level of band 3 tyrosine phosphorylation (Pearson's r = 0.80); **Figure 5.5**, B. Similarly, a significant (p=0.0003) and strong positive correlation (Pearson's r = 0.89) was observed between the extent of PTP1B cleavage and the percent Hb S in patients' blood, **Figure 5.5**, C. Because the cleavage of PTP1B correlates positively with degree of band 3 tyrosine phosphorylation, an inference is made that cleavage may cause a reduction in the function of PTP1B in SCD.

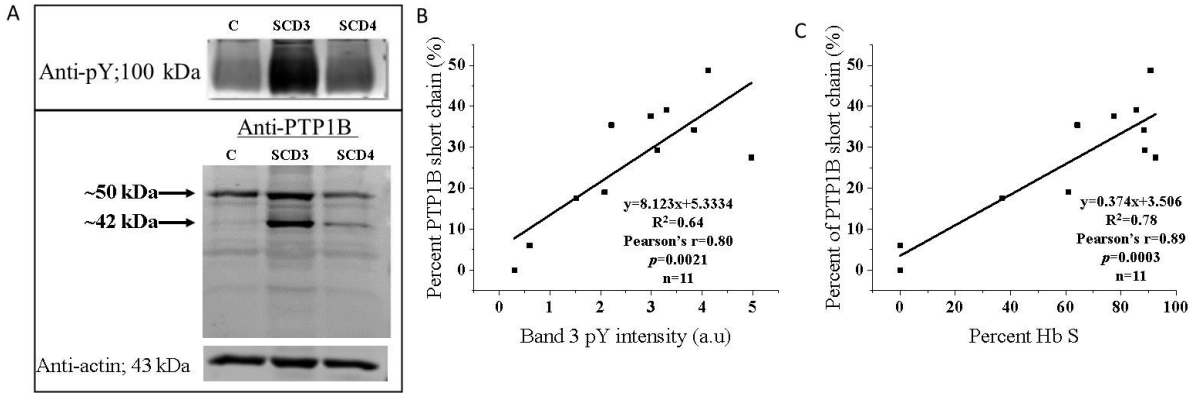


Figure 5.5 Assessment of PTP1B cleavage in red cells and its correlation with band 3 tyrosine phosphorylation and percent Hb S in blood. Healthy control (C), and two different sickle patients (SCD4 and SCD5) samples were subjected to SDS-PAGE electrophoresis and immunostained with mouse anti-phosphotyrosine (A, top panels) and anti-PTP1B antibody, and actin content was used as a loading control (A, bottom panels). Correlation of the percentage of PTP1B short chain with Hb S content in blood (B) and band 3 tyrosine phosphorylation (C).

5.4.5 Cleavage-induced alteration of PTP1B activity

Impact of cleavage on PTP1B activity was determined by dephosphorylating band 3 in KI-IOVs. Because the PTP enzymes had different activities based on the manufacturer certificate of analysis, experiments were optimized to use the number of enzymes that possess similar activity. Calculations of molarity of each phosphatase showed that the shorter chain enzyme is 3.25 times more concentrated than the full-length construct. Even though the short chain has more molecules per given volume, the ability to dephosphorylate band 3 in KI-IOVs by the two constructs is the same, **Figure 5.6**. That 3.25 molecules of the short chain form of PTP1B were required to obtain phosphatase activity level achieved with a single molecule of the full-length enzyme, is suggestive of partial reduction in activity of the truncated PTP1B.

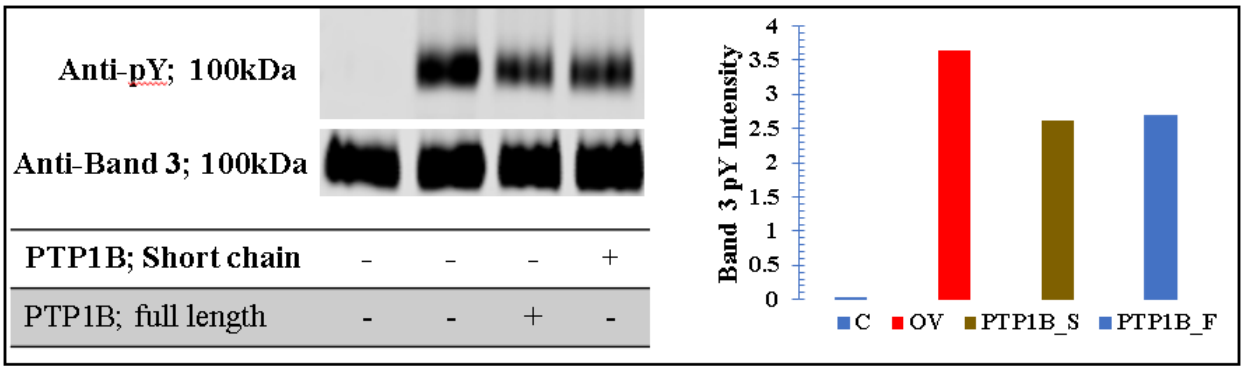


Figure 5.6 Impact of cleavage of phosphatase activity of PTP1B. Phosphorylated band 3 in KI-IOVs was dephosphorylated with PTP1B short (PTP1B_S) and full-length (PTP1B_F) constructs each at 6000 iu catalytic activity.

To affirm that role of cleavage of PTP1B on enzyme activity is important, the activity of the commercial enzymes was determined using p-nitrophenyl phosphate phosphatase assay. In this case, equimolar concentrations of both enzymes were used which showed that the relative activity of full-length PTP1B is at least 3 times that of short chain enzyme despite using equal moles, **Figure 5.7**. This confirms the preceding observation that 1 mole of the full length PTP1B displays similar activity to 3.25 moles of the short chain phosphatase.

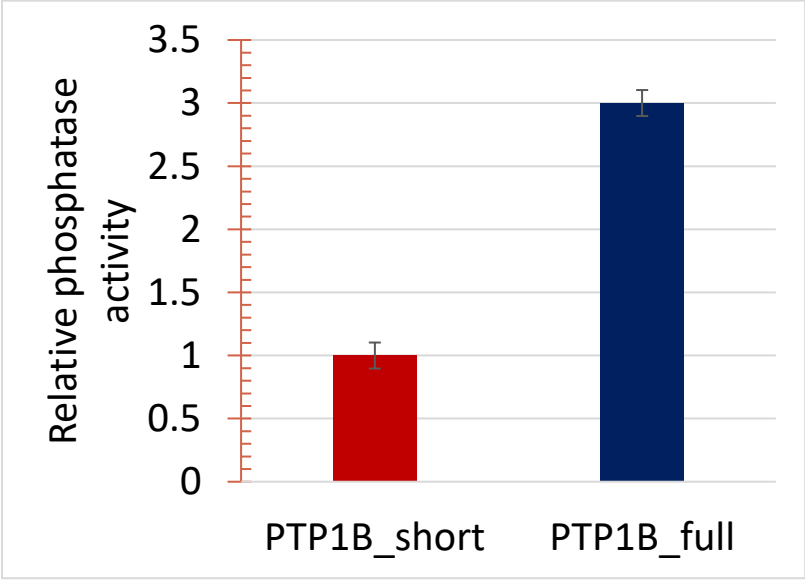


Figure 5.7 Relative phosphatase activity of PTP1B enzymes. (PTP1B_short and PTP1B_full stands for short and full chain PTP1B enzymes, respectively)

The working concentration/volumes of the enzymes were calculated as detailed in **Table 5.1** below. The percent purities of both enzymes, short and full chains, were assumed to be 90% and 50% respectively, i.e., the minimum amount of each enzyme deemed present based on Sigma's certificate of analysis.

Table 5.1 Calculation of equimolar working volumes of PTP1B enzymes

PTP1B Enzymes	Short chain (AA 1-321)	Full length (AA 1-435)
Concentration (mg/mL)	0.84	1.65
Purity (%)	≥90	≥50
Specific activity	742 pmole/min/µg	≥6,000pmole/min/µg
Molecular Weight (g/mol)	$63.3x10^3$	$76.0x10^3$
Amount of pure enzyme	(0.84mg/mL)x90/100 ≥0.756 mg/mL	(1.65mg/mL)x50/100 ≥0.825 mg/mL
Moles in 1 mL	$(0.756x10^{-3}g)/(63.3x10^3g/mol)$ $=0.01194x10^{-6}$ moles	$(0.825x10^{-3}g)/(76x10^3g/mol)$ $=0.0108x10^{-6}$ moles
Moles in 1 µL	$=0.01194x10^{-9}$ moles	$0.0108x10^{-9}$ moles
Working volumes while keeping full length PTP1B moles constant at 1µL volume	$=(0.0108x10^{-9}$ moles/µL$)/(0.01194x10^{-9}$ moles) =0.909 µL	$=(0.0108x10^{-9}$ moles/µL$)/(0.0108x10^{-9}$ moles) =1.0 µL

5.4.6 Co-immunoprecipitation of PTP1B with protein band 3

Cleavage of PTP1B initiated by $Ca2^+$/A23187 treatment was found to be responsible for displacement of the enzyme from band 3.[26] To investigate if a similar process was happening in sickled erythrocytes as well, PTP1B was immunoprecipitated using monoclonal antibody FG6 clone (sigma #MABS197) or with mouse monoclonal anti-PTP1B (ECM Biosciiences #pp2341) which recognize N-terminal or C-terminal of PTP1B, respectively The content of co-immunoprecipitated band 3 was examined with the aid of immunoblots. Data indicate that PTP1B co-immunoprecipitated with band 3 much more in healthy samples than in SCD patients' samples, **Figure 5.8**. Of note, is the almost absent band 3 bands on the SCD samples immunoprecipitated with the mouse antibody that recognizes the C-terminal. Although the phosphatase is not expected to be clamping on phosphorylated band 3 for too long, the co-immunoprecipitation possibly pulled down the proteins during their intermittent interactions. Since majority of the C-terminal was cut off in sickle erythrocytes and that the C-terminal domain which provide anchorage of the PTP to the membrane[49] is removed, it therefore follows that locational displacement of the PTP1B may

be another factor responsible in part for its inability to dephosphorylate band 3 in SCD as was observed in senescent erythrocytes.[26,49]

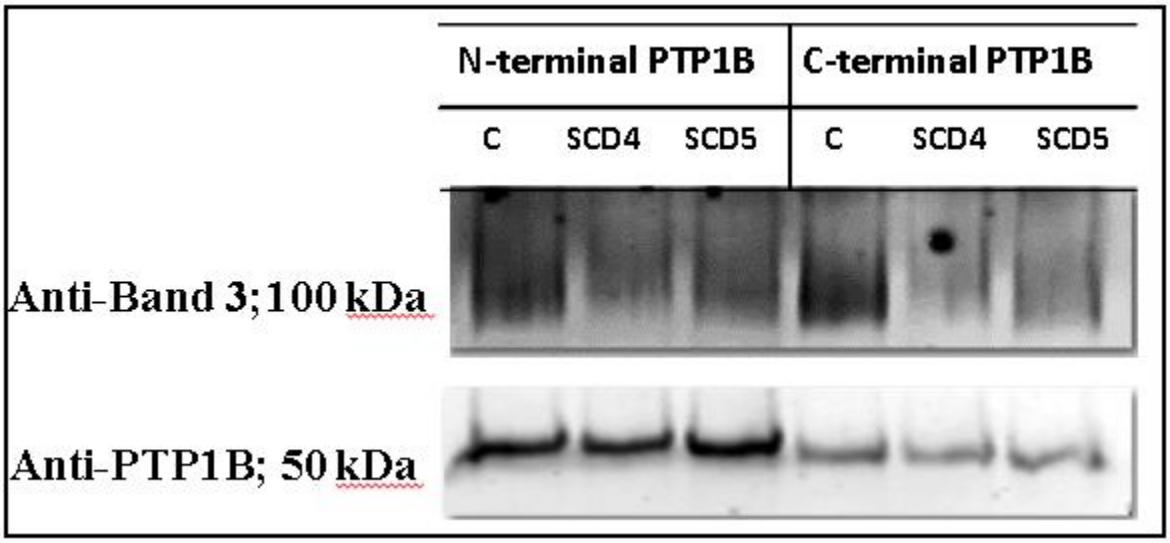


Figure 5.8 Co-immunoprecipitation of PTP1B and band 3 in healthy and sickled erythrocytes. Immunoprecipitation was carried out with anti-PTP1B antibodies raised against N- and C-terminals of PTP1B as indicated on the topmost part of the figure.

5.4.7 Probable mechanism of PTP1B cleavage

Previously, cleavage of PTP1B in red cells has been stimulated by treating healthy erythrocytes with Ca^{2+} in presence of calcium ionophore A23187.[22,26] To test reproducibility of this already proven process, erythrocytes from healthy individuals were treated with 1 mM Ca^{2+} and 5 µM ionophore A23187. As shown in **Figure 5.9** and in agreement with published reports,[26] the ex vivo assay triggered band 3 tyrosine phosphorylation as well as cleavage of PTP1B. Since there is enhanced influx of calcium ions into sickle cells due to activation of K-Cl ion channels,[47,48] it is highly probable that this is the process triggering proteolytic cleavage of PTP1B which may contribute to its diminished activity *in vivo*.

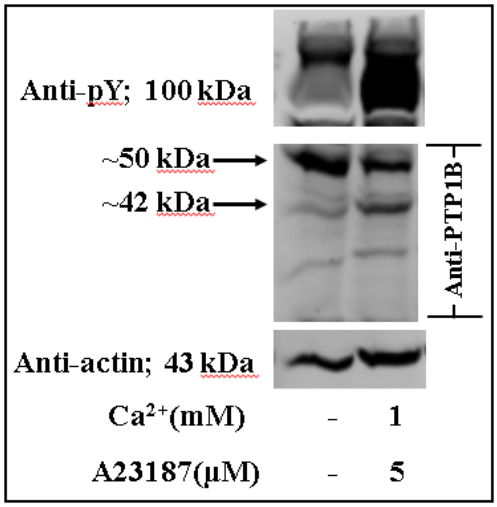


Figure 5.9 Stimulation of PTP1B cleavage and band 3 tyrosine phosphorylation with Ca^{2+}/A23187. Red cells suspended at 30% hematocrit were treated with 1mM Ca^{2+} in presence of 5 µM calcium ionophore A23187.

5.5 Discussion

Ciana *et al* demonstrated that PTP1B gets degraded as healthy erythrocytes age.[26] This degradation was accompanied by an increase in band 3 tyrosine phosphorylation and accumulation of dense cells. The authors hypothesized that PTP1B cleavage abrogates the C-terminal amino acids of the phosphatase which are necessary for effective interaction with band 3.[26] The data presented in this chapter agrees with these findings. Given that sickle cells survive in circulation for only 10-20 days, the present study suggest a possible role of PTP1B cleavage on the shortened lifespan of sickle cells. This data did not show any substantial cleavage of the enzyme in healthy cells, suggesting that the cleavage may be well pronounce at very late stages of erythrocytes' lifespan. In conjunction with increased band 3 tyrosine phosphorylation, the cleavage of PTP1B hints at large scale membrane destabilization of sickle erythrocytes.

Since PTP1B cleavage could be catalyzed with Ca^{2+}/A23187 in healthy RBCs and given the *in vivo* increased calcium influx in sickle erythrocytes, it unsurprising that this phenomenon was also observed in sickle cell erythrocytes. In other words, influx of calcium ions into sickle RBCs may trigger activation of the calpains (or Ca^{2+} stimulated proteases) which would then cause proteolysis of PTP1B. While further mechanistic investigations are warranted beyond those presented herein, the cleavage of PTP1B is well established to contribute to elevated band 3 tyrosine phosphorylation seen in SCD samples. Joiner *et al* explored the role of calpains in

dehydration of SCD erythrocytes and identified that calpastatin (an endogenous inhibitor of calpain-1[50,51]) is inhibited in dense sickle cells.[51] The researchers also discovered that treatment of murine SCD model with calpain inhibitor BDA-410 improved dehydration of RBCs by inhibition of calcium activated efflux of K^+ ions.[51] Because PTP1B is subject to proteolytic cleavage by calpain-1,[52,53] and that band 3 tyrosine phosphorylation causes membrane loss,[5,16] it is possible that cleavage of PTP1B was inhibited by BDA-410. In such a scenario, tyrosine phosphorylation of band 3 would be expected to be minimal.

The inception of Syk inhibitors as promising therapies for sickle cell disease warrants mechanistic investigations on causal stimuli of the phosphorylation. Because PTP1B cleavage is demonstrated to be a viable mechanism responsible for this process (i.e., band 3 tyrosine phosphorylation), it would be interesting to examine whether this cleavage could be prevented by Syk inhibitors treatment of patients with SCD during the ongoing initial proof-of-concept clinical trial of imatinib. Since tyrosine kinase inhibitors act upstream and may not directly impede calcium influx, the fact that they prevent membrane loss will unequivocally prevent potassium efflux, i.e., maintaining membrane fluidity and cells' normal density. In such a case, Ca^{2+} influx would presumably be curtailed thus preventing activation of calpain-1. As a result, intact PTP1B dominates and will likely remain more active which in turn provide an improved lifespan of erythrocytes by preventing stimulation of membrane fragmentation.

Beyond reliance on Syk inhibitors' upstream action to conserve the activity of PTP1B, other novel strategies for preventing cleavage of PTP1B or general inhibition of phosphatases are worthwhile. Interestingly, anti-oxidant supplements or therapies, such as dithiothreitol, quercetin, and N-acetylcysteine[54] and, L-glutamine,[55] have been tried to ease the SCD burden. Although their mechanism of action is thought to be countering oxidative stress and inhibition of cation channels[54] (hence preventing dehydration of cells), one may not rule out reductive reactivation of PTP activity as well as prevention of PTP1B cleavage. Given the improved membrane flexibility associated with the use of these anti-oxidants,[54] the concatenation of events may culminate in abolishment of band 3 tyrosine phosphorylation.

With increasing evidence that the elevated tyrosine phosphorylation of band 3 indeed constitutes a likely mechanism leading to the development of symptoms of sickle cell disease, I propose that selective inhibitors of the PTP 1B proteases could constitute an effective treatment for SCD.

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CHAPTER 6. IMATINIB AUGMENTS STANDARD MALARIA COMBINATION THERAPY WITHOUT ADDED TOXICITY

A portion of this chapter is under publication process and I acknowledge the involved authors listed below.

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6.1 Abstract

The malaria parasite, Plasmodium falciparum, replicates within the human erythrocyte during a 48-hour life cycle that culminates in parasite egress from the red blood cell. We have previously reported that activation of the erythrocyte tyrosine kinase Syk and the resulting destabilization of the erythrocyte membrane are required for parasite egress. Because imatinib mesylate, an FDA-approved tyrosine kinase inhibitor with minimal toxicity, exhibits off-target activity against Syk, we elected to determine whether the clinical dose of imatinib might have anti-malarial activity. Adult male patients with uncomplicated P. falciparum malaria from a region of Vietnam encumbered with delayed parasite clearance (DPC; continued parasitemia following 3 days of therapy) were treated with either the region's current standard-of-care (SOC; 40 mg dihydroartemisinin + 320 mg piperaquine/day for 3 days), imatinib alone (400mg/day), or imatinib+SOC. Patients were monitored for adverse events, changes in body temperature and parasite levels before, during, and after therapy. Consistent with previous clinical trials in Quang Tri Province, 1/3 of patients treated with SOC experienced DPC. Patients treated with imatinib alone experienced either a gradual decline in parasite density or transient increase followed by gradual decline, with no obvious drug-related toxicities. Patients treated with imatinib+SOC experienced no DPC, no drug-related toxicities, and a significantly accelerated decline in parasite density and pyrexia when compared to SOC-treated participants. This imatinib-derived

improvement was most pronounced in patients with high parasite density, where serious complications and death are most frequent. In conclusion, imatinib improves the efficacy of SOC malaria therapy, with no obvious drug-related toxicities.

