

Aspartic proteases and their potential for transmission blocking strategies

Kitsanapong Reaksudsan

ORCID ID: 0000-0001-8689-8226

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

December 2019

The University of Melbourne

Department of Medical Biology

The Walter and Eliza Hall Institute of Medical Research

Abstract

Sexual stage development in *Plasmodium* spp. is essential for transmission through the mosquito and to the human host. It represents objects to study a broad range of biological processes, including stage conversion and parasite/host co-adaptation. After the bloodmeal, male and female gametes emerge from intracellular gametocytes and zygote formation follows fertilization. Ookinetes develop from the zygote and traverse through the midgut epithelial cell layer to the basal lamina side of outer wall and develop into oocysts, the only parasite developmental stage that grows extracellularly and this growth and development creates thousands of sporozoites. Once fully developed and egressed, these sporozoites are released into the mosquito hemocoel and they migrate to the salivary gland ready to infect next mammalian host and continue their life cycle.

This sexual stage also represents a major bottleneck during the life cycle of *Plasmodium* as, in mosquito midgut, parasites have to persevere for up to 24 hours outside host cell, exposed themselves to various risk factors such as components of human immune system included within bloodmeal, natural midgut microbial flora in mosquito midgut, and mosquito innate immune system. This exposure can lead up to an approximate 300-fold decrease in parasite survivability during the transmission to mosquito. Due to this unique feature, sexual stage is prime target for transmission blocking intervention strategies aimed to inhibit spread of the disease by the mosquito.

Protease enzymes are essential during many steps of malaria parasite development in the blood and transmission stages and an important group of these enzymes are the plasmepsins, of which there are 10 in *Plasmodium* acting at various points through the life cycle. So far, only 4 plasmepsins are identified to be involved in critical processes and required for transmission. Firstly, plasmepsin VI is highly expressed during sexual stages and was previously shown to be involved in sporozoite development in *P. berghei*. Secondly,

plasmepsin VIII is expressed in mature sporozoite and responsible for sporozoite motility in *P. berghei*. Finally, PMIX and X are found to be essential in both blood and mosquito stages, making them stand out as promising drug targets.

In this study, we attempted to determine the biological functions of plasmepsin VI, IX, and X during transmission of malaria parasites. We found that plasmepsin VI is required for transmission of *P. falciparum* and might play an important role in sporozoite egress process instead of sporozoite development as observed in *P. berghei*. We also found that our dual inhibitor that targets both plasmepsin IX and X is able to block the transmission of *P. falciparum* to mosquito while another antimalaria compound that targets only plasmepsin X is enough to block transmission of *P. berghei* from mouse to mosquito suggesting that both plasmepsin IX and X are essential for transmission. Taken together, our data has identified 3 plasmepsins that play important roles in sexual stage of malaria parasites and more works are needed in order to determine the mechanism of action of these 3 proteases.

Declaration

This is to certify that this thesis comprises only my original work, except where indicated in the preface. Due acknowledgements have been made to individuals who contributed to the work in the preface and in the body of the thesis, including tables and figures. All work detailed herein was performed following the commencement of my PhD candidature on 15th May 2015. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies, and appendices.

Kitsanapong Reaksudsan

20 December 2019

Preface

The work described in this thesis was undertaken at the Walter and Eliza hall institute of Medical Research (WEHI) under the supervision of Prof. Alan Cowman and Dr. Tony Hodder. During the length of this work, the author was a recipient of the Victoria International Research Scholarship, through the University of Melbourne.

I duly acknowledge the important contributions of the individuals to the work presented within this thesis, as declared through the authorization of collaborators. Contributions are as listed :

Chapter 3:

Tony Triglia provided the 3' homologous flank for plasmepsin VI knock-out construct and plasmid for plasmepsin VI knock-in construct.

Danushka Marapana provided the plasmid for plasmepsin VI knock-out construct.

Michal Pasternak provided helps and supports with immunofluorescence assay.

Chapter 4:

Tony Hodder provided the *Pf* plasmepsin VI wild-type, mutated, *Pb* plasmepsin VI, *Pv* plasmepsin VI constructs for baculovirus transfection.

Chapter 5:

Paola Favuzza provided helps in preparation for mice treatment and infection with *P. berghei* parasites as well as all her data on the investigation of WM4 and WM382 effect during asexual stages life cycle.

Acknowledgements

My time at the Walter and Eliza Hall Institute of Medical Research has been nothing short of amazing experience and I am grateful for the support that I have been given from a multitude of people. I would like to thank my supervisors Alan Cowman and Tony Hodder for their guidance and support through this project.

Next I would like to thank Dr. Brad Sleebs and Assoc. Prof. Justin Boddey for sitting as members of my PhD committee. Our meetings were very useful for refining the scope of the project and keeping it on track.