6.2 Introduction

Malaria remains a serious health problem in much of the world today, with 228 million new cases and 405,000 deaths estimated in 2018.[1] Although artemisinin combination therapies (ACTs) continue to successfully treat most strains of P. falciparum malaria, new drug-resistance mutations leading to delayed parasite clearance (DPC; continued parasitemia following 3 days of standard therapy) have emerged, especially in Southeast Asia.[2–6] The presence of these artemisinin resistant parasites unfortunately suggests that current treatments may soon be inadequate. While artemisinin, the cornerstone of ACTs, is fast acting and effective, the duration of its efficacy is short, requiring a companion drug to achieve more prolonged activity.[7,8] Fortuitously, resistance to such companion drugs is also rising,[2] and while triple combination therapies are now under investigation for prevention of DPC,[9–11] the third components in most such ACTs are anti-malarials that have already failed due to decline in efficacy.[2] Taken together, these observations suggest that new approaches to treat P. falciparum malaria with orthogonal mechanisms of action are critically needed.

We have reported that tyrosine phosphorylation of the erythrocyte membrane protein 3 (band 3, AE1, SLC4A1) induces erythrocyte membrane weakening, leading to membrane vesiculation and fragmentation.[12–14] Because this phosphorylation dramatically increases during P. falciparum maturation in infected erythrocytes,[15–17] we have hypothesized that the resulting membrane weakening might contribute to erythrocyte rupture during merozoite egress from the infected red cell at the end of P. falciparum's life cycle.[16,18-20] Indeed, several studies have now demonstrated that blockade of band 3 tyrosine phosphorylation by a Syk tyrosine kinase inhibitor can prevent escape of P. falciparum from its erythrocyte host in vitro, thereby terminating the parasitemia.[18–20] The concomitant accretion of denatured hemoglobin within the parasitized erythrocyte is thought to further augment parasite killing by enhancing the redox-mediated activation of artemisinins.[21] Because imatinib, an FDA-approved tyrosine kinase inhibitor with an excellent safety profile,[22,23] has been found to exhibit off-target activity against Syk,[24] it seemed

reasonable to examine whether a clinically safe dose of imatinib might also exhibit anti-malarial activity in infected patients.

We describe here the results of two small clinical trials aimed at examining the safety and efficacy of imatinib in adult male P. falciparum malaria patients from a region of Vietnam where DPC is prevalent.[5,6] After evaluating the effect of imatinib as a monotherapy, we compare the potencies of the standard-of-care therapy in Vietnam (SOC; dihydroartemisinin plus piperaquine) with a combination of imatinib plus SOC (Im+SOC). We report that the usual clinical dose of imatinib (400 mg) causes no significant adverse events when administered once daily for three days either alone or in combination with SOC. We further demonstrate that although imatinib exhibits only moderate anti-malarial activity as a monotherapy, when administered in combination with SOC it significantly augments the potency of dihydroartemisinin plus piperaquine, not only by promoting a more rapid decline in pyrexia but also by accelerating elimination of the parasitized cells. Because imatinib functions via an orthogonal mechanism from current SOCs, and since its effective dose can be manufactured at minimal cost, we propose that imatinib should be evaluated as a third component of future triple combination therapies.

6.3 Methods

6.3.1 Study agents

Generic imatinib mesylate was purchased from TEVA Pharmaceuticals (Jerusalem, Israel) and provided to participants in two 200 mg tablets per treatment to conform with the typical dosage of imatinib indicated for treatment of chronic myelogenous leukaemia. CV-Artecan, the standard-of-care (SOC) for treatment of P. falciparum malaria in Vietnam, was purchased from OPC Pharmaceutical (Ho Chi Minh City, Vietnam) and administered to participants as a tablet containing 40 mg dihydroartemisinin plus 320 mg piperaquine phosphate.

6.3.2 Study participants

P. falciparum-infected males of age 16-55 years with no complicating co-morbidities that had not received an anti-malarial drug within the previous 4 weeks were eligible for both studies. Those individuals who met eligibility criteria and provided informed written consent were enrolled

in the trial (**Figure 6.1**). Women were not enrolled due to imatinib's unknown effects on pregnancy.

6.3.3 Study protocols

The phase 1/2 "imatinib monotherapy study" was an open-label trial aimed at determining the safety and tolerability of imatinib in adult male patients with uncomplicated P. falciparum malaria. Uncomplicated malaria was defined as a positive microscopy-confirmed P. falciparum infection in symptomatic patients with no complicating comorbidities and a malaria count <150,000 parasites/μL blood. Participants were randomly assigned to either a standard-of-care (SOC) control arm (n=8) in which they received 40 mg dihydroartemisinin plus 320 mg piperaquine phosphate orally twice (12 hours apart) on the first day and then once a day on the following 2 days, or an imatinib monotherapy arm (n=7) in which they received 400 mg of imatinib mesylate orally with a meal and full glass of water once a day for five days (**Table 6.2**).

Participants in the subsequent Im+SOC triple combination therapy trial were also randomly assigned to one of two cohorts, either the above SOC cohort (n=21) or an imatinib plus SOC cohort (n=20). The SOC cohort was dosed exactly as above, while the Im+SOC cohort was dosed as described above except each participant also received 400 mg of imatinib mesylate orally with a meal and full glass of water once a day for 3 days (Table 2).

During both trials, participant temperatures and peripheral blood parasite levels were monitored before, during and after the trial on days 0, 1, 2, 3, 5, 7, 28, and 42. Participants were also examined for the usual symptoms of P. falciparum malaria, including fever, chills, headache, fatigue, anorexia, and mild diarrhea. If any participant in either imatinib cohort was observed to exhibit either an increase in parasite density >150,000 parasites/μL or adverse symptoms exceeding those normally associated with malaria, the participant was transferred immediately to SOC.

Both trials were approved by the Vietnam Ministry of Health and the Institutional Review Board at the Hue University of Medicine and Pharmacy. The trials were conducted in the six Communes of the Lia Region Huong Hoa district (see map, Supplemental Fig. S1), Quang Tri province, due to the high levels of DPC present in this region of Vietnam.5,6 Both studies were registered with ClinicalTrials.gov (NCT02614404 and NCT03697668).

6.3.4 Randomization and Masking

Both clinical trials were open label, so no blinding of the attending physicians was performed. Participants, however, were randomly assigned to their cohorts by alternating their assignment based on the date and time of hospital admission, and all participants as well as microscopists who quantitated the parasite density were blinded to the treatment regimens.

6.3.5 Outcomes

Primary endpoints for both studies were safety and tolerability. Secondary endpoints were reduction in parasite density for the imatinib monotherapy study and both reduction in parasite density and decline in pyrexia for the Im+SOC study. Safety and tolerability were assessed at protocol-specified time points and adverse events were classified as toxicities not normally associated with P. falciparum malaria, including edema, rashes, severe diarrhea, etc. The severity of any adverse event was proposed to be classified as follows: i) Mild – events requiring minimal or no treatment that did not interfere with the participant's daily activities, ii) Moderate – events resulting in a low level of inconvenience or concern that may have caused some interference with the participant's daily functioning, and iii) Severe – events that interrupt a participant's daily activity and could be incapacitating or require medical intervention. Attribution of imatinib to adverse events was assessed using a 5-point scale: not, unlikely, possibly, probably, and definitely related. Primary endpoints would be met if the imatinib treatment groups exhibited an absence of any severe adverse events and an insignificant increase or actual decrease in moderate adverse events.

6.3.6 Statistical analysis

All authors had access to the primary clinical trial data. A mixed ANOVA was conducted to determine the effect of drug treatment on parasite density and pyrexia. Post-hoc multi-comparison testing was used to determine which time points were statistically different. Two-tail t-tests were used to determine statistical differences between means of independent groups. Significance was assumed for p-values <0.05. Data from all participants who received three doses of treatment drug(s) were included in the analysis. SOC treatment cohorts from each individual

trial were analyzed separately. Unless stated otherwise, all error bars represent standard error of the mean (SEM).

6.3.7 Data sharing

Data collected for this study will be made available to others upon publication and ending 36 months following article publication. De-identified datasets containing the variables analyzed for the primary and secondary objectives as well as other supporting documents such as the study protocol and informed consent will be made available. Investigators who seek access to individual-level data will need to contact the corresponding author PSL (plow@purdue.edu) to receive instructions on the formal request process. A data usage agreement will need to be signed by the respective institutions/individuals before data is transferred.

6.4 Results

To assess the safety, tolerability, and efficacy of imatinib in patients with P. falciparum malaria, an initial Phase 1/2 clinical trial was conducted where imatinib was administered to participants as a monotherapy and compared with a parallel cohort treated with standard-of-care (SOC) therapy in Vietnam. Although imatinib had already established a good safety record when administered in perpetuity to chronic myelogenous leukaemia cancer patients,[22,23] it had not been dosed in malaria patients prior to this study. Therefore, the primary endpoint for this trial was the safety and tolerability of imatinib in patients with P. falciparum malaria and the secondary endpoint was reduction in parasite density.

Prior to trial initiation, an appropriate site with endemic malaria and delayed parasite clearance (DPC) was to be identified. As noted in the introduction, DPC had been shown to be increasing in Southeast Asia, requiring treatment of patients well beyond the traditional three days of therapy.[5,6] Because any new remedy for malaria would have to demonstrate efficacy against these more refractory strains of P. falciparum malaria, we elected to conduct the initial clinical trial in the Quang Tri Province of Vietnam, where DPC had been documented by standard microscopy in 27.2% of the patients (39.3% when parasitemia was measured by PCR) and genetic markers of artemisinin and piperaquine resistance (K13 C580Y and PfPM2 multi-copies) had been identified in 1.2% of infected individuals.[5]

Table 6.1 Baseline characteristics of patients

	Imatinib only study cohorts			Imatinib + SOC study cohorts	
	SOC	**Imatinib only**		**SOC**	**Im+SOC**
	n = 8	n = 7		n = 21	n = 20
Age, years (SD; range)	20.3 (6.0; 18-35)	35.3 (12.7; 18-54)		29.1 (6.7; 18-38)	26.9 (9.4; 16-51)
Sex					
Female	0	0		0	0
Male	8 (100%)	7 (100%)		21 (100%)	20 (100%)
Race					
Southeast Asian	8 (100%)	7 (100%)		21 (100%)	20 (100%)
Data are mean (SD) or n (%) unless otherwise stated.					

To evaluate imatinib's safety and tolerability, participants (males, 18 to 54 years of age; **Table 6.1**) with uncomplicated malaria and no co-morbidities who had not taken an anti-malaria drug during the preceding month were randomized into one of two treatment cohorts (**Figure 6.1**). Following informed consent, participants were treated with either SOC for three days or a single daily dose of 400 mg imatinib (i.e., the recommended dose for treatment of chronic myelogenous leukemia patients) for five consecutive days, as described in Methods and **Table 6.2**. During and after the therapy, each participant was monitored for changes in hematology, blood chemistry, pyrexia, parasite level, and adverse events. Other than the expected symptoms of P. falciparum malaria, no other adverse events were observed except one case of mild abdominal pain that resolved spontaneously and is periodically observed in malaria patients receiving SOC. Analyses

of blood parameters and body temperatures also showed no evidence of drug-related toxicity. Although detection of toxicities could not be excluded with a larger number of patients examined, we nevertheless concurred that evaluation of imatinib's contribution to the efficacy of SOC in Vietnam was still warranted.

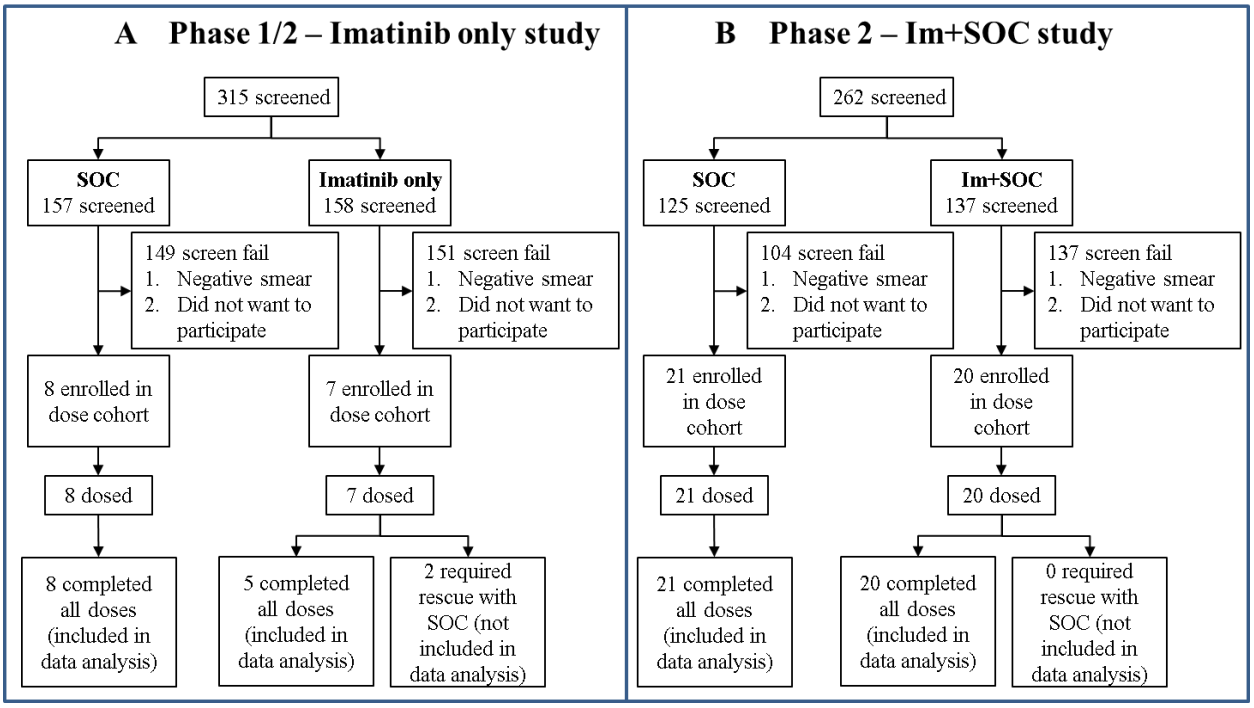

Figure 6.1. Overview of imatinib monotherapy (A) and triple combination therapies (B) clinical trials.

Table 6.2 Treatment schedules for trials

	Cohorts	0 hours	12 hours	24 hours	48 hours	72 hours	96 hours
Imatinib only Trial	SOC	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	NA	NA
	Imatinib	400 mg imatinib	NA	400 mg imatinib	400 mg imatinib	400 mg imatinib	400 mg imatinib
Im+SOC Trial	SOC	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ	NA	NA
	Im+SOC	40 mg DHA + 320 mg PPQ + 400 mg imatinib	40 mg DHA + 320 mg PPQ	40 mg DHA + 320 mg PPQ + 400 mg imatinib	40 mg DHA + 320 mg PPQ + 400 mg imatinib	NA	NA

SOC = Standard-of-care, DHA = dihydroartemisinin, PPQ = 320 mg piperaquine phosphate, and NA = not applicable, no drug administered at this time.

To determine whether imatinib alone might exhibit some indication of efficacy, we concurrently quantitated the parasite density in the peripheral blood of each participant before, during and after their course of treatment. As seen in **Figure 6.2 A**, a decrease in the number of parasitized cells per microliter of blood was observed in the imatinib-treated group. Although this decrease lagged behind the analogous response in the SOC group, when a main factors analysis using a mixed ANOVA was conducted, no statistically significant interaction was identified between treatment groups and the parasite density, indicating that both SOC and the imatinib monotherapy were capable of reducing parasite density in this patient population (p-value = 0.7736).

Unlike the SOC population, two of the seven participants treated with imatinib alone experienced a rise in parasite density and therefore had to be transferred to SOC (see representative time course in **Figure 6.2 B**). In contrast, the other patients in the imatinib monotherapy cohort

experienced either an irregular or steady decline in parasite density (**Figure 6.2 C**). In concordance with *in vitro* experiments showing that imatinib's efficacy depends on the stage of the parasite's life cycle during which it is first administered (i.e., owing to the fact that it primarily blocks parasite egress at the end of the parasite's life cycle),[18-20] the time-dependent efficacy of the imatinib monotherapy was predicted and could be hypothesized to derive from the stage of the parasite's life cycle when treatment was initiated. Finally, it was encouraging to note that the imatinib-treated cohort experienced a rapid decline in pyrexia not significantly different from that of SOC (P=0.70; **Figure 6.2 D**).

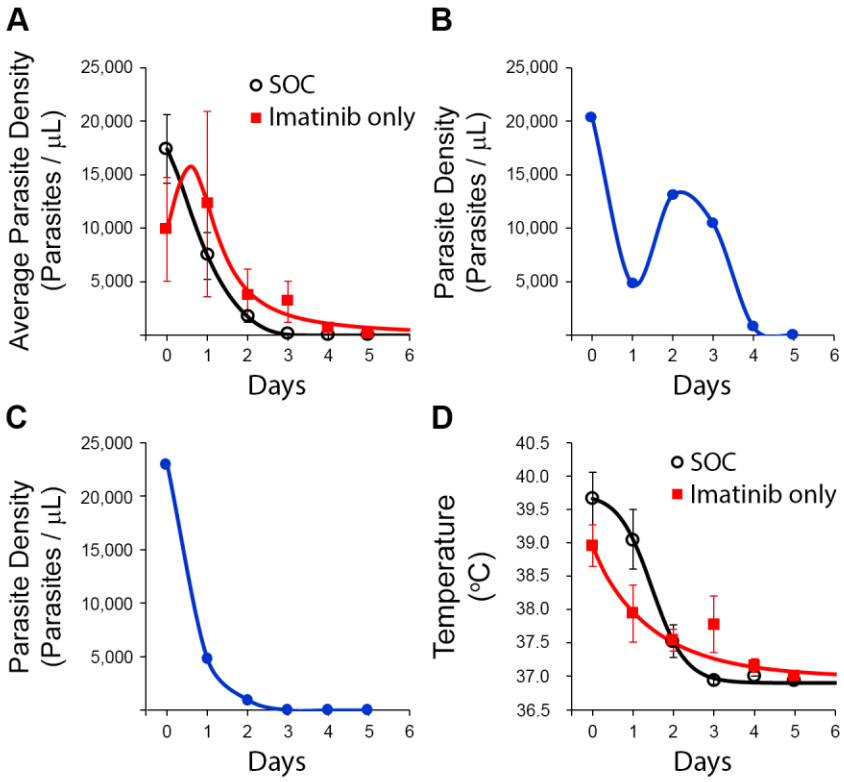

Figure 6.2 Comparison of the effect of imatinib and SOC treatment on the reduction of parasite density and pyrexia. (A) Participants were randomly assigned to receive either 40 mg dihydroartemisinin + 320 mg piperaquine phosphate (SOC; black circles) or 400 mg imatinib (red squares). The level of parsitemia was determined daily and their averages were plotted. (B) Representative time course of parasite density in a participant in the imatinib monotherapy arm that exhibited a temporary rise in parasite density. (C) Representative time course of parasite density in a participant in the imatinib monotherapy arm that exhibited a monotonous decrease in parasite density. (D) Average participant body temperatures were plotted for the SOC (black circles) and imatinib only (red squares) cohorts. Error bars expressed as SEM.

Encouraged that imatinib might have anti-malarial activity, we next decided to compare the safety and efficacy of SOC alone (40 mg/day dihydroartemisinin + 320 mg/day piperaquine) with the safety and efficacy of Im+SOC (i.e., 400 mg/day imatinib + 40 mg/day dihydroartemisinin + 320 mg/day piperaquine). For this purpose, 41 adult male participants with uncomplicated malaria were enrolled (**Figure 6.1**) from the same region of Vietnam and randomized into one of two cohorts that were treated for three consecutive days with either SOC or Im+SOC (**Table 6.2**). Evaluation of all adverse events revealed that the triple combination therapy was as safe as SOC, displaying no adverse events attributable to the added imatinib. Therefore, the primary endpoint of safety and tolerability was met for the Im+SOC triple combination therapy.

Although both cohorts entered the trial with similar levels of pyrexia (39.6 ±0.1°C vs 39.3 ±0.1°C for SOC vs Im+SOC; p=0.1425, NS; **Figure 6.3 A**), analysis of participant temperatures before, during and after the treatments demonstrated that body temperatures (i.e. a good measure of how a patient feels) returned to normal ~2 days faster (1.55±0.11 versus 3.57±0.26 days; p=0.00001) in the triple combination than SOC therapy (**Figure 6.3 B**). Moreover, while all participants treated with Im+SOC (**Figure 6.3 C** and **D**) experienced a monotonous decline in body temperature, >30% of participants in the SOC cohort (**Figure 6.3 C** and **D**) experienced a second increase in temperature during at least one day of their therapy or follow-up period (p=0.0191). Taken together, these data suggest that inclusion of imatinib with SOC may facilitate a faster resolution of pyrexia.

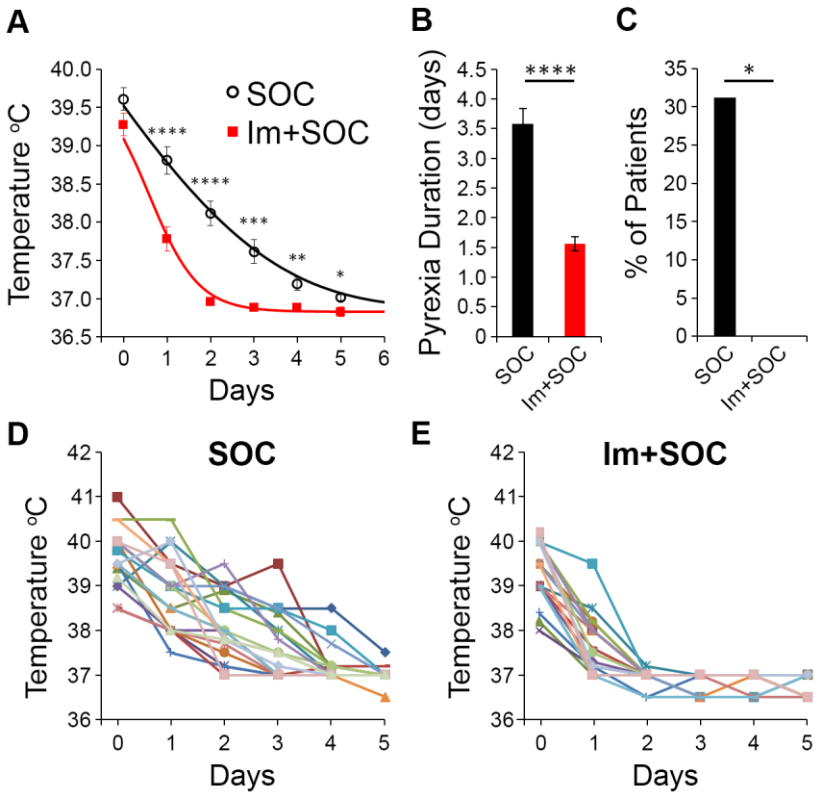

Figure 6.3 Changes in body temperature as a function of time on therapy. (**A**) Participants were randomly assigned to receive either 40 mg dihydroartemisinin + 320 mg piperaquine phosphate (SOC; black circles) or 400 mg imatinib plus SOC (red squares). Participant temperatures were measured daily and plotted. (**B**) Average duration of pyrexia in the two cohorts. (**C**) Percentage of participants that exhibited a second fever spike. (**D**) Plots of individual body temperatures versus time in the SOC cohort. (E) Plots of individual body temperatures in the imatinib + SOC cohort. Error bars expressed as SEM and * = p-value < 0.05, ** = p–value < 0.01, *** = p-value <0.001, and **** = p-value < 0.0001.

To assess whether the accelerated decline in body temperature might correlate with a more rapid reduction in parasite density, we next compared the number of parasites/μL of peripheral blood in the two treatment groups. Although the average initial parasite concentration in peripheral blood did not differ significantly between SOC and Im+SOC cohorts (16,601 ±2099 parasites/μL versus 31,380 ±13,351 parasites/μL, respectively; p=0.6762), parasite density decreased more rapidly in the triple combination than SOC cohort (**Figure 6.4**, panels **A**, **B**, **C**, **D**). Thus, 30% of participants receiving Im+SOC displayed no residual parasites at 24 hours post-ingestion of the initial dose of therapy (i.e., before receipt of their second dose), whereas none of the participants on SOC displayed an absence of parasites at the same 24-hour time point (**Figure 6.4 B**). Moreover, 90% of participants in the Im+SOC cohort were devoid of blood parasites by 48-hour post

initiation of therapy, with most of the remainder becoming parasite-free by day 3. In contrast, only 14% of participants in SOC group were devoid of parasites at the end of day 2 and one third still retained measurable parasites after the full 3-day course of therapy (**Figure 6.4 B**); i.e., confirming the established delayed parasite clearance in this region of Vietnam.[5] Therefore, the secondary endpoint of faster parasite clearance by the Im+SOC cohort was met.

Upon further scrutiny of the individual participant data in **Figure 6.4**, we noted that responses to the triple combination therapy were bimodal, with individuals initially diagnosed with high concentrations of parasites (panel **H**) responding more rapidly than participants initially diagnosed with a low parasite density (panel **F**). Indeed, all 10 participants presenting with >10,000 parasites/μL blood in the Im+SOC treatment group experienced a rapid decline in parasite density (~80% in 24 hours; **Figure 6.4 H**). In contrast, participants in the SOC treatment cohort showed no such trend, with participants presenting at diagnosis with either high or low parasite density responding similarly (**Figure 6.4**, panels **D**, **E**, **G**). Importantly, no participant in the triple combination treatment group experienced recrudescence following completion of the therapy (data collected for 42 days post therapy initiation).

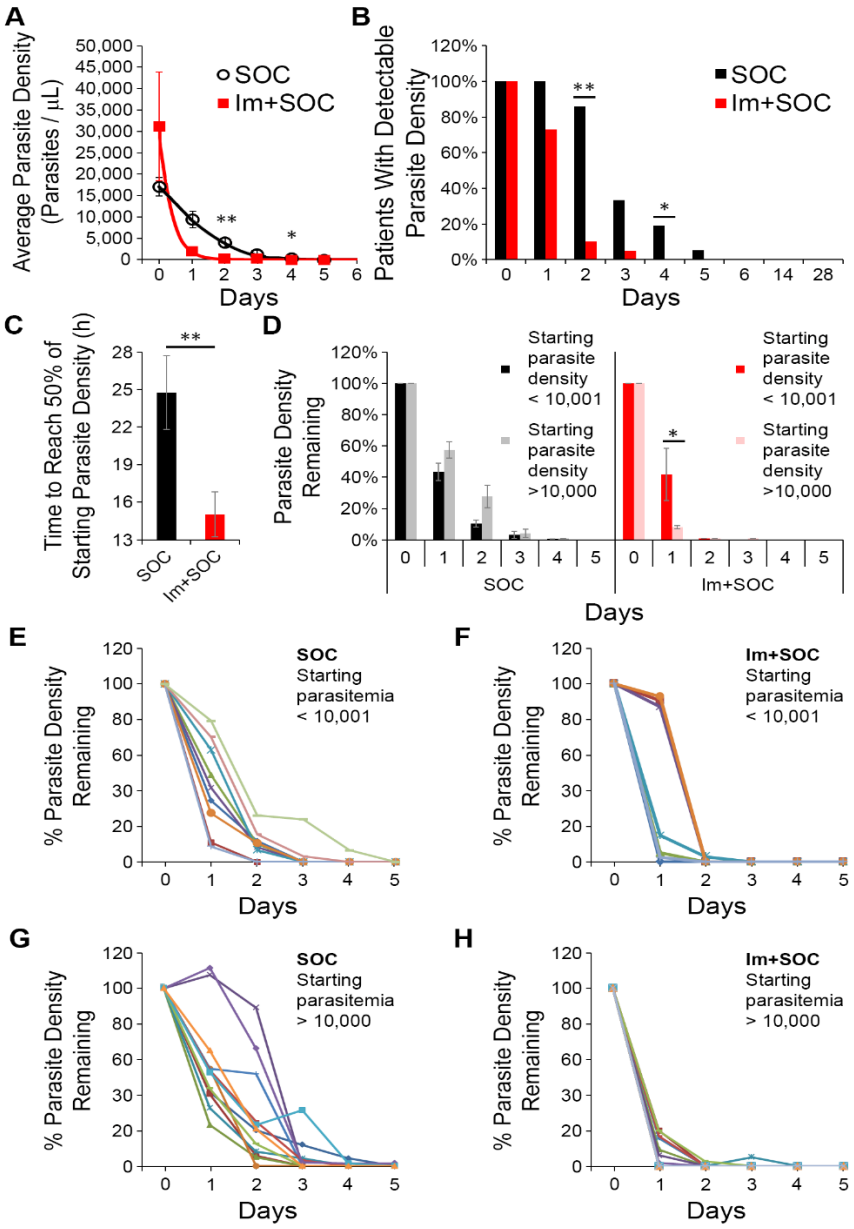

Figure 6.4 Comparison between imatinib and imatinib + SOC treatment on the reduction of parasite density. (**A**) Participants were randomly assigned to receive either 40 mg dihydroartemisinin + 320 mg piperaquine phosphate (SOC; black circles) or 400 mg imatinib plus SOC (red squares). The level of parsitemia was determined daily and averages were plotted. (**B**) The percentage of patients with detectable parasites on different days post initiation of therapy. (**C**) Average time for parasite density to decline to 50% of its starting level. (**D**) For analysis, treatment cohorts were split into two groups – participants who presented with an initial parasite density <10,001 and those who presented with >10,000 parasites/µL blood. The decrease in parasite density as a function of time was plotted separately for patients with <10,001 parasites/µL blood in the SOC arm (**E**) and Im+SOC arm (**F**) and for patients with >10,000 parasites/µL blood in the SOC arm (**G**) and Im+SOC arm (**H**). Error bars expressed as SEM and * = p-value < 0.05, ** = p–value < 0.01, *** = p-value <0.001, and **** = p-value < 0.0001.

6.5 Discussion

While the number of participants in these pilot clinical trials was relatively small, the data presented above support the conclusion that the safety and efficacy of the imatinib plus SOC combination therapy is superior to the current SOC for treatment of P. falciparum malaria. Thus, not only were no serious adverse events detected in participants treated with Im+SOC, but both parasite density and pyrexia subsided more rapidly in the Im + SOC than SOC cohorts. Because malaria-associated deaths occur most frequently in patients with high levels of parasite density,[25] the unexpectedly rapid rate of parasite clearance in the Im+SOC arm may also have the potential to save lives.

Imatinib has already established a good safety record in the treatment of chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST), allowing many patients to take imatinib chronically without serious adverse events.[22,23,26] While some CML and GIST patients indeed report limiting side effects such as abdominal pain, edema, nausea, muscle cramps, rashes, diarrhea, musculoskeletal pain, fatigue, joint pain, or headaches,[27] participants in the Im+SOC arm reported no more adverse events than those in the SOC arm, and in fact, they commonly reported feeling better sooner than those in the SOC cohort. Since clinical exams also revealed no obvious drug-related toxicities, we suspect that the cumulative toxicities that can arise from chronic dosing of imatinib do not normally emerge after only three doses of drug. However, due to the small number of participants in these pilot trials, larger studies will still need to be conducted before the safety of imatinib in malaria patients can be conclusively determined. Nonetheless, it is conceivable that imatinib can be used to enhance SOC potency without contributing significantly to its toxicity.

The ability of imatinib to improve the potency of dihydroartemisinin plus piperaquine supports our previous hypothesis that strengthening of the erythrocyte membrane can inhibit propagation of the malaria parasite.[18–20] Mechanistically, by blocking tyrosine phosphorylation of erythrocyte membrane band 3, imatinib prevents the prominent conformational change in band 3[12] that leads to dissociation of the membrane cytoskeleton from the lipid bilayer.[12,13] Blockade of this disjunction of the RBC membrane from its spectrin-actin cytoskeleton then inhibits the membrane weakening that induces both blebbing and fragmentation of the parasitized erythrocyte membrane. Because toxic hemichromes and oxidizing hemes are normally discharged within the blebbing membrane vesicles, the imatinib-induced retention of these oxidizing components within the

parasitized RBC must render the intra-erythrocytic environment increasingly toxic to the parasite.[21] Together with the fact that the parasite's egress from the erythrocyte is blocked, the parasite becomes trapped in its RBC host where the available food (i.e. hemoglobin) is largely consumed and toxic waste products (i.e. heme iron, etc.) are accumulating. Whether the resulting incarcerated parasite dies from starvation, toxification, or phagocytosis has not been determined, but the absence of recrudescence in any patients treated with the triple combination therapy indicates that all viable parasites are eliminated.

A major objective of any future malaria therapy will hopefully involve reducing the number of days a patient must be treated to experience a cure. Thus, drug resistant strains have been hypothesized to emerge because some patients feel better following intake of their first or second malaria pill and then decide to save the remaining one or two pills for a subsequent infection. Although a much larger clinical study will have to be performed before a conclusion can be proposed, the data in **Figure 6.3** and **Figure 6.4** suggest that addition of imatinib to DHA + PPQ could conceivably reduce the required days on therapy from three to two. Indeed, if the residual parasites detected in the 10% of participants at the end of day 2 were to prove to be inviable, it might be possible to optimize the Im+SOC therapy to eliminate all parasites following only two doses. Such a treatment would not only reduce the probability of patients saving pills for future infections, but also decrease the cost of the therapy, rendering it more affordable in indigent countries.

As noted by others,[11] a triple combination will generally outperform a dual combination therapy because multiple mechanisms of action are more difficult to evade than a single or dual mechanism of action. While this principle alone would argue that Im+SOC should prove more mutation resistant than SOC, we foresee still another mechanism by which the triple combination therapy should further suppress mutation-induced drug resistance. Thus, the target of imatinib is an erythrocyte tyrosine kinase (Syk[24]) that phosphorylates the protein band 3 in the human RBC.[28] Because the parasite's genome does not encode Syk,[29] it cannot mutate Syk to become imatinib resistant. While it is conceivable that the parasite could still mutate one of its own kinases to replace the function of erythrocyte Syk, this mutational strategy is also unlikely to succeed, since the *P. falciparum* genome contains no obvious tyrosine kinase.[29] Thus, evolution of mutations that could evade an Im+SOC combination therapy should be probabilistically more difficult.

Finally, there are still important issues that must be addressed before an imatinib + DHA + PPQ combination therapy can be considered a candidate for potential clinical use. These include an expanded evaluation of its safety and efficacy in larger patient cohorts that include women and children. An assessment of its potency against other species and strains of malaria along with an appraisal of its efficacy in other countries and patient populations will also be necessary. Finally, an analysis of its compatibility with other artemisinin combination therapies and an evaluation of strategies to render it affordable to patients in indigent countries will be similarly needed. Assuming that imatinib + DHA + PPQ passes these hurdles, it is hoped that the data obtained here will facilitate development of an improved therapy for treatment of this devastating disease.

Contributions

Conception of the study (PSL, FMT, AP); design of the clinical trial (FMT, HDC, AP, KRK, PSL); recruitment and treatment of patients (HDC, TAT, AP, FMT); verification, analysis, and interpretation of the data (FMT, PSL, PN, KSP); writing of the manuscript (PSL, PN, KSP).

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CHAPTER 7. QUININE AND OTHER QUINOLINE DERIVATIVES DISPLAY TYROSINE KINASE INHIBITORY EFFECTS AGAINST PROLIFERATION OF MALARIA PARASITE: A POSSIBLE ANTI-PLASMODIAL MODE OF ACTION

7.1 Abstract

Quinine and its closely related quinoline derivatives have been the pillar of malaria therapy for long. Quinine has been used to treat malaria for over 400 years, notwithstanding its low therapeutic index. The toxicities associated with quinine led research efforts to discovery of chloroquine, a less toxic and the then more efficacious quinoline derivative. However, malaria parasite quickly developed resistance to chloroquine following extensive use. While resistance and efficacy are clearly definitive, the mechanism of action of the quinolines has always remained elusive except for the well demonstrated inhibition of biocrystallization of the otherwise lethal parasite-digested hemoglobin to the less parasite-toxic hemozoin. This long-held tenet is convincing with respect to chloroquine, but there remain unresolved issues with respect to mechanism of action of quinine. Investigation of the role of membrane stability in preventing parasite egress, established a route through which tyrosine kinase inhibitors are toxic to Plasmodium *falciparum*. In a similar manner, the present study discovered that quinine blocks orthovanadate stimulated band 3 tyrosine phosphorylation. The study identified that quinine also inhibits parasite induced band 3 tyrosine phosphorylation and, prevent the concomitant discharge of hemoglobin and erythrocyte derived microparticles from malaria infected red blood cells. That the quinolines inhibits Syk in cell free assays, further support this mechanism.

7.2 Introduction

Malaria remains one of the deadliest parasitic diseases in much of the developing world, causing 405,000 deaths from 228 million global cases in just one year.[1] According to World Health Organization (W.H.O.) estimates, majority of the cases arise from the tropics,[2,3] with Africa alone accounting for over 90% of both incidence rates and death cases.[1] Since 2010, malaria prevalence has been on a sharp decline until 2014 after which it remained substantially unchanged through 2018.[1] Much of the decline has been occurring in Africa and South East Asia, however other regions, more so the Americas, have recorded an uptick in the case incidence between 2017 and

2018.[4] The interrupted trend of decline in malaria infection rates is a major cause of concern undermining the ongoing eradication efforts.[5]

Efforts to eliminate malaria worldwide have focused on both vector control and effective medicine ventures.[5–7] However, emergence of insecticide resistant mosquitoes[7] and the rising cases of parasite resistance to artemisinins,[8–10] glaringly compound these control efforts. To abate parasite resistance to drugs, elucidating drugs' mode of actions is an important aspect necessary to better understand molecular reasons underlying development of resistance. Although quinine has been in chemotherapeutic use against malaria since time immemorial, its mechanism of action has remained unclear, with reference to the chloroquine approach as a common mechanism to these cinchona alkaloids.[11–13]

In this chapter, a probable route through which anti-malarial efficacy of quinine may be based on, is presented. The data revealed that quinine and other quinoline derivatives display anti-spleen tyrosine kinase (Syk) activity. Because Syk and other tyrosine kinase inhibitors have been shown to impede parasite proliferation through inhibition of band 3 tyrosine phosphorylation, the data presented herein also show that quinine, mefloquine and chloroquine inhibit band 3 tyrosine phosphorylation, albeit with varying potency. Additionally, the three drugs decrease the rates of membrane fragmentation and discharge of free hemoglobin from parasite-infected red cells. That these drugs inhibit the activity of Syk in cell free assays, firmly support a possible alternative mechanism of action of quinine and the quinoline derivatives. For this reason, therefore, the hypothesis arose that quinine and quinolines derivatives could alternatively be impeding parasite proliferation by blocking its egress. This chapter provide possible foundations through which quinine and other quinoline derivatives may be acting against Plasmodium *falciparum* through inhibition of erythrocyte Syk kinase.

7.3 Methods

7.3.1 Malaria culturing and parasite's life cycle synchronization

Blood was collected from volunteers served with written informed consent and in accordance with Purdue University approved institutional board review (IRB) protocols, and the Good Clinical Practice Guidelines and the Declaration of Helsinki. The blood was centrifuged at 1250 rcf for 5 minutes and plasma aspirated. Pelleted erythrocytes were washed three times with

complete medium (CM) [RPMI 1640 medium (Invitrogen) supplemented with 2 mM glutamine, 25 mM HEPES, 20 mM glucose, 27 μg/mL hypoxanthine, and 32 μg/mL of gentamicin (Sigma) (pH 7.2)]. After the last wash, remaining red blood cells (RBCs) were suspended in CM at 50% hematocrit. The blood was used for culturing Plasmodium *falciparum* (Palo Alto strain) at hematocrit levels not exceeding 3%. In the event that synchronized culture is required, a fraction of the cultured cells was removed and synchronization conducted as explained previously with few modifications.[14] Briefly, infected cells with majority of the parasites at ring stage (~12 h post invasion) were incubated with 5% sorbitol for 5 minutes. The cells were vortexed for 15 seconds, centrifuged at 250 rpm for 5 minutes and thereafter washed three times with complete medium. To generate a tighter synchrony, the cells were incubated in complete media for 34 h and then subjected to magnetic separation. This approach would capture parasites that are at schizont/segmenter stage while the already egressed cells would elute out of magnetic columns due to their low hemozoin content.

7.3.2 Band 3 Tyrosine Phosphorylation

For sodium orthovanadate (OV) stimulation of band 3 tyrosine phosphorylation, fresh healthy blood was washed as described above except that phosphate buffered saline containing 5 mM glucose (PBS-G) was used in place of CM. Washed cells were suspended at 30% hematocrit in PBS-G, were separately incubated with quinine, mefloquine and chloroquine at 37 °C under 50 rpm shaking for 30 minutes followed by addition of 0.2 mM OV and then incubated under the same conditions for an additional 30 minutes. Addition of OV was omitted from the untreated control sample.