Every member of the Cowman lab would have been involved in this project in one way or another. For that, I am grateful that everyone has been so generous in the form of time and reagents. This gratitude extends to the rest of Infection and Immunity, where I have met really incredible people, many of whom have changed my life in one way or another. I am indebted to the many I have crossed paths with, but I want to thank Paola, Julie, Tony, Jenny, Danu, Wilson, Michal, Sash, Matt, Mel, Sara, and Stella in particular. Thank you, for always being there for me, as a friend, a colleague, and a mentor.

Lastly, and most importantly, I want to thank my mom and sister, for all your unconditional love and support. I know that you have all been worried about how things would work out at one point or another and I really need to thank you for showing me perspective when I most needed it. Most importantly, I have to thank you for always patiently waiting for me to make the right decision, for supporting those decisions and for always being there for me. With your love, it makes good days great and hard days good.

Thank you everyone, for believing in me.

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Abbreviations

Δ	Gene deletion
3D7	<i>Plasmodium falciparum</i> strain 3D7
3'HR	3' homologous region
3'UTR	3' untranslated region
3xHA	Triple haemagglutinin tag
5'HR	5' homologous region
ACT	Artemisinin-based combination therapy
AD	Alzheimer's disease
Amp	Ampicillin
AQ	Amodiaquine
ART	Artemisinin
ATQ	Atovaquone
bp	base pair
BSA	Bovine serum albumin
BSD	Blasticidin
BV	Baculovirus
Cas9	CRISPR-associated protein 9
CQ	Chloroquine
CRISPR	Clustered regularly interspaced short palindromic repeats
Da	Dalton
DAPI	4', 6'- diamidino-2-phenylindole
DiCre	Dimerisable Cre-recombinase
DPBS	Dulbecco's phosphate-buffered saline
DSB	Double-stranded break
E64	N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FACs	Fluorescence activated <i>cell sorting</i>

G6PD	Glucose-6-Phosphate Dehydrogenase
gDNA	Genomic DNA
GFP	Green fluorescent protein
GlcN	Glucosamine
HA	Haemagglutinin
HAP	Histo-aspartyl protease
hr	Hour
hDHFR	Human dihydrofolate reductase
HIV	Human immunodeficiency virus
hpi	Hours post infection
HRP	Horseradish peroxide
IFA	Immunofluorescence assay
iKD	Inducible knockdown
iKO	Inducible knockout
IP	Immunoprecipitation
iRPMI	incomplete RPMI
Kan	Kanamycin
KD	Knockdown
kDa	Kilodalton
KO, K/O	Knockout
LLINs	Long-lasting insecticide treated nets
MB	Methylene blue
MFQ	Mefloquine
min	Minute
mL	Millilitre
mM	Millimolar
MW	Molecular weight
NEB	New England Biolabs
NF54	<i>Plasmodium falciparum</i> strain NF54
nM	Nanomolar
OD ₂₈₀	Optical density at 280 nm
OD ₄₅₀	Optical density at 450 nm

P1-3	Cell passage 1, 2, 3
PAM	Protospacer-adjacent motif
PbDHFR	<i>P. berghei</i> dihydrofolate reductase
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.1% Tween 20
PCR	Polymerase chain reaction
PDB	Protein databank
PEMS	Parasitophorous vacuole-enclosed merozoite structures
PEXEL	<i>Plasmodium</i> export element
Pf	<i>Plasmodium falciparum</i>
PfAMA1	Pf Apical membrane antigen 1
PfCSP	Pf Circumsporozoite protein
<i>Pfdhfr</i>	Pf dihydrofolate reductase gene
<i>Pfdhps</i>	Pf dihydropteroate synthase gene
PfKAHRP	Pf Knob-associated histidine rich protein
pI	Point of ionisation
PI3K	Phosphatidylinositol-3-kinase
PMs	Plasmepsins
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RT	Room temperature
S-broth	Super broth
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE asia	Southeast Asia
Sf21 cells	<i>Spodoptera frugiperda</i> IPLB-Sf21-AE cells
sgRNA	Single guided RNA
WEHI	Walter and Eliza Hall Institute of Medical research
WHO	World Health Organization
WR	WR99210
WT	Wild-type
μL	Microlitre
μM	Micromolar

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Chapter 1 – Literature review

Malaria

Malaria is an important parasitic disease present in many parts of the world but especially in developing countries located in tropical and subtropical regions (Fig 1), causing not only enormous health issues but also tremendous social and economic losses (1, 2). The disease malaria is an overwhelming problem especially among children and pregnant women, there are more than 200 million clinical cases worldwide every year, 88% of fatalities occur in the African region where the disease kills more than 400,000 children annually report (WHO, 2018). Whilst people living in endemic areas are at risk of being infected by parasites, that cause malaria, it also poses an intimidating risk to travellers and immigrants which, in turn, can import disease to non-endemic areas.