To prepare samples for Western blotting, 10 μL of each sample was dissolved in 100 μL of 4x SDS-PAGE sample buffer based on Cold Spring Harbor recipe,[15] then heated to 95 °C for 5 minutes. Proteins were resolved using SDS-PAGE electrophoresis through a 10% SDS-PAGE gel as explained in chapter 5 section 5.3.1. The proteins on the gel were then transferred to a nitrocellulose membrane at 100 V for 2 hours and immunoassayed with mouse anti-phosphotyrosine (Santa Cruz Biotechnology #SC-7020), and rabbit anti-actin (Sigma) at 1: 1000 and 1:20000 concentrations, respectively. After an overnight primary antibody incubation, membranes were washed 3 times with TBST (tris buffered saline with 0.1% v/v Tween-20) and then incubated with respective anti-mouse or anti-rabbit secondary antibodies conjugated with

IRDYE detectable at 700-800 nm of infrared region. The membranes were washed 2 times with TBST followed by 2 more washes with PBS and then immediately imaged using LI-COR scanner.

7.3.3 Quantitation of erythrocyte-derived microparticles (MPs) and free hemoglobin (Hb)

At ~34 h post invasion (i.e., time at which enhanced membrane fragmentation starts), malaria infected RBCs were washed two times with CM, re-suspended at 2% hct and treated with varying concentrations of quinine, chloroquine, and mefloquine for 18 hours (i.e., until egress stage of the parasites). The treated infected cells were centrifuged at 500 rcf for 5 minutes to separate the media (containing released hemoglobin (Hb) and MPs) from packed red cells. The supernatant was centrifuged twice at 2500 rpm for 15 minutes each time, to remove any residual erythrocytes. After each centrifugation cycle, the supernatant was pipetted off into new 1.5 mL micro-centrifuge tubes while leaving behind the formed pellet. The pellet was bleached and discarded into a biohazardous waste container. For flow cytometry analysis, 100 µL of the supernatant was incubated for 20 minutes with BV421 mouse anti-glycophorin A antibody (BD biosciences #562938). After the incubation, 1000 µL of stain buffer (BD biosciences #554656) was added to the samples and mixed well. Finally, 1000 µL of the diluted samples was transferred to Trucount^{TM} tubes ((BD biosciences #340334) and analyzed on the Attune NxT Flow Cytometer[16] to obtain the number of erythrocyte-derived microparticles released during parasite maturation to egress.

The content of Hb in the supernatants was quantified using Cayman Hb colorimetric assay (Cayman Chemical #700540) according to manufacturer's instructions.

7.3.4 Assessment of life stage of malaria impacted by quinolines

With > 60% of parasite at ring stage, infected red cells (iRBCs) were treated with either quinolines or imatinib and incubated for 24 h in 48-well plate. The cells were re-suspended and transferred into tubes, centrifuged at 500 rcf for 5 minutes and washed 2 times with sterile PBS-G. Washed iRBCs were suspended in 1 mL PBS-G and stained with SYBR Green I (Invitrogen #S7563), incubated at room temperature for 20 minutes and then the cells were washed 2 times with PBS-G. The parasite growth was analyzed using Attune NxT Flow Cytometer.

7.4 Results

7.4.1 Synchronized parasite cultures

Malaria parasite development within red blood cells undergoes three stages; ring, trophozoite and schizont stages, **Figure 7.1**, A. Ring stage involves parasites that are at the point of invasion (entry into the host cell) and up to 24 hours after invading the red cell, i.e., 24 hours post invasion (hpi) trophozoites and schizonts are 24-40 hpi and 40-48 hpi, respectively. To ensure that the parasites under investigation are in the same developmental stage, the cells were synchronized as explained in methods section. Synchronization by 5% sorbitol yielded parasites that are mostly in the ring and young trophozoite stages, **Figure 7.1**, B and C. Magnetic separation in the last step was able to generate a tightly synchronized culture that consisted exclusively of schizont/segmenter stage parasites, panel D.

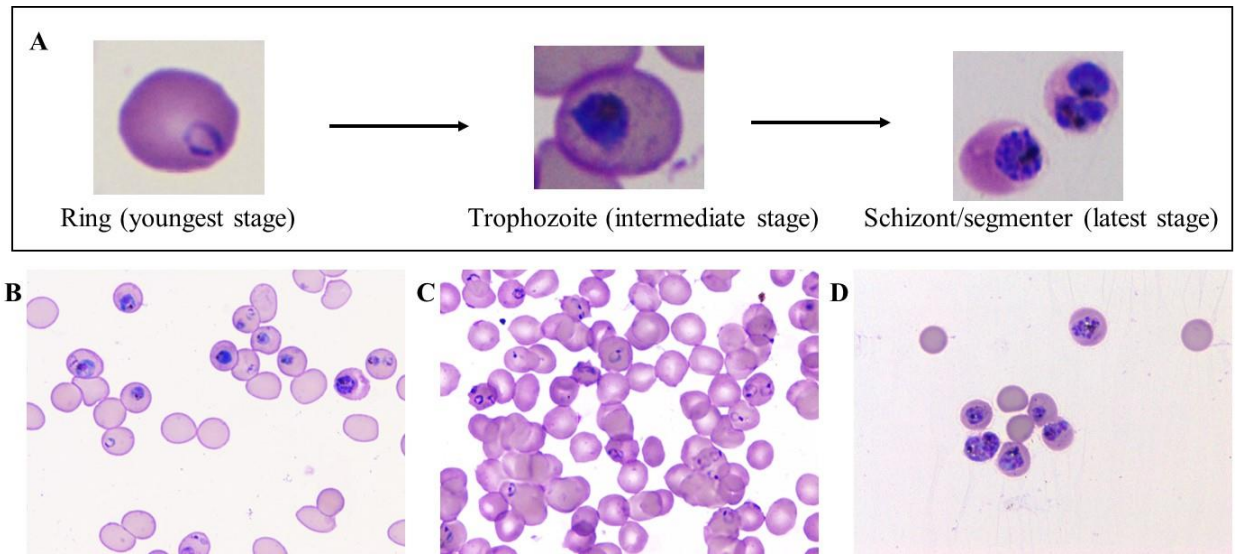

Figure 7.1 Illustration of malaria parasite synchronization steps and life cycle stages. Unsynchronized parasites, **A**, displaying all life cycle stages, were incubated with aqueous solution of 5% sorbitol to lyse infected red cells with late-stage parasites, **B**. After 34 hours of incubation with culture media, the loosely synchronized cells in **B** were subjected to magnetic separation to eliminate red cells containing parasites in younger stages of development thus leaving behind schizonts/segmenters only (**C**). Panel **D** illustrates developmental steps of malaria parasite (**D**) within erythrocytes.

7.4.2 Inhibition of orthovanadate-induced band 3 tyrosine phosphorylation

Following previous observations that quinine suppressed generation of MPs. in healthy RBCs stimulated with Ca^{2+} influx, special interest arose about the exact target of this drug. While the mechanism explored therein was based on inhibition of calcium ionophore channel (A23187) and hence blockade of calcium influx into the cell, nothing much have been proposed regarding inhibition of tyrosine kinase Syk.[17] To examine whether quinine and other quinolines display kinase inhibition effects, healthy cells were treated with 0.2 mM OV and the extent of inhibition of band 3 tyrosine phosphorylation was used as a measure of kinase inhibition. Surprisingly, quinine and mefloquine were found to abrogate band 3 tyrosine phosphorylation, **Figure 7.2**. Chloroquine, however showed weaker effects on band 3 tyrosine phosphorylation. At this juncture, it became apparent that quinine and mefloquine may be weak Syk kinase inhibitors thus the drugs may act against proliferation of malaria parasite through inhibition of Syk kinase in a manner similar to the well-characterized mode of action of the more specific Syk inhibitors R406c and the off-target anti-Syk drug imatinib.[14]

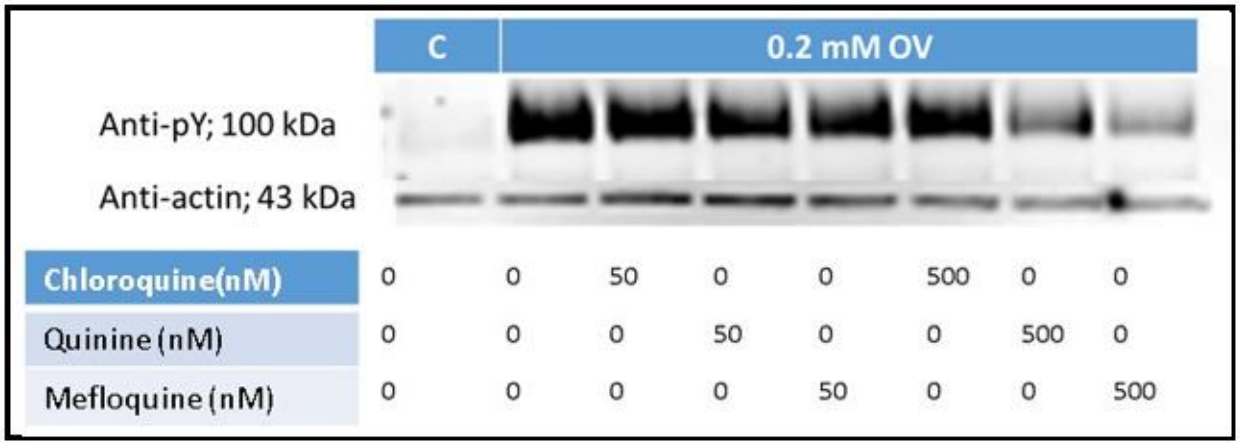

Figure 7.2 Inhibition of band 3 tyrosine phosphorylation by quinolines. Healthy 30% hematocrit blood (in PBS-glucose) was incubated with respective drugs for 30 minutes at 37 °C and 50 rpm shaking prior to addition of 0.2 mM OV and a further 30-minute incubation. A volume of 10 µL of each sample was dissolved in 100 µL of SDS-PAGE sample buffer and subsequently used for western blot.

7.4.3 Inhibition of Plasmodium falciparum-induced band 3 tyrosine phosphorylation

To investigate whether the quinolines could inhibit parasite induced band 3 tyrosine phosphorylation, quinine was used as a representative. Immunoblots revealed that quinine is indeed able to block parasite induced band 3 tyrosine phosphorylation, **Figure 7.3**. The immunoblot lays a strong foundation that quinine could be killing the parasite through inhibition of tyrosine kinase.

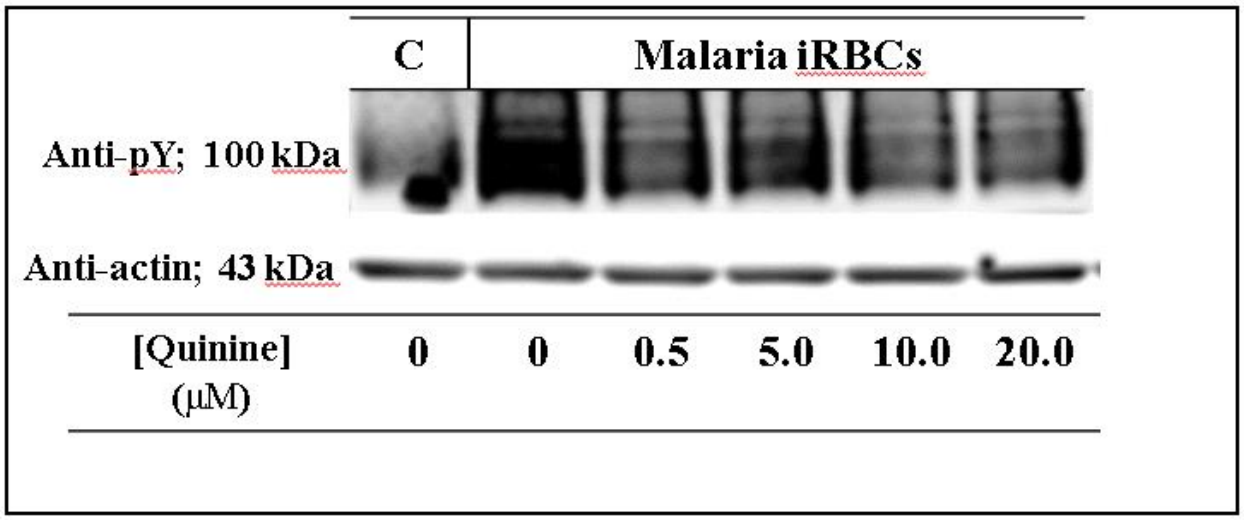

Figure 7.3 Inhibition of band 3 tyrosine phosphorylation by quinine. Infected cells at ~24 h post invasion, were incubated with quinine for an additional 18 h. Band 3 tyrosine phosphorylation was examined after the treatment.

7.4.4 Quinine halt parasite egress

Inhibition of band 3 tyrosine phosphorylation has been proposed to block parasite egress.[18] Because quinine was found to be a probable tyrosine kinase inhibitor, it was tested whether it could halt parasite egress. After 18 h incubation of iRBCs with quinine, thin blood smears were prepared and visualized under light microscopy on a Nikon microscope. As seen in **Figure 7.4**, most of the parasites are in schizont/segmenter stage in the treatment group while the untreated control has none.

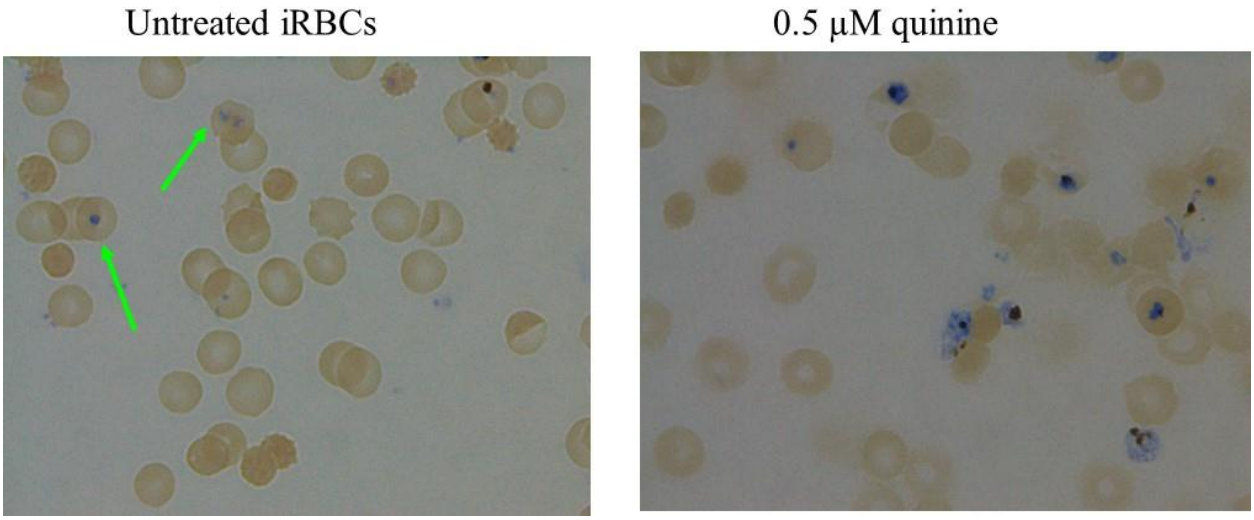

Figure 7.4 Quinine halt parasite egress. Thin blood smears were prepared after 18 h incubation of iRBCs with quinine. The light green arrows on the untreated thin blood smear slide indicates newly formed rings (<4 h post invasion).

7.4.5 Quinolines inhibit discharge of MPs. and free Hb

Because band 3 tyrosine phosphorylation cause membrane weakening through the shedding of MPs. and release of free Hb, quinolines were assessed if they could inhibit the process in subject. **Figure 7.5Figure 7.5** shows that the quinolines significantly block fragmentation of iRBCs which agrees with the smears shown in the preceding section.

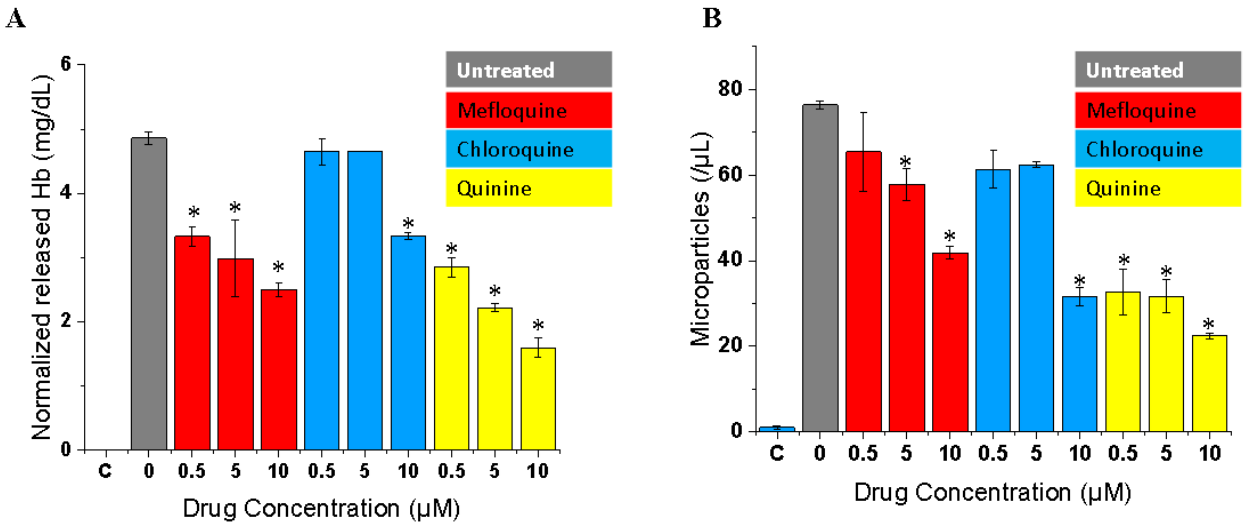

Figure 7.5 Inhibition of membrane fragmentation by quinolines. Malaria infected cells were incubated with the drugs at the indicated concentrations. Treatment was initiated at trophozoite stage (~ 32 h post invasion) and continued for 20 hours.

7.4.6 Assessment of impact of quinolines on malaria parasite maturation

Thin blood smears provide a visual perspective of the developmental stage of malaria parasite. For a thorough investigation on impact of quinolines on parasite maturation, flow cytometry was used to gauge the impact of parasite on transition from one life stage to another. This approach would allow for inter-stage transition observation because each life stage has a different mean fluorescent intensity (MFI) due to progressive increase in the DNA synthesis by the parasite.[14,18] In this case, the ring and schizont/segmenter stages have the least and highest MFI, respectively.

Before addition of drugs to the infected cells, flow cytometry shows relative abundance of parasite stages as 62% rings, 26% trophozoites and 12% schizonts, **Table 7.1** and **Figure 7.6**.

Table 7.1 Relative abundance of Plasmodium falciparum developmental stages following treatment with quinolines and imatinib. The concentration of quinine, mefloquine and chloroquine was set at 500 nM while imatinib concentration was 2.5 µM.

Stage	0 h	24 hours after treatment				
	Untreated	Untreated	Quinine	Mefloquine	Chloroquine	Imatinib
Rings	62	35	28	24	62	31
Trophozoites	26	43	57	71	35	50
Schizonts	12	22	15	5	3	19

After 24 hours, the untreated control consisted of 35% rings, 43% trophozoites and 22% schizonts while quinine-treated cells contained 28% rings, 57% trophozoites and 15% schizonts. The positive control, imatinib treatment yielded 31% rings, 50% trophozoites and 19% schizonts. This observation suggests that, quinine does impact parasite maturation in a way similar to imatinib (inhibition of parasite egress). Parasites treated with chloroquine experienced a stalled development at ring stage, i.e., rings remained at 62% after 24 hours of drug incubation which implies a different mode of action from that of quinine. Moreover, mefloquine halted parasite's transition into schizont stage but the rings were able to progress to trophozoites.