Malaria is a mosquito-borne infectious disease caused by protozoan parasites from genus *Plasmodium*. There are five parasite species that can infect humans: *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium knowlesi*. Among these five species, *P. falciparum* causes the most mortalities and also significant morbidity (1, 2). Symptoms of malaria can vary from fever, nausea, and headache to severe anemia and cerebral malaria which, if not treated properly, can result in coma and death (3-5). The pathology of the disease is complex but has been directly associated with increased parasite load due to cycles of host erythrocyte invasion during the asexual blood stage. The parasite developing inside the erythrocyte substantially modifies the surface of the host cell thus mediating its ability to sequester in vital organs, presumably to prevent passage through and clearance in the spleen. The ability of parasite infected-erythrocytes to adhere to endothelial cells in the microvasculature can interfere with blood circulation which is linked to disease syndromes such as cerebral malaria (6, 7).

Artemisinin is currently the gold standard for malaria treatment. However, despite the fact that artemisinin is still the best option against malaria, resistance toward its partner drugs is increasing, affecting the overall efficiency of treatment. The highest levels of resistance of *P. falciparum* parasite to artemisinin can be found in South east Asia

especially in the border between Myanmar and Thailand. Due to the resistance of parasites to antimalarial drugs and resistance of mosquitoes to insecticides, new efficacious vaccines and drugs are needed in order to lessen malaria transmission along with its associated burden (8, 9).

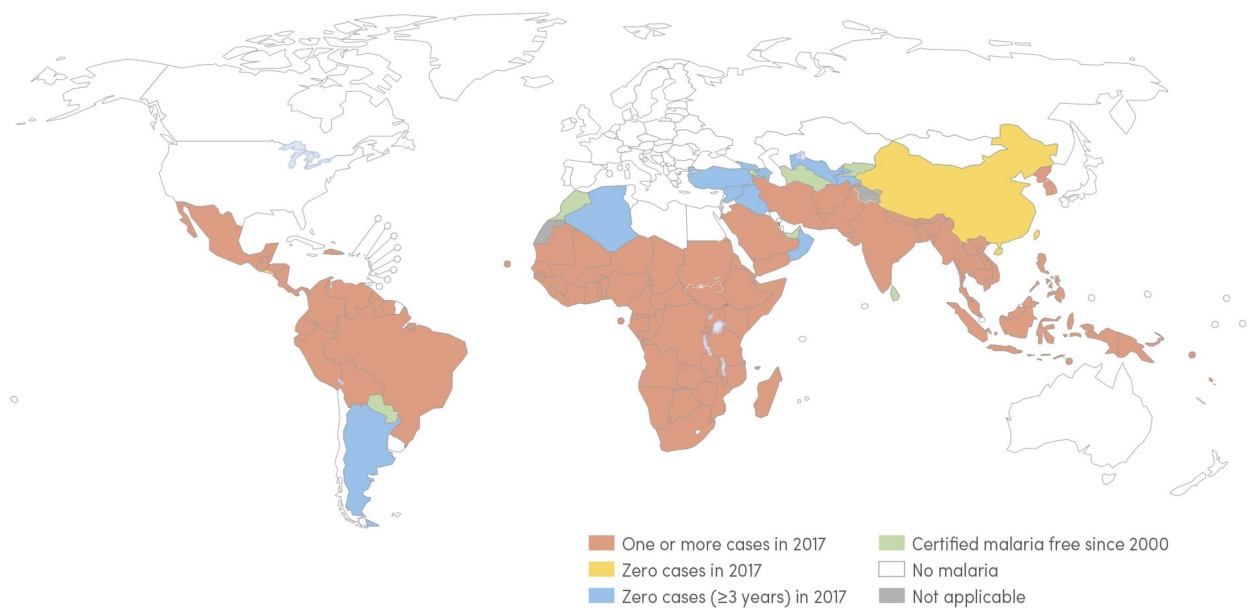


Figure 1. Malaria Elimination in the years 2000-2017. Countries with malarial cases in 2000 and their status in 2017.

Countries with zero cases over at least 3 consecutive years are considered as malaria free (WHO malaria report, 2018).

Plasmodium falciparum life cycle

The *P. falciparum* life cycle remains one of the most complex and fascinating of any organisms and is characterized by both intracellular and extracellular forms that develop between the definitive vector, the female *Anopheles* mosquito, and the human host (reviewed in (10) (Fig 2)). This complicated life cycle of the parasite is due to its ability to change its cellular and molecular makeup to develop as an extracellular form as well as to establish intracellular niches within the human host and mosquito vector.

The parasite life-cycle starts in the human host when a blood feeding *Anopheles* mosquito injects salivary-gland resident sporozoites through the skin dermis layer (11-13). Once these sporozoites enter the host circulation, they migrate quickly to the liver where they traverse Kupffer cells until finally invading an hepatocyte which provides a nutrient rich environment and presumably a place to hide from host immune responses. Once in the hepatocyte, the parasite activates development and replication and differentiates into a liver trophozoite eventually developing into 1,000s of liver merozoites. Liver infection does not result in any clinical symptoms but leads to more than a 10,000 fold amplification of parasite numbers. The accumulated merozoites in the hepatocyte are released into the blood circulation by budding of merozoites that are essentially packets of merozoites. These rupture to release merozoites that then invade circulating erythrocytes which activates the start of the asexual blood stage of the parasite life-cycle.

The newly invaded parasite develops within a parasitophorous vacuole, from the ring to trophozoites, the stage when DNA replication occurs, and, finally to schizont with development of 16-32 merozoites. The newly formed merozoites egress from the infected erythrocyte, into the blood circulation where they can interact with and invade new erythrocytes to continue the asexual blood stage life cycle. Within the erythrocyte, the parasite sequentially progresses through rings, trophozoites, and schizonts before rupturing and reinventing a new erythrocyte. The full asexual blood stage cycle takes approximately 48 hours for *P. falciparum*.

The malaria parasite life cycle has a sexual stage that is required for transmission to mosquitoes. This begins when a differentiation process is activated in some merozoites to develop into gametocyte forms. These can be taken up by a feeding female *Anopheles* mosquito and once the cells are ingested in the vector gut along with the bloodmeal, gametogenesis is triggered. This stage can be activated by three main factors; firstly, a shift in temperature; secondly, changes in pH, and, thirdly; a mosquito derived molecule, xanthurenic acid (reviewed in (14)). Once triggered, male gametocytes undergo exflagellation while the female gametocyte differentiates to a macrogamete. This important process enables the haploid male and female gametocytes to develop into gametes which eventually fuse and create a zygote. Soon after zygote formation, meiosis occurs with associated genetic recombination. The zygote then undergoes multiple morphological changes and transforms into an ookinete positioned within the lumen of mosquito midgut. Once fully developed, the motile ookinete penetrates the midgut epithelium to reach its destination at the basal lamina located in the outer wall of midgut, where it will further develop into an oocyst. In heavily infected mosquitoes, oocysts can be found distributed over the whole external side of midgut epithelium (15)(Fig 3). Immature oocysts are covered by the overlying basement membrane, this membrane will gradually stretch out as parasite grows showing a cobweb-like appearance between the oocyst and the midgut membrane.

Approximately at day 7 after the bloodmeal, sporulation, a budding of young sporozoite from the sporoblast, occurs within the oocyst. This process continues as the oocyst grows resulting in a thinner sporoblast and development of thousands of slender sporozoites resembling a sea anemone-like structure within the mature oocyst. The oocyst ruptures as a passive result from over expansion due to the accumulation of thousands of sporozoite during sporulation (16). The sporozoites subsequently egress from the oocyst and are released into the hemocoel where they can be carried by the hemolymph circulation to all tissues within the mosquito. The hemocoel sporozoites eventually reach the basal lamina of mosquito's salivary gland where host receptors are recognized by parasite ligands and allow the sporozoite to attach specifically to the salivary glands. Attached sporozoites traverse through the basal lamina

membrane and invade the salivary gland secretory acinar cells (17). During the mosquitoes' next bite of the mammalian host, infectious sporozoites are injected from salivary gland, and these make their way to the liver where they invade hepatocytes and ultimately complete the life cycle.