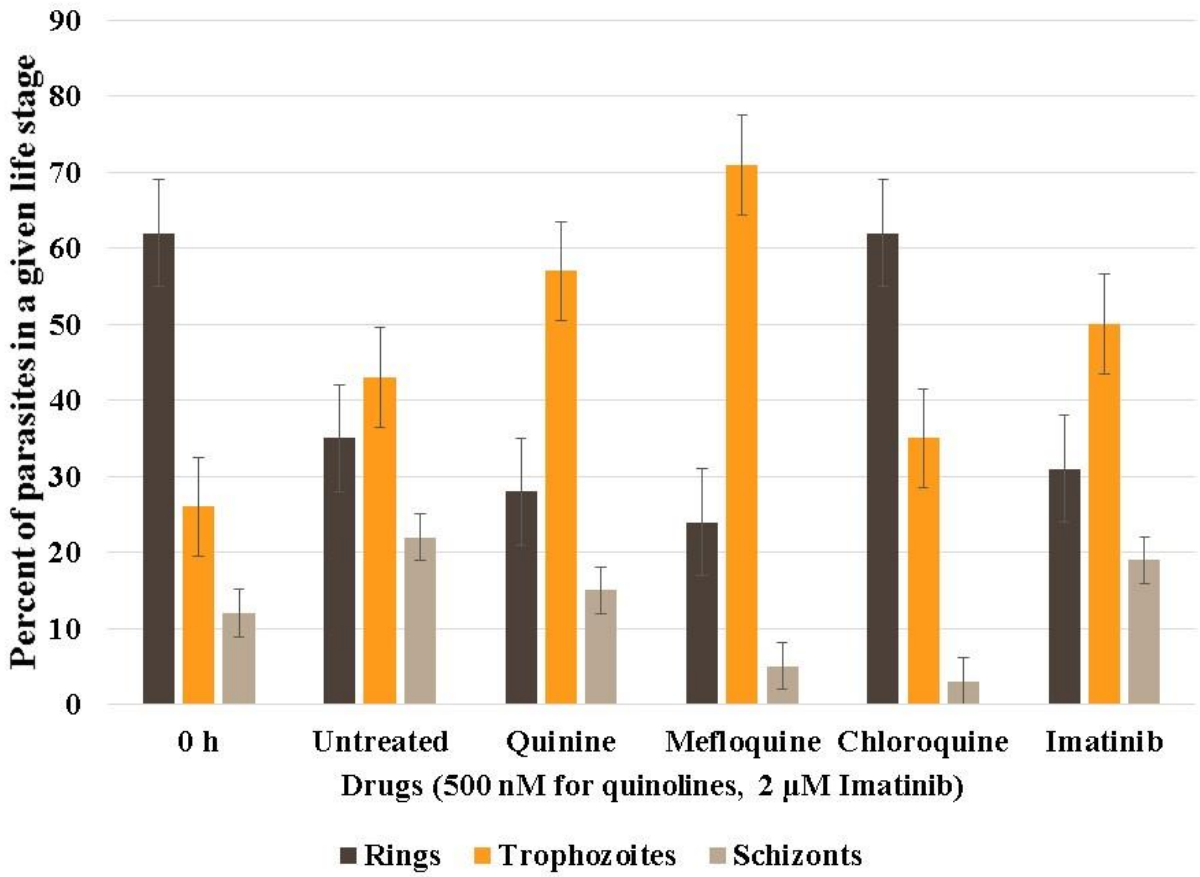

Figure 7.6 Assessment of impact of quinolines on malaria parasite development. Imatinib was used as a positive control or reference to the impact of Syk kinase inhibitors on malaria parasite development.

7.4.7 Inhibition of Syk in cell free assays

Since the quinolines especially quinine inhibit parasite egress, the next course of action was to probe if these drugs inhibit Syk in cell free assays. Promega™ ADP-Glo assay was employed to determine the half maximal inhibitory concentration (IC_{50}) of the drugs. As shown in **Figure 7.7** below, quinine, mefloquine and chloroquine display anti-Syk activity with IC_{50} values of 49 µM, 75 µM and 393 µM, respectively. The data herein present additional positive grounds that quinine and possibly other quinolines may be impacting parasite proliferation through inhibition of Syk leading to entrapment of parasites inside red cells thereby preventing new invasion.

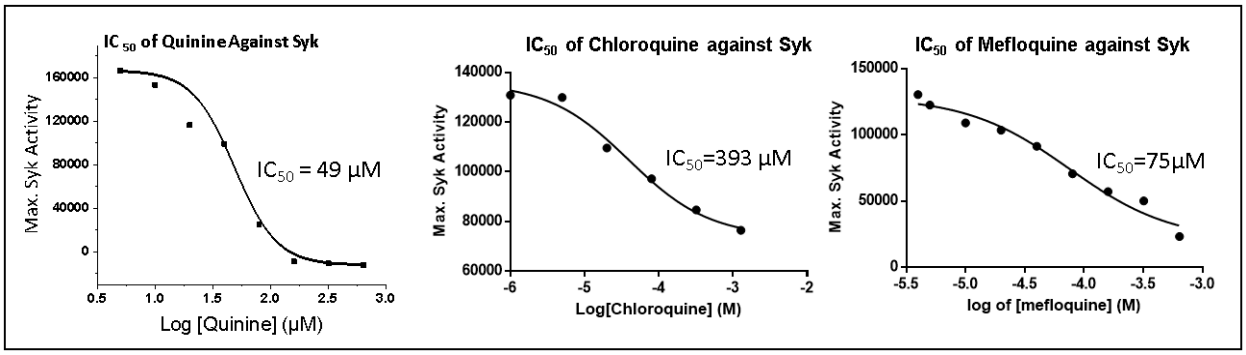

Figure 7.7 Inhibition of Syk by the quinolines in cell-free assay using ADP-Glo. The kinase inhibition was conducted according to manufacturer's instructions.

7.5 Discussion

It is hypothesized that quinine works in a mechanism similar to that of the well-characterized chloroquine, but this has not been proven.[11] The fact that widespread resistance to chloroquine[19,20] but not to quinine[11] has developed in malaria endemic regions over time, is suggestive that quinine uses a different mechanism of action. Previously, several publications have demonstrated that rupture of erythrocyte's membrane can be triggered by band 3 tyrosine phosphorylation,[14,21,22] a protein modification process driven by imbalance between the constitutive action of protein tyrosine phosphatases and protein tyrosine kinases.[23] During digestion of Hb by the parasite, produced reactive oxygen species induce oxidative stress in the host cell. The oxidants elicit oxidative inhibition of erythrocyte protein tyrosine phosphatases that would otherwise maintain low levels of band 3 tyrosine phosphorylation.[24–26] Additionally, Syk is progressively activated as the parasite matures pointing out at a necessary role that is played by the kinase during malaria parasite proliferation.[18,27,28]

That Syk inhibitors treat malaria,[14,18] and that quinine is a potent inhibitor of band 3 tyrosine phosphorylation strongly suggest that quinine is acting by inhibiting tyrosine kinases which in turn prevent parasite proliferation. Other quinolines, mefloquine and chloroquine, were also found to inhibit Band 3 tyrosine phosphorylation albeit with varying concentration. Of note, is the much higher concentration of chloroquine necessary to inhibit band 3 tyrosine phosphorylation. While the quinolines' concentrations used in this study may be a little bit higher than the clinical range of 0.1-0.3 µM,[29] it is worth noting that the weak base effect of these drugs modulate excessive intra-erythrocytic gradient causing accumulation of chloroquine 1000 folds and 70 fold for quinine/mefloquine within infected erythrocytes. During convalescence, plasma

concentrations of the quinine peaks at concentration as high as 37µg/mL of plasma (i.e., 116 µM).[29] While the minimum required therapeutic concentration of quinine is 4.5µg/mL of plasma (i.e., 14 µM).[29] Such conditions would increase intracellular concentrations of these drugs to levels above their IC_{50} values against Syk (**Figure 7.7**) within the infected erythrocytes.

It should be noted that the parasite itself is the sole reason for increased accumulation of quinolines in iRBCs. Although targeting band 3 tyrosine phosphorylation may delay emergence of kinase inhibitors' resistant strains of malaria parasite, the parasite have evolved ways to exclude intra-erythrocyte accumulation of chloroquine but not quinine.[19,20] Exclusion of quinolines from entry into infected red cells would certainly reduce the kinase inhibitory efficacy of the drugs. This would be unexpected with respect to neutral Syk inhibitors such as R406 and imatinib. This study therefore has revealed that quinine and possibly other quinolines may kill the malaria parasite by blocking band 3 tyrosine phosphorylation and thus trapping the parasite to death within the cell. The study further provides more grounds that band 3 tyrosine phosphorylation is an important target for malaria therapeutic interventions and such a mutation-resistant approach could be the reason for the relentless efficacy of quinine against malaria. Even though malaria therapies are running away from quinine use due to its toxicity, this study support the use of Syk kinase inhibitors for the fight against malaria as potential mutation-resistant antimalarials.

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CHAPTER 8. CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions and further hypotheses

The dissertation has extensively explored the role of band 3 tyrosine phosphorylation and provided evidence-based ideas that tyrosine phosphorylation of band 3 is a druggable target worth considering for SCD and malaria therapies. Figure 8.1 below summarizes the concept of inhibiting band 3 tyrosine phosphorylation with respect to sickle cell disease. Briefly, inhibiting band 3 tyrosine phosphorylation will prevent membrane destabilization and, as a result, reduce the content of free heme, free plasma hemoglobin and microparticles which are largely responsible for triggering vaso-occlusive crisis.

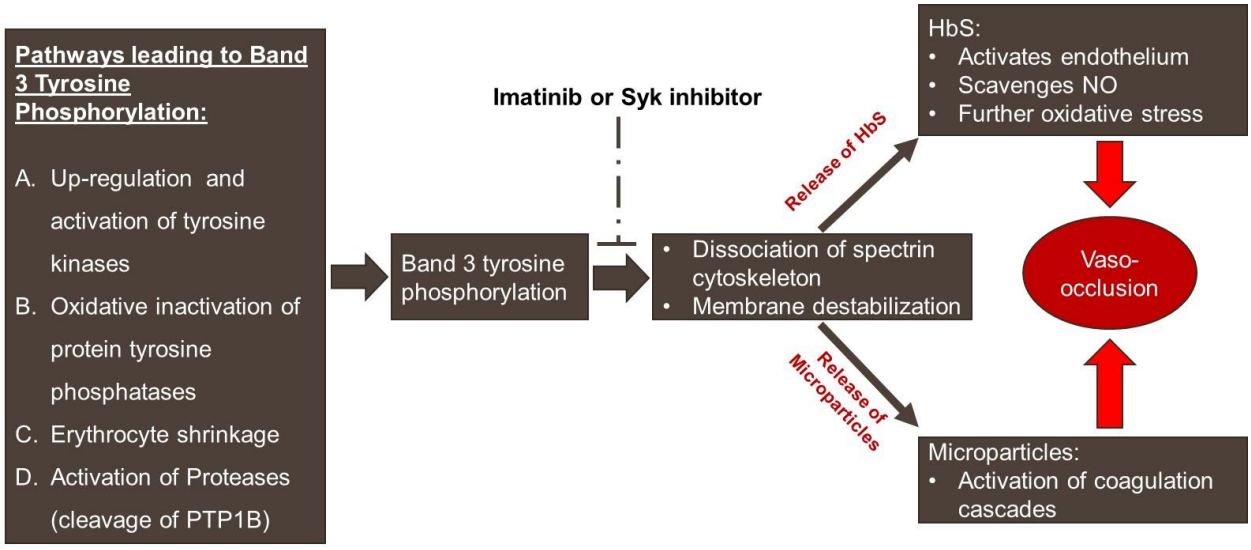

Figure 8.1 Pathways involved in triggering band 3 tyrosine phosphorylation and its consequential link to vaso-occlusive crisis. Imatinib or a Syk inhibitor block band 3 tyrosine and hence help prevent vaso-occlusive crisis as at the downstream level.

Concerns on the use of kinase inhibitors for children affected by SCD are legitimate and new drugs that do not target growth receptors are worth research efforts to provide an all-inclusive treatment regimen. However, tyrosine kinase receptors responsible for growth are similar in structure which compounds selectivity of kinase inhibitors.[1] Nonetheless, efforts to design more selective Syk kinase inhibitors are ongoing in Low lab and elsewhere, with significant progress.[2,3] Since sickle cell disease is unique in the essence that growth, development and angiogenesis

(required due to recurrent tissue injury) are necessary for a better life, such factors have to be at the forefront of new drug designs. At the bare minimum, a good Syk inhibitor for use in sickle cell therapy should have the following properties:

i. kinase inhibitors for sickle cell treatment should not inhibit receptors responsible for growth, e.g. HGFR, IGFR and EPGR.[1,4]
ii. kinase inhibitors that minimize impairing the function of receptors that are critical for angiogenesis especially vascular endothelial growth receptor VEGFR and to a lesser extent, the platelet derived growth factor (PDGFR).[4–6]
iii. must be cell permeable to penetrate through erythrocyte cell membrane.
iv. must display high potency against Syk over other growth receptors as opposed to a very potent Syk inhibitor with little selectivity. In this aspect, the IC_{50} value maybe in the micromolar range but if IC_{50} of the drug against other kinases is higher (say >10 times), the drug should be given consideration.

With a clinical trial ongoing, new insights on efficacy of imatinib on sickle cell disease will be evaluated. It should be noted that, imatinib is a weak Syk inhibitor with IC_{50} in cell free assays of ~5 µM.[7] Imatinib is also not selective with regards to growth receptors and this aspect has led to stunted growth in children suffering from chronic myelogenous leukemia (CML) who have been prescribed to the drug.[8,9] Additionally, imatinib also inhibits VEGFR,[5] but banking on its ability to stop the upstream tissue injury stimulants (i.e. release of microparticles, free heme and hemoglobin) cast a silver lining on its efficacy.

With respect to malaria, imatinib has already demonstrated high efficacy under triple combination therapy with a potential of resolving pyrexia in 1.5 days on average. However, its monotherapy displayed reduced efficacy and inability to quickly clear parasitemia when compared to standard of care therapy in South East Asia (Vietnam). Considering that nearly all anti-malaria drugs have one weakness e.g. recrudescence for artemisinins and toxicity for quinolines,[10] and that imatinib is a weaker Syk inhibitor as stated above (with other more potent inhibitors such as fostamatinib already approved by FDA[11]), the apparent and most promising aspect is the advent of a new readily available class of drugs that can be added to our therapeutic arsenal against malaria. A much larger clinical trial in other regions will still have to be performed for more confidence on reliability of imatinib in treatment of malaria.

The data on the mechanism of action of quinine and other quinoline derivatives as inhibitors of band 3 tyrosine phosphorylation (or inhibitors of Syk, a tyrosine kinase), posits the said phosphorylation is a reliable druggable target that could further be investigated. That quinine has not experience global parasite resistance, may be an indication that the drug could be targeting a human-based enzyme necessary for parasite proliferation. In other words, Syk inhibitors may turn out to be game changers with respect to preventing development of drug resistant strains of malaria parasites.

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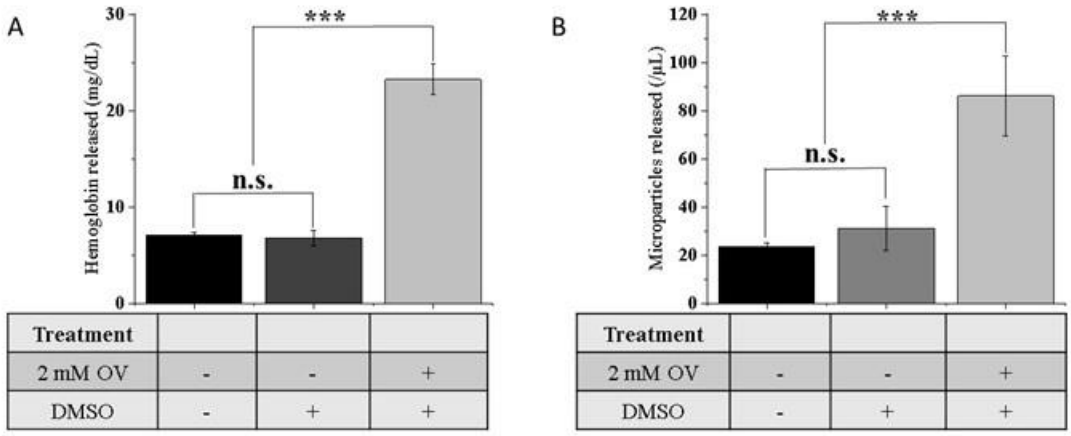
APPENDIX A: SUPPLEMENTAL INFORMATION FOR CHAPTER 2

Quantitation of Band 3 Tyrosine Phosphorylation

Washed RBCs were suspended at 30% hematocrit (Hct.) in PBS-G and treated with either 5 μM drug (imatinib, PRT062607 or R406) or vehicle (control) for 4 hours at 37°C under 50 rpm shaking. RBC ghosts were prepared by mixing 150 μL packed RBCs with 1500 μL ice-cold ghost buffer (5 mM Na_2HPO_4, 1 mM EDTA, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride and 1% phosphatase cocktail inhibitors 2 and 3) and incubated on ice for 30 min. Samples were centrifuged at 13000 rpm for 15 min at 4 °C and supernatants were removed. Ghost pellets were washed an additional 3x, after which 10 μL was removed for determination of protein concentration using Pierce™ Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, A53226). Residual ghosts were solubilized in 4x Laemmli buffer (Bio-Rad) at a concentration of 1μg protein/μL buffer and warmed to 45 °C for 15 min prior to storage at -20 °C until use.

Band 3 tyrosine phosphorylation was quantitated by loading sample onto a 10% SDS-PAGE gel and separating at 90, 120 and 150 V for 30, 45 and 20 minutes, respectively. Proteins were transferred to a nitrocellulose membrane at 100 V for 2 hours, after which membranes were immunostained with anti-phosphotyrosine (1:1000, Cell Signaling Inc., #9411S) or anti-actin (1:20000; Sigma Aldrich #A2103) antibodies dissolved in TBST (25 mM Tris, 140 mM NaCl, 3 mM KCl, 0·05% v/v Tween-20) containing 5% w/v milk. After washing, membranes were incubated in TBST containing anti-mouse-horseradish peroxidase or anti-rabbit-horseradish peroxidase antibody conjugates, then washed 4x in TBST, and incubated with SuperSignal™ West Pico chemiluminescent substrate (Thermo Scientific™) prior to imaging with a Chemidoc XRS+ (BioRad). Band 3 tyrosine phosphorylation intensity was quantitated using image J software.