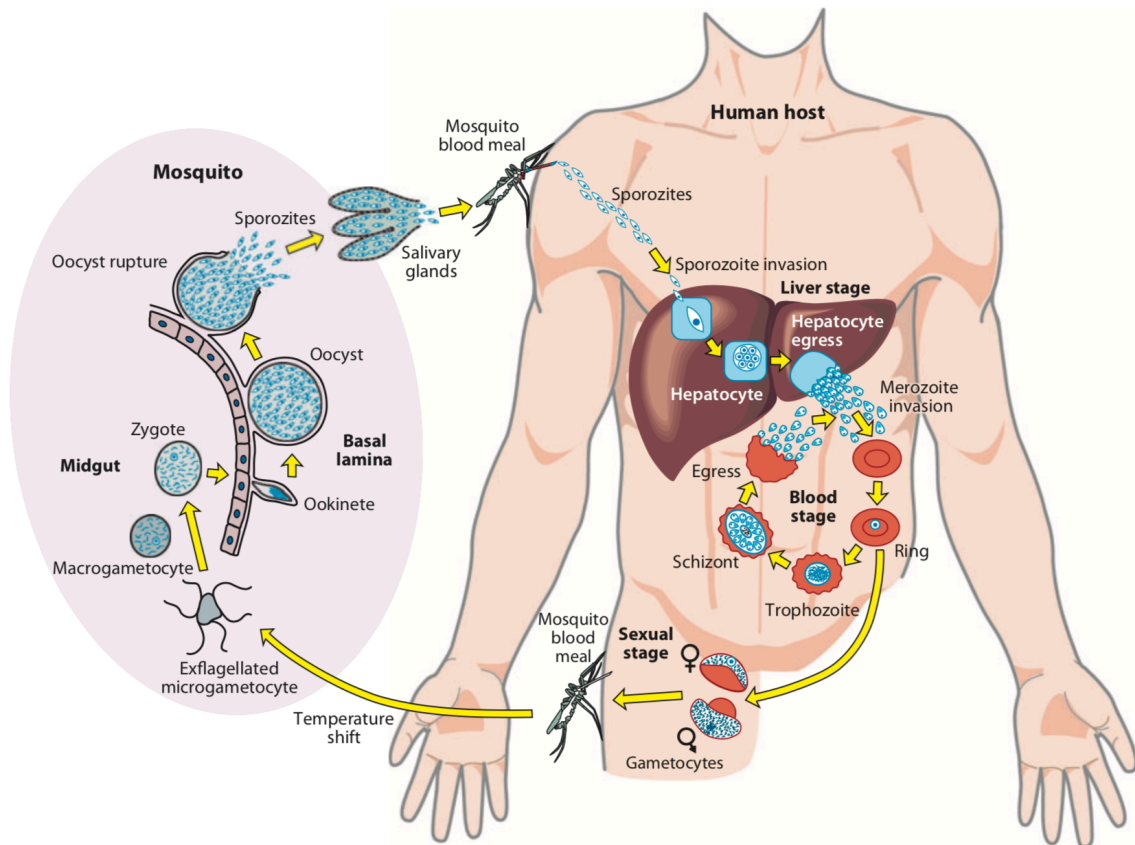


Figure 2 *Plasmodium falciparum* life cycle

The life cycle of *P. falciparum* consists of three separate stages. Once the human host is bitten by a mosquito, sporozoite forms are injected and these migrate to the liver and traverse Kupffer cells and then invade an hepatocyte which initiates the liver stage. The parasite then develops and replicates into daughter merozoites which, once they egress, invade host erythrocytes to start the asexual blood stage. The sexual/mosquito stage starts when male and female gametocytes are taken up by female mosquito where they can further develop into gametes before differentiate into oocyst and sporozoite later on. The sporozoite burst out of midgut and migrate to mosquito's salivary gland, ready to infect next human host and continue its life cycle (reviewed in (10)).

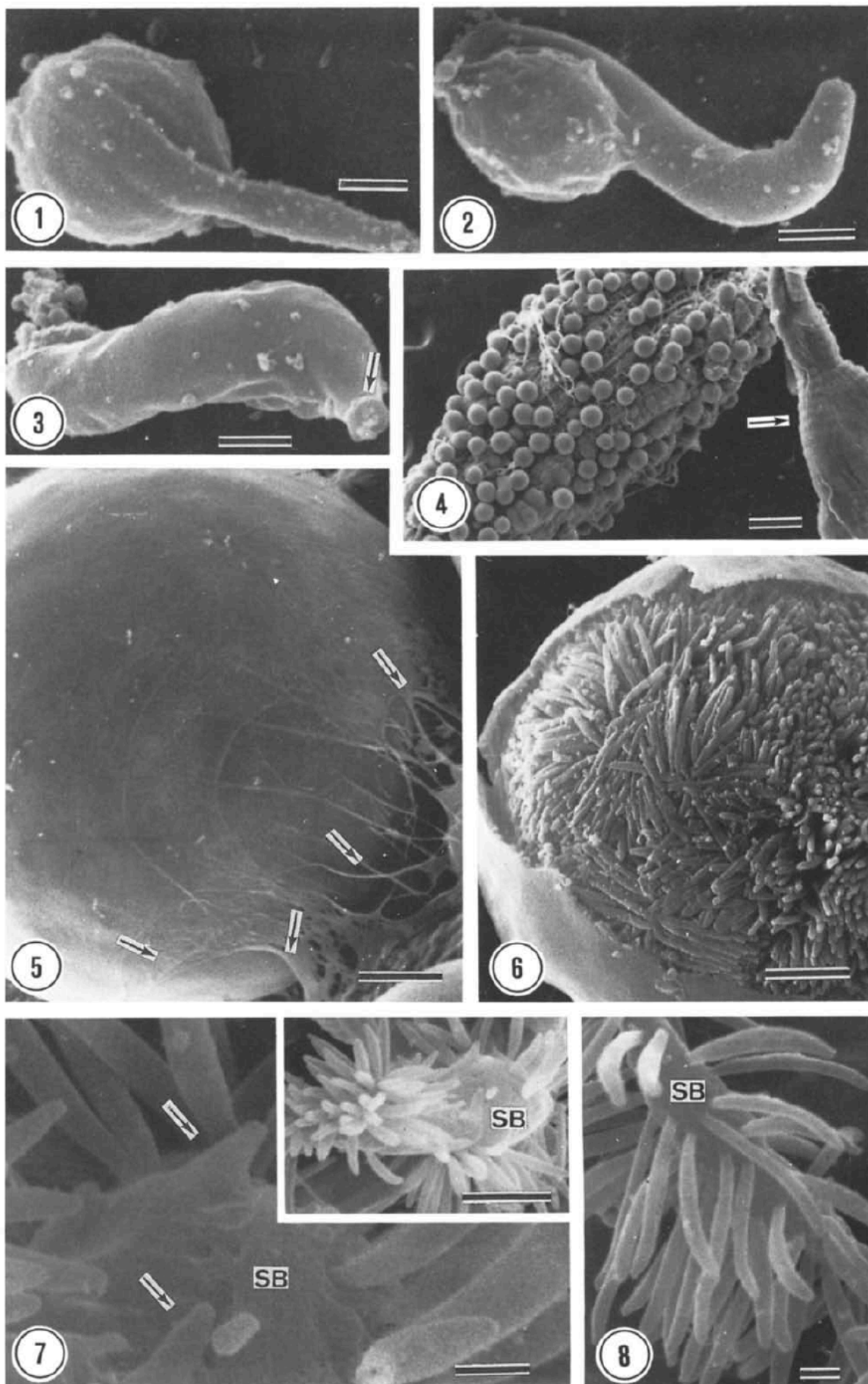


Figure 3 Electron microscopy of oocyst and sporozoite development

The process by which oocyst and sporozoites are formed has been observed by electron microscopy. After a blood meal, *P. falciparum* zygotes transform into retort forms (1, 2) before full development into ookinete (3). The ookinete then transverse through midgut epithelial and further develop into an oocyst at the basal lamina on midgut outer wall (4). Mature oocysts are closely attached to the midgut wall by a cobweb-like overlying membrane (5). Thousands of sporozoites form in the fully developed oocyst as a result of sporulation (6). This process occurs within oocysts and can be seen as budding of young sporozoites from the sporoblast (SB)(7), the sporoblast gets thinner and slender sporozoites are formed as the budding process continues resulting in a sea anemone-like structure (reviewed in (15)).

Pathogenesis of malaria

The symptoms of malaria occur during the blood stage asexual lifecycle of the *Plasmodium* parasite. Moreover, the involvement of the erythrocyte makes malaria potentially a multisystem disease, as every organ of the body is connected by the blood circulation (18, 19). All types of malaria manifest with common symptoms such as fever while some patients may progress into severe malaria. Although severe malaria can be usually found in those infected with *P. falciparum*, complications and even deaths have also been reported in non-falciparum infections.

At the completion of schizogony within infected erythrocytes, each cycle lasts approximately 24 to 48 hours depending on the *Plasmodium* spp., newly developed merozoites are released by the lysis of infected erythrocytes along with numerous known and unknown waste substances such as erythrocyte membrane products, hemozoin pigment, and other toxic factors such as glycosylphosphatidylinositol (GPI). Once released into blood circulation, these products, particularly GPI, activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumour necrosis factor, interferon- γ . Interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage colony stimulating factor, lymphotoxin, and superoxide and nitric oxide (NO) (5, 20). Many studies have implicated GPI, which is common to several merozoite surface proteins such as MSP1, MSP2, and MSP4 as a key parasite toxin (5, 20-24). The systemic manifestations are largely caused by various cytokines released in response to these parasites and erythrocyte membrane products (25). In addition to these factors, parasite DNA is highly proinflammatory and can induce cytokinemia and fever. During parasite development, parasite DNA binds to hemozoin and intracellularly interacts with the Toll-like receptor 9 within infected erythrocyte. This process lead to the release of proinflammatory cytokines which, in turn induce COX-2, upregulating prostaglandins and result in the induction of fever (26, 27). Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (28, 29).