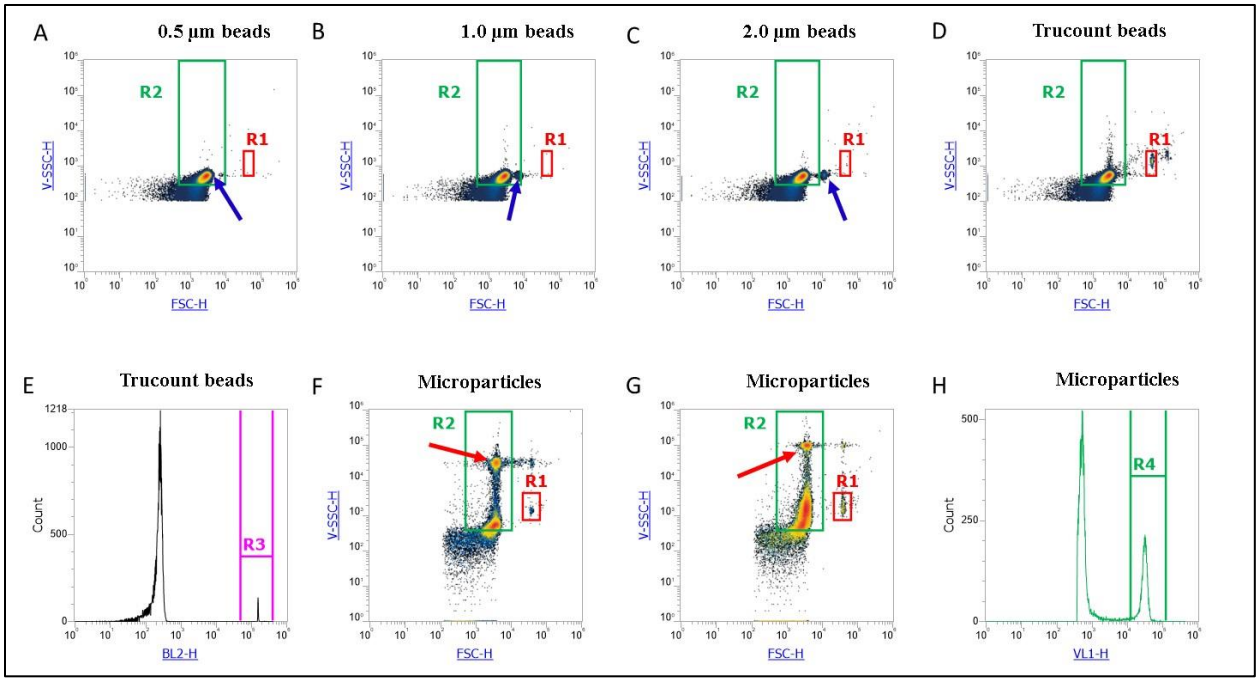
To induce Band 3 tyrosine phosphorylation and membrane fragmentation in healthy erythrocytes, blood from healthy donors was washed and cells were suspended at 30% Hct in PBS-G prior to incubation with imatinib or dimethyl sulfoxide (DMSO) at 37 °C for 1 h. After addition of 2 mM sodium orthovanadate, cells were incubated for 4 h at 37 °C while shaking at 1400 rpm.To minimize impact of vehicle (DMSO) on the cells, the volume of DMSO added to the cells was kept at ≤0.5% of the total test volume. Figure 1 below illustrates that DMSO does not impact hemolysis (Panel A) or microparticle formation (Panel B)



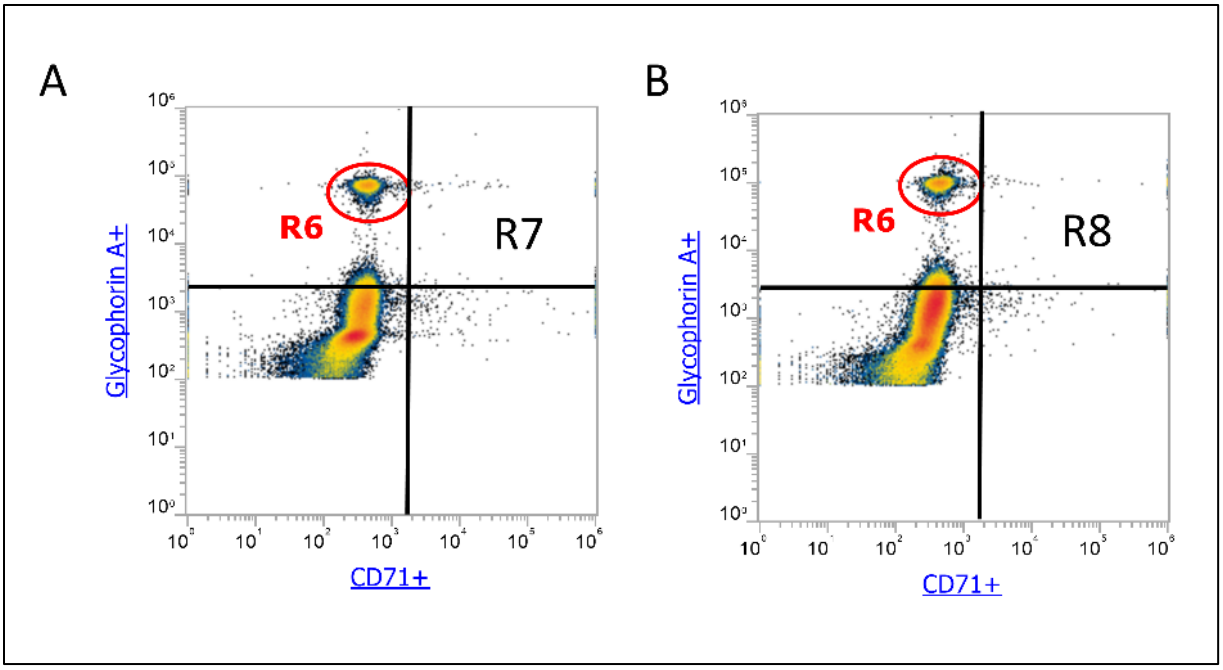
Supplemental Figure 1: Effect of DMSO on release of hemoglobin;Hb (Panel A) and microparticles; MPs. (Panel B). Erythrocytes suspended at 30% hct were treated with0.5% v/v of DMSO and/or Band 3 tyrosine phosphorylation stimulant (sodium orthovanadate; OV) prior to analysis of the amount of Hb and MPs. released.

Microparticle quantitation

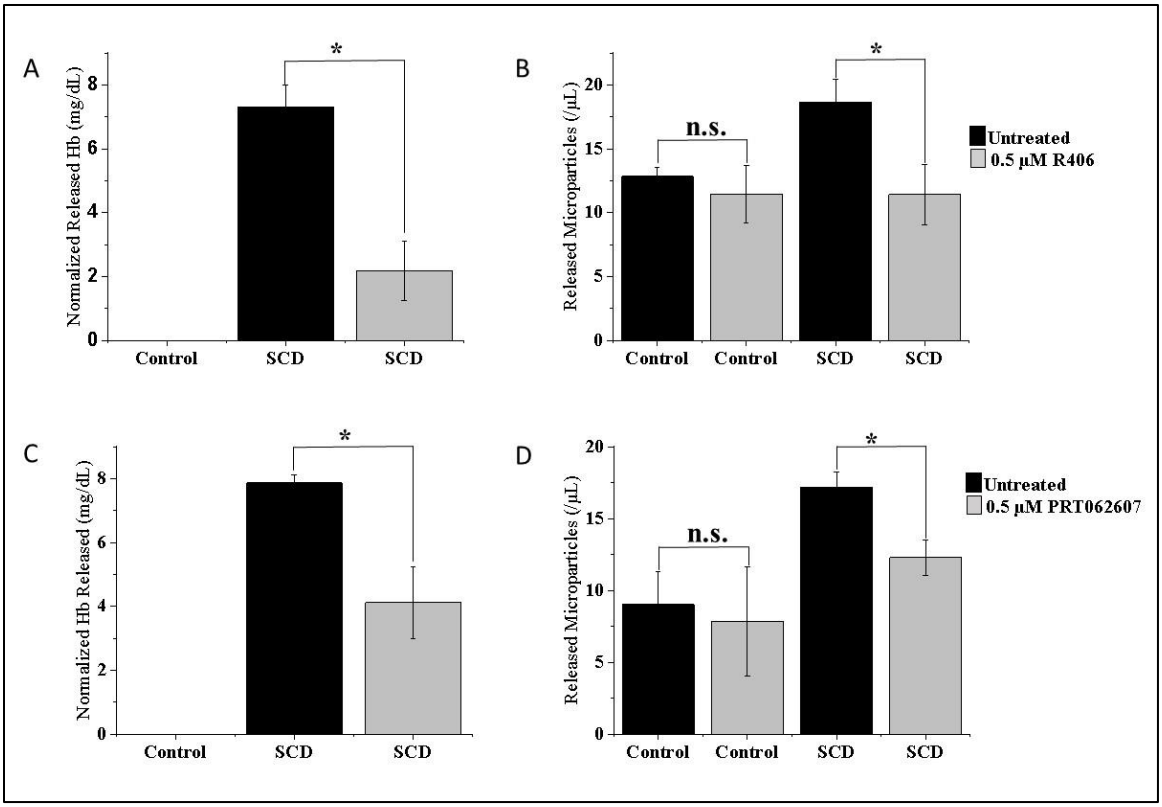
Microparticles were identified as glycophorin A positive particles in the size range of 0.1-1.0 µm. The size range was determined using flow cytometry sub-micron particle size reference beads (Thermo Fisher # F13839) as illustrated in Supplemental Figure 2 panels A, B and C for 0.5 µm, 1.0 µm and 2.0 µm diameter beads respectively, on V-SSC-H (violet side scatter; for better small-particle resolution) vs FSC-H (forward scatter) dot plot. Gate R2 (green) was set as the region for microparticles/events smaller than 1.0 µm diameter. Blue arrows in panels A-C indicate the positions of 0.5 µm, 1.0 µm and 2.0 µm diameter beads respectively, while Figure 2 panel D shows Trucount counting beads (~4.2 µm diameter) which are gated in R1. As can be seen in panels C and D, 2.0 µm beads falls outside gate R2 as do the Trucount beads. Trucount beads were detected in the blue fluorescence detector (488 nm) as shown in Figure 2 panel E, gated R3. Figure 2 Panel F and G show glycophorin A positive erythrocyte-derived microparticles (red arrows) in the plasma from healthy and sickle cell patients, respectively (gate R2). Figure 2 Panel H represents events within gate R2 where glycophorin A positive events (erythrocyte-derived microparticles) are gated in R4 (which constitute the number of erythrocyte-derived microparticles reported in this article).



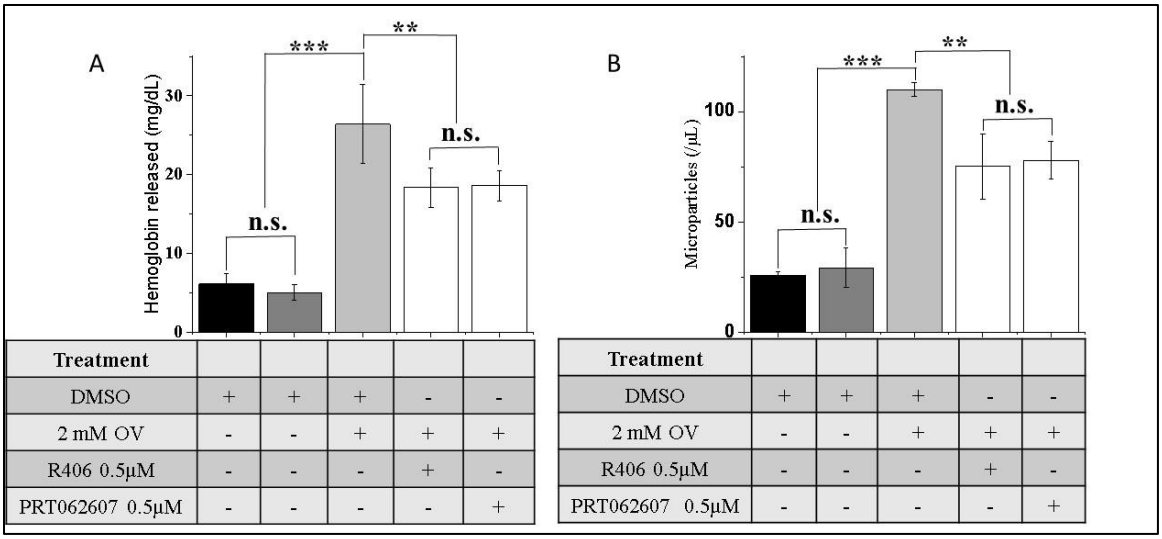
Supplemental Figure 2. Quantitation of erythrocyte-derived microparticles by Attune NxT Flow Cytometer.



Supplemental Figure 3: Identification of surface markers of erythroid derived Microparticles. Microparticles were stained with 0.5 µL of mouse anti-human glycophorin A antibody and 1.0 µL of CD71 antibody (BD Biosciences #12-0711-82) and incubated for 20 minutes on ice prior to analysis with Attune NxT flow cytometer. Glycophorin A positive microparticles (circled R6) from healthy control (Panel A) and from a non-transfused SCD patients (panel B) turned out to be CD71 negative (quadrant R7 and R8).



Supplemental Figure 4: Inhibition of hemolysis and microparticle formation by well-established Syk inhibitors R406 (panels A and B) and PRT062607 (panels C and D). Washed sickle erythrocytes suspended in PBS-G at 30% Hct were incubated with 0.5 μM of either Syk inhibitor or an equivalent volume of vehicle (DMSO) at 37°C for 4 h under 1400 rpm shaking. (n=3; Error bars are expressed as standard error of the mean (SEM); * denotes $p<0.05$, and n.s. denotes not statistically significant)



Treatment					
DMSO	+	+	+	-	-
2 mM OV	-	-	+	+	+
R406 0.5μM	-	-	-	+	-
PRT062607 0.5μM	-	-	-	-	+

Treatment					
DMSO	+	+	+	-	-
2 mM OV	-	-	+	+	+
R406 0.5μM	-	-	-	+	-
PRT062607 0.5μM	-	-	-	-	+

Supplemental Figure 5: Effect of treatment of sickle cells with Syk inhibitors on inhibition of: A) release of free Hb, and B) discharge of erythrocyte-derived microparticles. Both Syk inhibitors significantly reduced the amount of Hb and MPs. released following treatment with OV suggesting that Syk is likely the predominant tyrosine kinase that phosphorylates Band 3. (n=3; Error bars are expressed as standard deviation; *** denotes $p<0.001$, ** denotes $p<0.01$ and n.s. denotes not statistically significant).

RBC Exit Velocity Methods

Microfluidic capillary devices were fabricated with polydimethylsiloxane (PDMS) using a standard soft photolithographic technique. Capillaries were constructed with a straight channel (w = 75 μm, h = 6.5 μm) containing a constriction (w_c = 5 μm and h = 6.5 μm) in the middle. To control oxygen level inside the channel, the microfluidic device was submerged in a customized glass chamber filled with sodium sulfite solution (0M, 0·01M, 0.1M or 1M) (Sigma-Aldrich). The oxygen level was calibrated using an O_2 indicator (tris(2,2'-bipyridyl) dichlororuthenium(II) hexahydrate (Sigma-Aldrich)), and fluorescence intensity was converted into a pO_2 value using the Stern-Volmer equation, $I^0 / I = 1 + pO_2 \times K_q$, where I^0 is the maximum fluorescence intensity and K_q is the quenching constant.

Sickle cell blood samples were obtained from five patients (n = 5, age 4-9 years old) and RBCs were isolated by washing three times at 500 rcf for 1.5 min in PBS-G and the resuspended to 1% v/v for microfluidic measurements. The RBC suspension was injected into the microfluidic capillary at a constant pressure of 1.6 psi and the velocity of RBCs in the constriction was recorded using a high-speed video camera (Phantom Miro M120, 1900 frames per second) mounted on an inverted microscope (Leica DMI 6000B). Data were analyzed using Phantom Camera Control

software. For quantitation of RBC velocity in the presence or absence of imatinib, 10 µL of 0.5 mM imatinib dissolved in DMSO was added to 30 µL packed RBCs in the presence of 960 µL PBS-G. RBC suspension was then incubated for 4 hours at 37˚C and used for RBC capillary velocity measurements.

Microfluidic Endothelial Adhesion Study Methods

Sample collection

Venous blood samples from 10 subjects with homozygous SCD (HbSS) were obtained from University Hospitals Seidman Cancer Center's Adult Sickle Cell Disease Clinic with standard laboratory procedures approved by the IRB. Upon collection, the blood samples were drawn into vacutainer tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA), and were stored at 4°C. All the experiments were performed within 48 hours of blood collection.

Endothelialization of microfluidic channels

Microfluidic channels were fabricated using a lamination technique, as described in our previous studies.(Alapan *et al*, 2016) Briefly, a top polymethyl methacrylate (PMMA) cap and a double-sided adhesive (DSA) film were laser micro-machined and assembled with a bottom microscope glass slide (Gold Seal, coated with APTES, 3-Aminopropyl Triethoxysilane, Electron Microscopy Sciences, Hatfield, PA), forming a uniform flow domain (Fig. 1A). 25 mg of 4-Maleimidobutyric acid N-hydroxysuccinimide ester (GMBS, ThermoFisher Scientific, Waltham, MA) was dissolved in 0.25 mL of dimethyl sulfoxide (DMSO) and was diluted with pure ethanol to at 0.28 % v/v to obtain the GMBS working solution. The assembled microchannels were rinsed with PBS and pure ethanol and incubated with GMBS for 15 minutes at room temperature. Thereafter, the microchannels were rinsed again with pure ethanol and PBS before loading the microchannels with a fibronectin (FN) solution at a concentration of 0.2 mg/mL. FN-loaded microchannels were incubated at 37ºC for 1 hour to complete FN-immobilization in the microchannels. To endothelialize the microchannels, HUVECs were seeded into FN-immobilized microchannels at an initial density of 1×10^6 cells/mL and cultured under flow with a flow rate of 100 µL/min at 37ºC and 5% CO_2 for 48-72 hours until a sufficiently confluent monolayer over the surface was achieved, based on our earlier work.(Kucukal *et al*, 2018)

Heme activation of endothelial cells

Prior to the flow adhesion assay, immobilized HUVECs were incubated with 40 μM heme-containing basal medium (Lifeline, Frederick, MD) for 2 hours in 37ºC. The control microchannels were loaded with heme-free basal medium and incubated under the same conditions. Heme stock solution was prepared by dissolving bovine hemin in 0.5 M NaOH solution to obtain a final heme concentration of 20 mM. Next, the stock solution was mixed with 0.5 M HCl solution to adjust pH and further diluted with basal medium to obtain a final concentration of 40 μM.

Imatinib Treatment of sickle RBCs

Imatinib (Sigma-Aldrich, St. Louis, MO) was dissolved into DMSO to obtain a stock solution with the concentration of 2.5 mg/mL and stored at 4ºC. Whole blood samples from subjects with HbSS were centrifuged at 500g for 5 minutes under room temperature to isolate RBCs. Isolated RBCs were washed in PBS for 2 times and re-suspended in PBS-G (PBS containing 5.5 mM glucose). Thereafter, RBC samples were treated with either 5 μM imatinib or vehicle (DMSO) as Control at 30% hematocrit for 4 hours at 37°C under 50 rpm shaking prior to the flow adhesion assay.

Fabrication of the micro-gas exchanger

To assess sickle RBC adhesion to heme-activated endothelium under physiological hypoxia, we fabricated a micro-gas exchanger to facilitate blood deoxygenation. Briefly, the micro-gas exchanger is composed of a gas permeable inner tubing (Silastic Silicone Laboratory Tubing, Dow Corning, Auburn, MI) placed within a gas impermeable outer tubing (Cole-Parmer, Vernon Hills, IL). The blood flow exchanged oxygen through the permeable tubing wall with 5% CO_2 and 95% N_2-controlled gas inside the impermeable tubing by diffusion (Fig. 1B). Simulation results in our previous study showed that the gas exchange results in an SpO_2 of 83%.(Kim *et al*, 2017)

Flow adhesion assay under physiological hypoxia

Treated RBCs were isolated and re-suspended in basal medium supplemented with 10 mM HEPES containing 5 μM imatinib or vehicle (DMSO as Control) at the same hematocrit. A total sample volume of 15 μL was perfused through the microchannel using a syringe pump (NE300, New Era Pump Systems, Farmingdale, NY) at a shear stress of 1 dyne/cm^2, corresponding to a

typical value observed in human post-capillary venules. Non-adherent RBCs were washed of by injecting medium at 1 dyne/cm^2.

Image processing and quantification

Bright field images of microfluidic channels were obtained using an Olympus long working distance objective lens (20×/0.45 ph2) and a commercial software (CellSense Dimension, Olympus Life Science Solutions, Center Valley, PA). Microchannel images were processed using Adobe Photoshop software (San Jose, CA). An area of 32 mm^2 at the center of the microfluidic channel was selected to quantify cell adhesion events.

Statistical analysis

Data acquired in this adhesion assay study were reported as mean ± standard error of the mean (SEM). All statistical analyses were carried out using Minitab 19 Software (Minitab Inc., State College, PA). Data were initially analyzed for normality, which was followed by paired t test. Statistical significance was set at 95% confidence level for all tests ($p<0.05$).