Infection of host erythrocytes by malaria parasites, especially *P. falciparum*, result in progressive and dramatic structural, biochemical, and mechanical modifications of the erythrocyte that can play a role in the pathogenesis and symptoms of malaria. While the vast majority of severe malaria and related mortality are caused by *P. falciparum* infection, complications can also occur in non-falciparum infections as well. In recent years, several cases of severe infection and even deaths caused by *P. vivax* and *P. knowlesi* infections have been reported (30-37). Several pathophysiological factors such as the parasite biomass, malaria toxin, inflammatory response, cytoadherence, rosetting, sequestration, altered deformability, fragility of parasitized erythrocytes, endothelial activation, dysfunction and injury, and altered thrombostasis have been found to be involved in the development of severe malaria. All these factors are more profound and wide spread in *P. falciparum* infection compared to infections caused by other *Plasmodium* species. As a result, except for severe anemia, complications such as cerebral malaria, hypoglycemia, metabolic acidosis, renal failure, and respiratory distress are more commonly seen in *P. falciparum* infections (35, 38, 39).

Role of cytokines in severe malaria

Cytokines of the proinflammatory cascade like tumour necrosis factor, interleukins, interferon- γ , and nitric oxide are highly involved in the pathogenesis of malaria (reviewed in (40)). Cytokines act as homeostasis agents and an early proinflammatory cytokine response helps in limiting the infection, allow cytokines to inhibit the growth of malarial parasites (25, 41). However, failure to down-regulate this inflammatory response can also result in progressive immune pathology and leads to more complications. Moreover, excessive levels of cytokines can also lead to many complications such as: decreased mitochondrial oxygen use and enhanced lactate production, increased cytoadherence which results in microvascular obstruction and more hypoxia, disturbed auto-regulation of local blood flow leading to poor circulation and further tissue hypoxia, dyserythropoiesis, poor erythrocyte deformability and multifactorial anemia, reduced gluconeogenesis and hypoglycaemia, myocardial depression and cardiac insufficiency, loss of endothelial integrity and vascular damage in the lung and brain, selective upregulation of vascular and intercellular adhesion

molecules (ICAMs), especially in the brain and placenta leading to cerebral malaria and placental dysfunction, activation of leukocytes and platelets, promoting procoagulant activity (5, 19, 20, 27, 38, 42-47). Taken together, the outcome of malaria infection is determined by the balance between the pro- and anti- inflammatory cytokines (19, 20, 43).

Some of the complications detected in *P. vivax* infection may be related to cytokine-mediated injury. *P. vivax* has been found to induce a greater inflammatory response than *P. falciparum* (with equal or greater parasite load), resulting in higher levels of cytokine release. The pyrogenic threshold is also lower in *P. vivax* infections, resulting in fever at lower levels of parasitemia. The differences in pyrogenicity between these two species might be caused by structural differences in *P. vivax* GPI, that may make it more pyrogenic and/or greater concentrations of Toll-like receptor 9 stimulating motifs within *P. vivax* hemozoin (35). A cholesterol/triglyceride(s) containing lipid, that has greater activity than GPI-like phospholipids, has also been proposed as a putative malaria toxin unique to *P. vivax* and may also contribute to the greater pyrogenicity of *P. vivax* (48).

Cytoadherence, sequestration, and rosetting

Structural changes in the infected erythrocytes leading to increases in rigidity and adhesiveness are major contributors to the virulence of *P. falciparum* infection (49, 50). Due to increased adhesiveness, the erythrocyte infected with late stages of *P. falciparum* parasites (during the second half of 48 hour life cycle) adhere to the capillary and post capillary venular endothelium in the deep microvasculature (cytoadherence). The infected erythrocytes can also adhere to uninfected erythrocytes leading to the formation of erythrocyte rosettes (rosetting), a cytoadherence responsible for the sequestration of the parasites in various organs such as heart, lung, brain, liver, kidney, intestines, adipose tissue, subcutaneous tissues, and placenta. Sequestration of the growing *P. falciparum* parasites in these deeper tissues provides them with the microaerophilic venous environment that is more appropriate for their maturation. Moreover, the adhesion to endothelium allows parasites to escape

clearance by the spleen and hide from host immune system. These factors allow the parasite to occupy protective niches in the circulation and major organs and multiply, thereby increasing the parasite load.

Due to sequestration of the growing parasites in deeper vasculature, only ring stage trophozoites of *P. falciparum* can be found circulating in peripheral blood, while the more mature trophozoites and schizonts are more likely to be seen in the deep microvasculature, hence seldom on peripheral blood examination. If the cytoadherence/rosetting/sequestration of infected erythrocytes in the vital organs goes on uninhibited, it can ultimately block some blood flow, limit the local oxygen supply, obstruct mitochondrial ATP synthesis, and stimulate cytokine production. All these factors mentioned above contribute to the development of severe disease (20, 35, 38, 39, 45, 51-54).