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PUBLICATIONS

1. **Noomuna, P.;** Risinger, M.; Zhou, S.; Seu, K.; Man, Y.; An, R.; Sheik, D.A.; Wan, J.; Little, J.A.; Gurkan, U.A.; Turrini, F.M.; Kalfa, T. and Low, P.S. Inhibition of Band 3 tyrosine phosphorylation: a new mechanism for treatment of sickle cell disease. Br J Haematol, **2020**, 190: 599-609. doi:10.1111/bjh.16671

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5. **Panae Noomuna**, Ruhani Sansoya, Theodosia Kalfa, Mary Risinger, Philip S. Low Rapid Degradation of Protein Tyrosine Phosphatase 1B in Sickle Cells: A Possible Role in Vaso-occlusion. *(in preparation).*

bjh research paper

Inhibition of Band 3 tyrosine phosphorylation: a new mechanism for treatment of sickle cell disease

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Summary

Many hypotheses have been proposed to explain how a glutamate to valine substitution in sickle haemoglobin (HbS) can cause sickle cell disease (SCD). We propose and document a new mechanism in which elevated tyrosine phosphorylation of Band 3 initiates sequelae that cause vaso-occlusion and the symptoms of SCD. In this mechanism, denaturation of HbS and release of heme generate intracellular oxidants which cause inhibition of erythrocyte tyrosine phosphatases, thus permitting constitutive tyrosine phosphorylation of Band 3. This phosphorylation in turn induces dissociation of the spectrin-actin cytoskeleton from the membrane, leading to membrane weakening, discharge of membrane-derived microparticles (which initiate the coagulation cascade) and release of cell-free HbS (which consumes nitric oxide) and activates the endothelium to express adhesion receptors). These processes promote vaso-occlusive events which cause SCD. We further show that inhibitors of Syk tyrosine kinase block Band 3 tyrosine phosphorylation, prevent release of cell-free Hb, inhibit discharge of membrane-derived microparticles, increase sickle cell deformability, reduce sickle cell adhesion to human endothelial cells, and enhance sickle cell flow through microcapillaries. In view of reports that imatinib (a Syk inhibitor) successfully treats symptoms of sickle cell disease, we suggest that Syk tyrosine kinase inhibitors warrant repurposing as potential treatments for SCD.

Keywords: anion exchanger 1, erythrocyte membrane, haemoglobinopathy, sickle cell disease, tyrosine phosphorylation.

Introduction

Although most symptoms of SCD are thought to be caused by occlusion of the microvasculature,[1–4] the mechanistic steps underpinning these vaso-occlusive events are still debated. The most cited mechanisms include: i) loss of erythrocyte viscoelastic properties deriving from sickle haemoglobin (HbS) polymerisation, red blood cell (RBC) dehydration, and membrane rigidification,[1,3,5–9] ii) activation of adhesion receptors on the vascular endothelium and/or

doi: 10.1111/bjh.16671

erythrocyte membrane,[10 13] and iii) initiation of thrombosis by RBC-derived microparticles (MPs) and the subsequent activation of platelets by thrombin and other coagulation factors.[12 17] Vaso-occlusive processes may result in tissue hypoxia, ischaemia-reperfusion injury, organ damage and associated morbidities, and debilitating pain which results in significant suffering and may require medical treatment/hospitalization.[18,19] Sickle cell haemolysis, reduced sickle red cell lifespan, and anaemia can further aggravate clinical symptoms.[20,21]

While RBC dehydration,[3,5,6,22] loss of membrane deformability[3,23] and increased RBC/endothelial cell adhesion[10 12] undoubtedly contribute to SCD, an increasing number of researchers now propose that additional pathologic sequelae may arise from the weakening of the erythrocyte membrane, leading to discharge of MPs[12,14,15,17] and free haemoglobin (Hb).[15,24] In this hypothesis, accelerated denaturation of HbS[25] hemichrome formation[26,27] and release of heme may collectively induce oxidative stress within the RBC.[26,28,29] Increased oxidative stress can then cause inhibition of RBC tyrosine phosphatases which normally prevent constitutive Band 3 tyrosine phosphorylation.[30 33] Upon inhibition of these phosphatases, over-phosphorylation of Band 3 then induces global destabilisation of the erythrocyte membrane,[34,35] accelerating intravascular haemolysis and MP release. The increased plasma haemoglobin and heme can 'activate' the vascular endothelium, causing expression of adhesion receptors (e.g., p-selectin, E-selectin and von Willebrand factor),[10 12] as well as sequestration of the vasodilator (NO),[11,36 38] while the release of MPs can trigger intravascular thrombosis via activation of prothrombin.[12,14,15,17] When initiated concomitantly with loss of RBC deformability and enhanced vaso-adhesion, the sequelae associated with membrane weakening can aggravate an already compromised blood flow, leading to micro-emboli and progressive tissue damage.

In this paper, we explore the role of Band 3 tyrosine phosphorylation and consequent membrane weakening in the development of the symptoms in SCD. We first show that the extent of tyrosine phosphorylation of Band 3 correlates with the percentage of HbS in sickle erythrocytes, the concentration of cell-free Hb in the patient's plasma, and the number of RBC-derived MPs in a patients' peripheral blood. We next document that the blockade of tyrosine phosphorylation of Band 3 with tyrosine kinase inhibitors prevents the release of cell-free Hb and the discharge of RBC-derived MPs, while concomitantly enhancing sickle cell deformability, reducing sickle cell sickling at lower O_2 pressures, and enhancing sickle cell flow through microcapillaries. Finally, we also establish that imatinib treatment suppresses adhesion of erythrocytes to heme-activated endothelium. Based on evidence from several labs that MPs,[12,14 17] cell-free Hb,[36,39,40] reduced sickle cell deformability, and enhanced sickle cell adhesion to the endothelium[10 12,23,41] contribute to the pathology of SCD, we argue that Band 3 tyrosine phosphorylation inhibitors could constitute a potent therapy for treatment for SCD.

Methods

Processing of SCD blood samples

All sickle cell blood samples were obtained following informed consent using procedures approved by the local institutional review boards (IRBs). Venous blood was collected from patients (genotype SS or $S\beta^0$) and healthy volunteers in EDTA-containing vacutainer tubes and maintained at 4°C until use. Samples were centrifuged at 800 rcf (relative centrifugal force) for ten minutes and plasma was removed for analysis of MPs and cell-free Hb. RBC pellets were washed three times with phosphate buffered saline, pH 7·4, containing 5 mM glucose (PBS-G) and the buffy coat was aspirated after each wash cycle.

Quantitation of Band 3 tyrosine phosphorylation

Washed RBCs were suspended at 30% haematocrit (Hct) in PBS-G and treated with either 5 μM of a drug (imatinib, PRT062607 or R406) or vehicle (control) for 4 h at 37°C, shaking at 50 rpm. The 5 μM drug concentration was determined from the minimum concentration of imatinib required to completely reverse Band 3 tyrosine phosphorylation within 4 h. RBC membranes were prepared and processed for western blotting as described in supplemental information. Band 3 tyrosine phosphorylation intensity was quantitated using image J software. To induce Band 3 tyrosine phosphorylation and membrane fragmentation in healthy erythrocytes, blood from healthy donors was washed and cells were suspended at 30% Hct in PBS-G, containing either 5 μM imatinib or a vehicle of dimethyl sulfoxide (DMSO); ≤0·5% v/v to minimise impact of the vehicle on cells (see Fig S1), at 37°C for 1 h. After the 1h incubation, 2 mM sodium orthovanadate (OV) was added to both imatinib-treated and untreated cells, and cells were incubated for 4 h at 37°C, while shaking at 1400 rpm.

Quantitation of erythrocyte-derived microparticles and free plasma haemoglobin

Plasma from sickle cell samples was centrifuged two times at 2500 rcf for 15 min to remove platelets, and 100 μl supernatant, containing MPs, was incubated for 20 min on ice with 0·5 μl mouse anti-human glycophorin A antibody (BD Biosciences #562938). To test if the MPs are CD71 positive, 1·0 μl of CD71 antibody (BD biosciences #12-0711-82) was added alongside the glycophorin A antibody. Samples were diluted with 1 ml stain buffer (BD Biosciences #554656), transferred to BD Trucount™ tubes (BD Biosciences #340334) and analysed on Attune NxT Flow Cytometer, utilising a violet fluorescent trigger channel.[42] The absolute

number of MPs was calculated as follows:

$$\text{Absolute microparticles count} = ((\text{Glycophorin A positive events})/(\text{bead events})) \times ((\text{Number of trucount beads})/\text{Volume})$$

For evaluation of the effect of tyrosine kinase inhibitors on release of MPs, 500 μL sickle cells, suspended at 30% Hct in PBS-G, were treated for 1 h with the desired tyrosine kinase inhibitor or vehicle control, and shaken at 1400 rpm for 4 h. Newly released microparticles were separated from residual RBCs by centrifuging at 800 rcf for 10 min, collecting the supernatant, and quantitating the MPs as described above.

Cayman's haemoglobin colorimetric assay was used to quantitate cell-free Hb according to manufacturer's instructions.

Measurement of red blood cell deformability

Erythrocyte deformability was measured using Technicon™ Ektacytometer and plotted as elongation index versus shear stress. Data were acquired while accelerating the ektacytometer from 0 to 250 rpm and shear stress was calculated:

$$\text{Shears stress} = (2\pi \times \text{viscoscity(poise)} \times \text{rpm} \times \text{radius of cylinder(cm)})/(60 \times \text{gap between the two cylinders (cm)})$$

Measurements of RBC flow through microcapillaries

The effect of imatinib on the rate of sickle cell flow through 5 μm × 6·5 μm microcapillaries at different oxygen pressures was measured using a high speed camera mounted onto an inverted microscope, focused on a microfluidic device through which sickle blood was flowed at constant pressure (1·6 psi), as described in Supplement Information and reference.[43]

Oxygen gradient ektacytometry (oxygenscan)

To determine the point of sickling (PoS) upon deoxygenation, oxygenscans were performed using a Laser Optical Rotational Red Cell Analyzer, as described.[44] Patient samples were washed and resuspended at 20% haematocrit in HBSS, modified with 10 mM HEPES and 10 mM $MgCl_2$, and incubated with 5 μM imatinib or DMSO for 4 h at 37 °C. RBCs of 300×10^6 were added to 5 ml OxyIso solution (RR Mechatronics, Zwaag, the Netherlands) and loaded into the Lorrca where they were subjected to constant shear stress (30 Pa). Deformability was measured while partial pressure of oxygen (pO_2) was gradually decreased from 150 mmHg to < 20 mmHg before reoxygenation with ambient air. The PoS was determined as the pO_2 at which samples reached 95% of their initial deformability and began to sickle.

Measurements of RBC adhesion to endothelial cell functionalised microchannels

Microfluidic channels were fabricated and incubated with fibronectin prior to coating with a monolayer of human umbilical vein endothelial cells (HUVEC) and human pulmonary microvascular endothelial cells (HPMECs), as described earlier.[16,45] Two hours prior to analysis of RBC adhesion, adherent HUVECs and HPMECs were activated with 40 μM heme to induce expression of adhesion receptors.[16] Freshly isolated sickle cells were simultaneously incubated in basal medium for 4 h and perfused through the microchannels at a physiological shear stress level of 1 dyne/cm^2 in precisely controlled physiological hypoxia (SpO_2 of 83%).[16] The SpO_2 level was chosen to be pathologically relevant to SCD, based on clinical studies.[46] Non-adherent RBCs were washed away and adherent erythrocytes were counted (see details in Supplemental Information).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) and F-test on linear regression. Statistical significance was set at a 95% confidence level for all tests ($P < 0{\cdot}05$).

Results

To test the hypothesis that sickle cells are distinguished by increased tyrosine phosphorylation of Band 3, membrane weakening, and release of both cell-free Hb and MPs, we focused studies on RBCs from non-transfused children (age range 3 20 years, mean 9·3 years; $n = 48$) with SCD all undergoing hydroxycarbamide treatment. As shown in Fig. 1A, the concentration of cell-free Hb in patient plasma was more than twice that of healthy volunteers, i.e. in agreement with previous studies.[12,15,24,47] Moreover, the number of MPs, identified as glycophorin A positive particles of 0·1 1·0 μm diameter (Figure S2), were also more than twice as abundant in patients than in healthy volunteers (panel B), and therefore also consistent with previous observations[14] by[12,15,48] It is worth noting that the glycophorin A positive microparticles identified herein are CD71 negative (Figure S3), which indicates that the MPs observed are mainly derived from mature erythrocytes. Because release of MPs would be expected to render the membrane-depleted erythrocytes less deformable (due to their smaller surface to volume ratios and higher Hb concentrations[2,3,5,23,41]), the reduced deformability of SCD blood versus normal controls (panel C) was expected. Importantly, the nearly linear correlation between MP count and cell-free Hb in each patient's blood sample (panel D) suggested a possible relationship between cell-free Hb and RBC-derived MPs. The weakening of the membrane by tyrosine phosphorylation of Band 3 could account for this correlation.

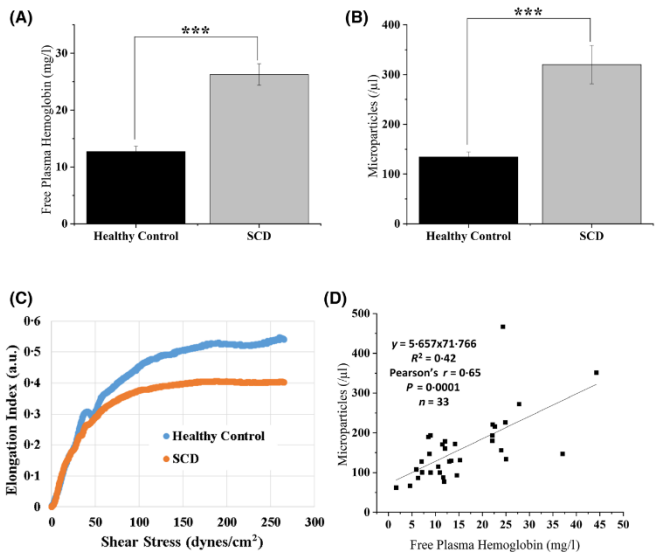

Fig 1. Quantitation of cell-free Hb and erythrocyte membrane derived MPs in blood from patients with SCD. The concentration of cell-free Hb in the plasma (A) and numbers of erythrocyte membrane-derived MPs (B) were evaluated in both patients with SCD and healthy controls (n = 48). Erythrocyte elongation index as a function of increasing shear stress (i.e. deformability) was also compared for SCD and control blood samples (C, representative sample of three different healthy controls and five different SCD patients). A plot of free plasma Hb versus MP count was constructed to explore a possible common mechanism leading to their production (D). (Error bars are expressed as SEM, *** denotes $P \leq 0{\cdot}005$ computed from one-way ANOVA or student t-test; Pearson's r = 0·65, and P = 0·0001 in panel D). (Hb, haemoglobin; MP, microparticle; SCD, sickle cell disease; SEM, standard error of the mean).

It has been frequently reported that oxidative stress leads to inactivation of erythrocyte tyrosine phosphatases,[30,31,49] which in turn allow unimpeded tyrosine phosphorylation of Band 3 by constitutively active tyrosine kinases.[30,35,50] Because this tyrosine phosphorylation induces an intramolecular interaction in Band 3, which causes dissociation of the spectrin-actin cortical cytoskeleton from the membrane,[34,35] we hypothesised that oxidative stress deriving from premature HbS denaturation[25,28,29] might initiate a phosphorylation cascade, which would lead to dissociation of the spectrin-based cytoskeleton from the membrane, causing membrane destabilisation and fragmentation. To test this hypothesis, we compared tyrosine phosphorylation of Band 3 in sickle cells and healthy controls. As shown in Fig. 2A, tyrosine phosphorylation of Band 3 in healthy cells was almost undetectable, whereas phosphorylation in sickle cells was prominent. Evidence that phosphorylation in sickle cells was dependent on their levels of HbS is provided in panel B, where a positive correlation (Pearsons r = 0·70) and a significant linear 'relationship (P = 0·008) was observed between Band 3 tyrosine phosphorylation and the percentage of HbS in each patient's sample.

Documentation that tyrosine phosphorylation of Band 3 was likely also related to release of Hb into plasma was demonstrated by a significant correlation (P = 0·02, Pearson's r = 0·63) between these two parameters (panel C). Furthermore, an indication that Band 3 tyrosine phosphorylation was related to the release of MPs is shown in panel D (Pearson's r = 0·72, P = 0·01). These data suggest that elevated tyrosine phosphorylation of Band 3 in sickle cells is related to the membrane destabilisation that causes release of MPs and free Hb.

To further test the hypothesis that Band 3 tyrosine phosphorylation might promote release of Hb and MPs from sickle erythrocytes, we explored the effect of imatinib on tyrosine phosphorylation of Band 3. As shown in Fig. 3A, healthy erythrocytes displayed low levels of Band 3 phosphorylation, whereas sickle erythrocytes exhibited higher levels of phosphorylation. Moreover, treatment of sickle erythrocytes with 5 µM imatinib lowered their Band 3 tyrosine phosphorylation to levels similar to control cells, demonstrating that imatinib can inhibit the natural tyrosine phosphorylation of Band 3 in sickle cells. As documented in panels B and C, imatinib treatment also reduces the release of cell-free Hb and MPs, suggesting that Band 3 phosphorylation is directly related to both characteristics of sickle blood. Importantly, although the tyrosine phosphorylation of Band 3 and accompanying Band 3 conformational changes are readily reversible, Hb and MP release are not reversible.[33 35]

Because imatinib inhibits several kinases besides Syk,[51] the question arose whether other more Syk-specific inhibitors might similarly suppress Band 3 tyrosine phosphorylation in sickle cells. As shown in Fig. 3, incubation of sickle cells with either 5 µM PRT062607 (panel D) or R406 (panel E) resulted in an analogous diminution of Band 3 tyrosine phosphorylation. Moreover, the same Syk-specific inhibitors also suppressed MP and Hb release from both sickle cells (Figure S4) and o-vanadate-treated healthy cells (Figure S5). These data demonstrate that Syk-specific inhibitors also suppress the tyrosine kinase that phosphorylates Band 3 in SCD, suggesting that at least one of the kinases that phosphorylates Band 3 in SCD is Syk.

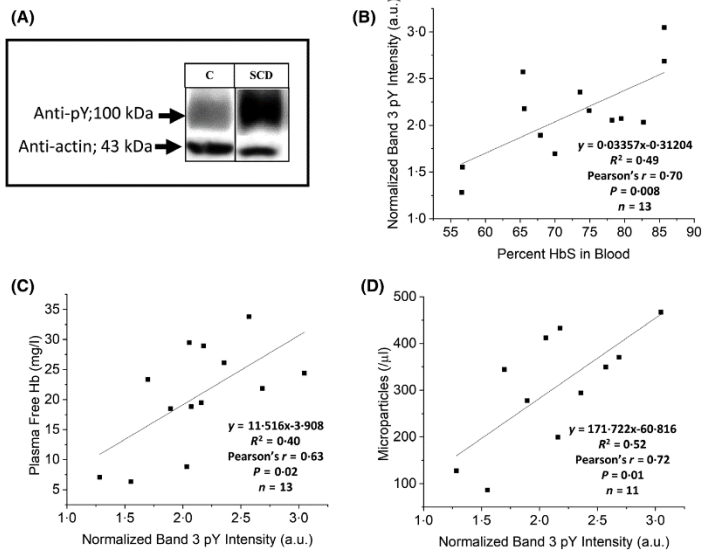

Fig 2. Analysis of the relationship between Band 3 tyrosine phosphorylation, cell-free Hb and MP count in sickle cell blood samples. A) Representative anti-phosphotyrosine immunoblot of Band 3 in erythrocyte membranes from a healthy control (C) and a sickle cell patient (SCD) treated with hydroxycarbamide. The correlation between Band 3 tyrosine phosphorylation and percentage of HbS in the blood sample (B), cell-free Hb in the plasma (C), and RBC membrane-derived MPs (D), is also plotted. (Statistical data from F-tests are as indicated on the respective figures). (Hb, haemoglobin; MP, microparticle; HbS, sickle haemoglobin; RBC, red blood cell).

Because defects in molecular bridges connecting the erythrocyte membrane to its cortical spectrin-actin cytoskeleton have been shown to compromise erythrocyte deformability,[34,35] we next examined whether inhibition of Band 3 tyrosine phosphorylation might restore the disrupted bridges and thereby improve sickle cell deformability. Firstly, sickle blood samples were incubated for 4 h in the presence or absence of 5 μM imatinib and then examined by ektacytometry for changes in cell deformability. As shown in Fig. 4A, although the deformability of sickle cells was lower than that of healthy controls, incubation with imatinib improved their deformability. Secondly, the deformability of sickle erythrocytes under constant shear stress was examined during deoxygenation and re-oxygenation of the sickle cells.[44] As shown in Fig. 4B, sickle RBCs pre-incubated with imatinib exhibited higher baseline deformability, initiated sickling only when exposed to lower pO_2 (point of sickling 5%), and displayed improved minimal deformability (Elmin, panel B) compared to untreated cells from the same patient. Thirdly, because pO_2-regulated RBC capillary flow velocity relates mechanistically to cell deformability,[43] we studied the flow of sickle erythrocytes through microcapillaries at controlled pO_2 (panel C). Relative to untreated cells, imatinib-treated cells were found to experience a significant increase in capillary velocity, which improved as the concentration of imatinib was increased (panel C). Since similar observations were obtained at all other O_2 pressures examined, we conclude that imatinib improves the flow of sickle cells through microcapillaries.

To directly demonstrate that tyrosine phosphorylation of Band 3 promotes membrane weakening and release of cell-free Hb and RBC membrane-derived MPs, we induced tyrosine phosphorylation of Band 3 in healthy erythrocytes by treatment with the tyrosine phosphatase inhibitor, orthovanadate, and then examined release of cell-free Hb and MPs in the presence and absence of imatinib. As shown in Fig. 5, treatment of control RBCs with orthovanadate induced tyrosine phosphorylation of Band 3 (panel A), as well as the release of cell-free Hb (panel B) and the discharge of MPs (panel C) in a manner that could be inhibited by imatinib. These data demonstrate that tyrosine phosphorylation of Band 3 constitutes the cause of Hb and MP release and that imatinib prevents these pro-embolic processes by inhibiting Band 3 tyrosine phosphorylation.

Finally, we examined the effect of imatinib on the adhesion of flowing sickle cells to heme-activated endothelial cells. As seen in representative images of adherent RBCs in Fig. 6, untreated sickle cells (panels A and B) are more adherent to heme-activated HUVECs and HPMECs under hypoxia than imatinib-treated sickle cells (panels C and D). The mean adhesion of naïve sickle cells was 383 ± 57 (control), compared to 171 ± 30 for imatinib-exposed sickle cells

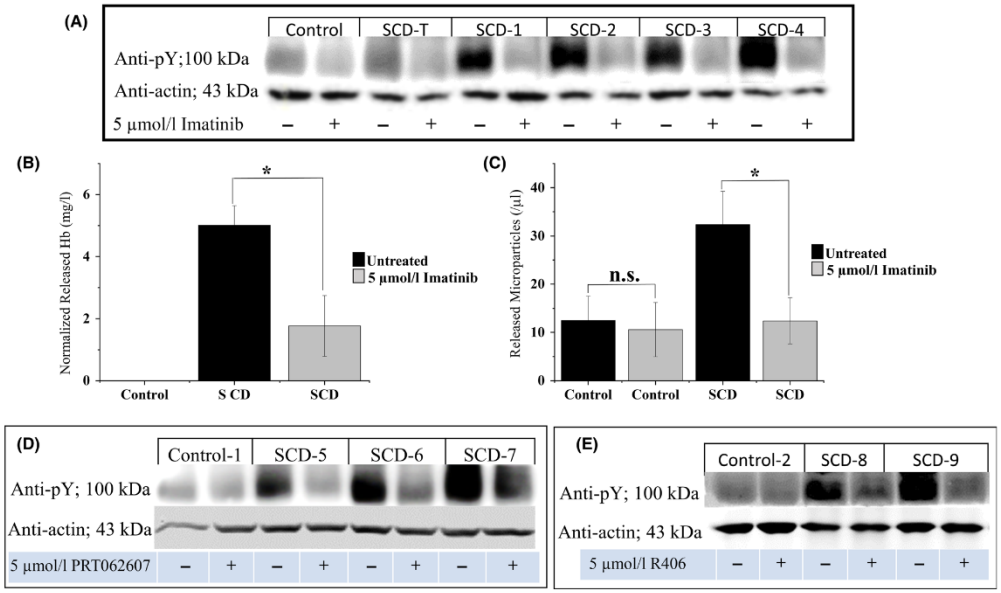

Fig 3. Effect of treatment of sickle cells with imatinib or other tyrosine kinase inhibitors on: A) tyrosine phosphorylation of Band 3 in healthy erythrocytes (control), erythrocytes from a SCD-T patient, and erythrocytes from non-SCD-T patients treated with hydroxycarbamide, B) release of cell-free Hb ($n = 5$), and C) discharge of RBC membrane-derived MPs from non-transfused sickle cell blood samples *in vitro* ($n = 6$). Panels D and E illustrate the effect of two Syk-specific inhibitors on tyrosine phosphorylation of Band 3 in erythrocytes from healthy controls, and non-SCD-T patients on hydroxycarbamide. Whole blood samples from healthy controls or SCD patients were washed three times in PBS-G and incubated for 4 h at 37°C in PBS-G, containing or lacking 5 µM imatinib, or 5 µM PRT062607, or 5 µM R406 while shaking at 50 (A), or 1400 (B-E) rpm prior to analysis. Note that the sickle cell patient receiving transfusions (SCD-T) exhibits no increase in Band 3 tyrosine phosphorylation. (Error bars are expressed as SEM, *denotes $P \leq 0{\cdot}05$, computed from one-way ANOVA). (SCD-T, transfused sickle cell disease; Hb, haemoglobin; RBC, red blood cell; MP, microparticle; PBS-G, phosphate-buffered saline with glucose; SEM, standard error of the mean).

(panel E, $n = 13$ patients; $P < 0{\cdot}001$, paired t-test). These data suggest that imatinib can further reduce vaso-occlusive events by suppressing adhesion of sickle cells to activated endothelial cells.

Discussion

Multiple publications have reported that oxidative stress is elevated in sickle cells,[11,28,29] that this oxidative stress inhibits erythrocyte tyrosine phosphatases,[26,30,31,50] and that inhibition of erythrocyte tyrosine phosphatases leads to elevated tyrosine phosphorylation of Band 3.[34,34,35] We document here that elevated tyrosine phosphorylation of Band 3 causes destabilisation of the membrane, promoting the release of both MPs and cell-free Hb.[34,35,52] Recognising that erythrocyte-derived MPs[14] (as previously observed by [12,15,48]) and cell-free Hb[12,15,47] are pro-embolic, we formulated the hypothesis that elevated oxidative stress in sickle cells should sequentially induce: i) heightened tyrosine phosphorylation of Band 3,[30,32,34,53,54] ii) destabilisation of the sickle cell membrane,[34,35] iii) release of MPs and cell-free Hb,[4,12,14,15,47] iv) activation of adhesion receptors on the vascular endothelium by the released cell-free Hb and heme,[10,11,14,16] v) stimulation of micro-embolisms by the prothrombotic MPs,[4,12,14,15] vi) enhancement of adhesive properties of sickle cells,[10 13,39] and vii) induction of vaso-occlusive events due to concurrent activation of the above processes. The data presented here provide strong evidence that these sequelae do in fact occur in SCD and that Band 3 tyrosine phosphorylation is critical for their occurrence.

In addition to the effects of inhibitors of Band 3 tyrosine phosphorylation on SCD symptoms outlined above, we also envision that these inhibitors may exert other unanticipated positive effects on SCD. Although inhibition of MP and HbS/heme release can be predicted to reduce micro-embolic events, the concomitant reduced blebbing/loss of the erythrocyte membrane area should also improve sickle cell deformability by maintaining a higher cell surface to volume ratio, thereby improving the flow of sickle erythrocytes (Fig. 4A, C). This maintenance of sickle cell volume should also suppress the cell's tendency to sickle, since the delay in sickling is related to the 30th power of HbS concentration (i.e. a

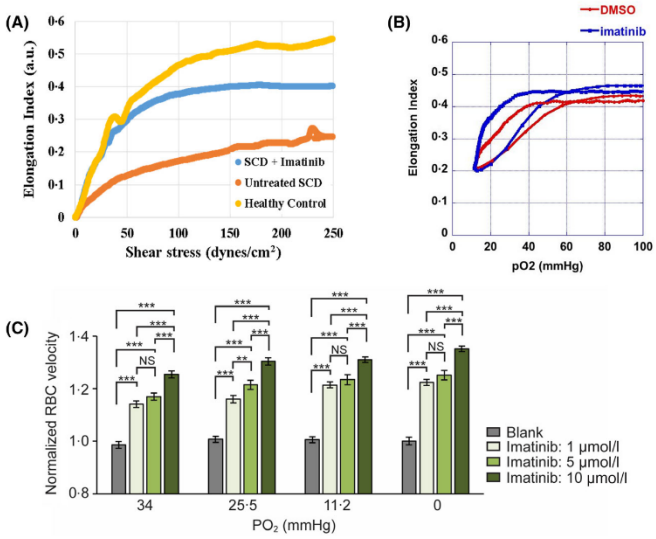

Fig 4. Effect of imatinib on the deformability and rheology of sickle cells at different oxygen pressures. A) The deformability of healthy erythrocytes and washed sickle cells is determined by ektacytometry in the presence and/or absence of 5 μM imatinib. B). A representative scan of inhibition of sickling after treatment of blood from SCD patients with imatinib or DMSO (vehicle control) as detected by Oxygenscan. Elmax, point of sickling (PoS5%) and Elmin are recorded as the sample is deoxygenated and then reoxygenated after 4 h incubation with 5 μM imatinib or an equal volume of DMSO carrier. Note both the left-shift in PoS5% and higher Elmin in sickle RBCs following incubation with imatinib, which are indicative of improved deformability of the cells. C) Washed sickle or healthy cells were incubated for 4 h at 37°C in the indicated concentrations of imatinib and then equilibrated at the indicated partial pressures of oxygen, prior to analysis of flow rates. [***denotes $P \leq 0{\cdot}05$, figures are representative of three (panel A) and five (panels B and C) different patient samples]. (SCD, sickle cell disease; DMSO, dimethyl sulfoxide; Elmax or Elmin, Eadie-Hofstee linearisation maximum or minimum deformability).

change in only 8% in RBC volume will cause a 10-fold change in the lag time before sickling[8,9]), and prevention of membrane loss will prevent the associated increase in cytoplasmic HbS concentration (Fig. 4B). The observed decline in sickle cell adhesiveness upon treatment with imatinib (which must derive from an effect on the erythrocyte membrane since the endothelial cells were not exposed to imatinib) should also improve sickle cell flow through the vasculature, and this improved flow should reduce the time each sickle cell remains deoxygenated, thereby further decreasing the tendency to sickle (Fig. 6).[8,9] Furthermore, while our studies did not examine sickle cell lifespan, it's predictable that inhibition of Band 3 phosphorylation should also improve sickle cell survival, since prevention of membrane loss should prolong maintenance of RBC flexibility and thereby reduce its susceptibility to phagocytosis by macrophages[55,56] and hence improve SCD-associated anaemia.

Erythrocyte deformability is thought to depend on three parameters: i) the cell's surface to volume ratio, ii) the viscosity of the cell's cytoplasm (which is determined by the concentration of Hb), and iii) the intrinsic deformability of the RBC plasma membrane[3,5] As shown in Fig. 4, both sickle cell deformability and sickle blood rheology are improved within 4 h of exposure to imatinib. Because significant changes in either RBC volume or surface to volume ratio did not occur over this short time span, the rapid improvement in RBC rheology must have derived from an enhancement in membrane deformability. These data therefore suggest that restoration of the disrupted bridges between Band 3 and the spectrin-actin cytoskeleton by imatinib can improve membrane deformability. The fact that exposure of the isolated sickle cells to imatinib also reduced their tendency to bind heme-activated human endothelial cells (Fig. 6) also suggests that imatinib has a positive effect on sickle cell membrane properties.

With more potent kinase inhibitors readily available,[34] the question naturally arises why we selected imatinib to test involvement of Band 3 phosphorylation in SCD. Following initial observations that inhibition of Band 3 phosphorylation by tyrosine kinase inhibitors suppressed release of cell-free Hb and MPs, it seemed logical to explore whether any SCD patients might have fortuitously been treated for another disease with such inhibitors. Upon screening FDA-approved tyrosine kinase inhibitors for inhibition of Band 3 tyrosine phosphorylation, we found that imatinib was an

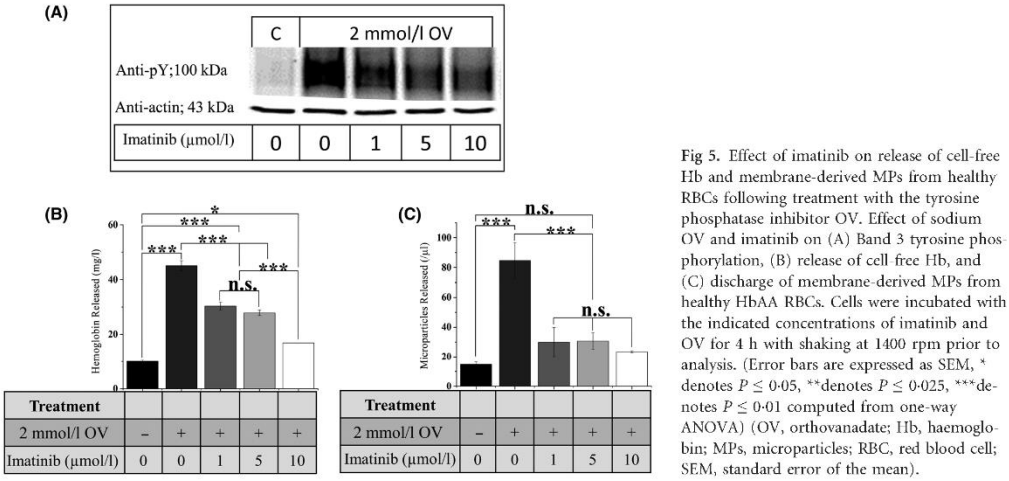

Fig 5. Effect of imatinib on release of cell-free Hb and membrane-derived MPs from healthy RBCs following treatment with the tyrosine phosphatase inhibitor OV. Effect of sodium OV and imatinib on (A) Band 3 tyrosine phosphorylation, (B) release of cell free Hb, and (C) discharge of membrane-derived MPs from healthy HbAA RBCs. Cells were incubated with the indicated concentrations of imatinib and OV for 4 h with shaking at 1400 rpm prior to analysis. (Error bars are expressed as SEM, * denotes $P \leq 0{\cdot}05$, **denotes $P \leq 0{\cdot}025$, ***denotes $P \leq 0{\cdot}01$ computed from one-way ANOVA) (OV, orthovanadate; Hb, haemoglobin; MPs, microparticles; RBC, red blood cell; SEM, standard error of the mean).

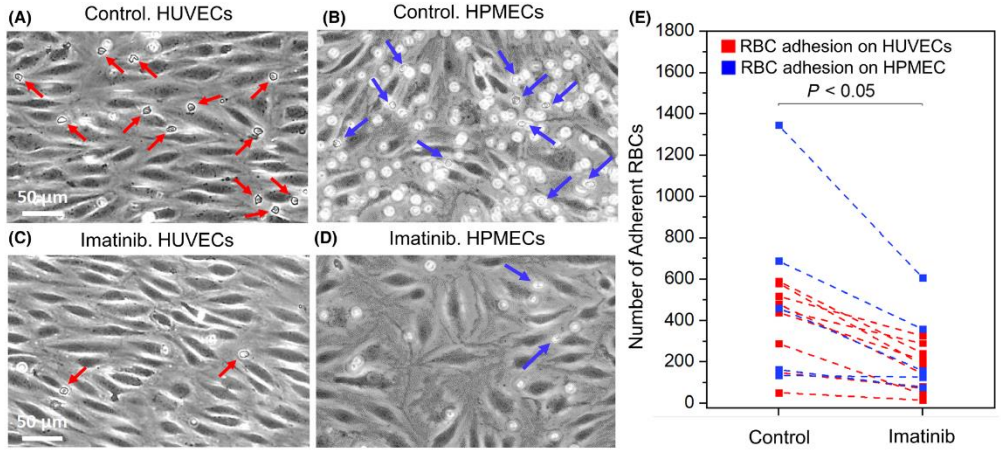

Fig 6. Sickle RBC adhesion to heme-activated endothelial cells under physiologic hypoxia in microfluidic channels *in vitro*. Representative images of adherent RBCs to heme-activated endothelial cells are also shown in the control group (A, B on HUVECs and HPMECs) and in the imatinib-treated group (C, D on HUVECs and HPMECs). Arrows indicate RBCs adherent to endothelium. (E) Sickle RBC adhesion to heme-activated endothelial cells is significantly reduced by imatinib (5 μM) treatment, compared with control (vehicle, DMSO) treatment (N = 13 subjects, mean adhesion of untreated vs. imatinib-treated sickle cells ± SEM = 383 ± 57 vs. 171 ± 30, $P < 0{\cdot}001$, paired *t*-test).). (RBC, red blood cell; HUVEC, human umbilical vein endothelial cells; HPMEC, human pulmonary microvascular endothelial cells).

effective inhibitor at clinically relevant concentrations. We then looked for reports in the literature where chronic myelogenous leukaemia (CML) patients who coincidentally suffered from SCD might have been treated with imatinib. We found two anecdotal publications that essentially reported the same observation, namely that administration of imatinib successfully treated the symptoms of SCD in their CML patient.[57,58] Although neither author linked his/her findings to any RBC property, their results nevertheless suggest that an inhibitor of Band 3 tyrosine phosphorylation could constitute a therapy for SCD. While chronic use of imatinib should not be considered for the treatment of SCD in children because it can stunt a child's growth,[59,60] a well-designed short term clinical evaluation of imatinib in a more mature population could provide a proof-of-concept test which would inform whether a search for a more selective

inhibitor of Band 3 tyrosine phosphorylation might be worthwhile.

Acknowledgements

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Authorship contributions

PSL and FMT conceived of the study. PSL helped design and supervise the study, and contributed to interpretation of data and writing the paper. PN assisted with the design of the study, and executed most of the experiments (microparticles and free plasma haemoglobin quantitation, western blots and deformability scans). PN also helped with interpretation of the data and writing of the manuscript, in some cases with the help of DAS. TK, KS and MR consented patients, analysed haematologic parameters, performed oxygenscans, interpreted data and contributed to revision of the manuscript. SZ and JW conducted and interpreted erythrocyte microcapillary flow studies and contributed to writing and revising of the manuscript. YM, RA, JAL, and UAG performed the endothelial cell adhesion studies and wrote the associated text for the Methods.

Conflict of interest disclosures

The authors declare no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Effect of DMSO on release of Hb (panel A) and MPs (panel B). Erythrocytes suspended at 30% Hct were treated with 0.5% v/v of DMSO and/or a Band 3 tyrosine phosphorylation stimulant, OV, prior to analysis of the amount of Hb and MPs released. (DMSO, dimethyl sulfoxide; Hb, haemoglobin; MPs, microparticles; Hct, haematocrit; OV, sodium orthovanadate.)

Fig S2. Quantitation of erythrocyte-derived microparticles by Attune NxT Flow Cytometer.

Fig S3. Identification of surface markers of erythroid-derived MPs. MPs were stained with 0.5 μL of mouse anti-human glycophorin A antibody and 1.0 μL of CD71 antibody (BD Biosciences #12-0711-82) and incubated for 20 min on ice prior to analysis with Attune NxT flow cytometer. Glycophorin A positive MPs (circled R6) from healthy control (panel A) and from a non-transfused SCD patient (panel B) turned out to be CD71 negative (quadrant R7 and R8). (MPs, microparticles; SCD, sickle cell disease.)

Fig S4. Inhibition of haemolysis and microparticle formation by well-established Syk inhibitors R406 (panels A and B) and PRT062607 (panels C and D). Washed sickle erythrocytes suspended in PBS-G at 30% Hct were incubated with 0.5 μM of either Syk inhibitor or an equivalent volume of vehicle (DMSO) at 37°C for 4 h under 1400 rpm shaking. [$n = 3$; error bars are expressed as SEM; * denotes $P < 0.05$; n.s., not statistically significant). (PBS-G, phosphate-buffered saline with glucose; Hct, haematocrit; DMSO, dimethyl sulfoxide; SEM, standard error of the mean.)

Fig S5. Effect of treatment of sickle cells with Syk inhibitors on inhibition of: A) release of free Hb, and B) discharge of erythrocyte-derived MPs. Both Syk inhibitors significantly reduced the amount of Hb and MPs released following treatment with OV, suggesting that Syk is likely the predominant tyrosine kinase which phosphorylates Band 3. ($n = 3$; error bars are expressed as standard deviation; *** denotes $P < 0.001$, ** denotes $P < 0.01$ and n.s. denotes not statistically significant). (Hb, haemoglobin; MPs, microparticles; OV, sodium orthovanadate.)

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