Certain proteins expressed on the surface of infected erythrocytes mediate the adhesion of parasitized erythrocytes to the endothelium and to uninfected erythrocytes. One of the most important protein is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), an antigenically diverse protein family that is expressed in the thousands of knob-like protrusions on the surface of erythrocyte infected with *P. falciparum* trophozoites and schizonts (55-58). PfEMP1 is anchored at the erythrocyte membrane skeleton by the knob-associated histidine-rich protein (59). PfEMP1 can be found on the surface of the *P. falciparum* infected erythrocytes about 16 hours after the invasion and is responsible for cytoadherence to receptors on the microvasculature (39, 53, 58).

Different PfEMP1 types can bind to several adhesion receptors expressed on the endothelial cells such as thrombospondin, CD36, ICAM-1, vascular cell adhesion molecule 1, platelet/EC adhesion molecule/CD31, neural cell adhesion molecule, P-selectin, E-selectin, integrin $\alpha_v\beta_3$, globular C1q receptor (gC1qR)/hyaluronan binding protein 1/p32, chondroitin sulfate A (CSA), and hemagglutinin ((5, 38, 45)). Whereas ICAM-1 and CD36 are more commonly used receptors, CSA acts as the receptor for binding in the placenta (60). Activation of endothelial cells by cytokines and infected

erythrocytes increases the expression of adhesion promoting molecules and then further promotes cytoadherence (61), differences in binding to these receptors (CD36 and ICAM-1) may determine the virulence of *P. falciparum* (39, 51).

PfEMP1 also mediates formation of rosettes created by adherence of *P. falciparum*-infected erythrocytes to uninfected erythrocytes (55-57, 59). PfEMP1 has a large N-terminal extracellular region comprising an N-terminal segment (NTS), and several Duffy-like (DBL) domains and cysteine-rich interdomain regions (CIDR) (62-64). DBL and CIDR domains can be assigned to a small number of sequence classes. The arrangement of these domains is modular but most variants begin with NTS followed by α class DBL domains. It is mediated by binding of PfEMP1-DBL α on the surface of infected erythrocytes to complement receptor 1, CD31, and heparin sulfate-like glycosaminoglycans of uninfected erythrocytes (51, 52, 65). Rosetting is found to be less in blood group O erythrocytes compared with group A, B, and AB, and thus patients with blood group O may be protected from severe malaria (66, 67). Cytoadherence, sequestration, rosetting and aggregation of leukocytes have been reported in *P. vivax* infections as well. However, these are less in magnitude and extent in comparison to *P. falciparum* infections and their roles in pathophysiology of complications still remain unclear (35).

Erythrocyte membrane rigidity and deformability

Altered erythrocyte membrane rigidity and deformability also contribute to the pathogenesis of severe malaria. In patients with severe malaria caused by *P. falciparum*, the entire erythrocyte mass, comprising mostly of uninfected and infected erythrocytes, becomes rigid (68-70). Several mechanisms such as hemozoin-induced oxidative damage of the erythrocyte membrane, alteration in the phospholipid bilayer and attached spectrin network by the proteins transported to the erythrocyte membrane, thermally driven membrane fluctuations due to fever, and inhibition of the Na⁺/K⁺ pump on the erythrocyte membrane by nitric oxide (NO), may be responsible for the increase in rigidity and reduction in deformability of the *P. falciparum* infected erythrocytes (20, 68, 70). Increased rigidity can also be caused by the parasite

infection itself. Parasite proteins such as knob associated histidine rich protein (KAHRP) must traverse several membranes to reach their destination at the highly organized erythrocyte membrane skeleton structure (49). Once reached, KAHRP bound to the membrane skeleton leads to an increased rigidity contributing to blockage of blood vessels and a reduction in normal blood flow (50). Reduced erythrocyte deformability results in increased splenic clearance and loss of erythrocytes leading to anemia. Hemolysis, suppression of erythropoiesis by cytokines, and hemozoin induced apoptosis in developing erythroid cells also contribute to the development of anemia in severe malaria (20, 28, 70). Compared to infection with *P. falciparum*, in which erythrocyte deformability is reduced, it is increased in *P. vivax* infection. While this may enable *P. vivax* infected erythrocytes to survive passage through the splenic sinusoids, the accompanying increase in fragility of both infected and noninfected erythrocytes may contribute to severe malaria in *P. vivax* malaria. Increased deformability of *P. vivax* infected erythrocytes also makes sequestration and obstruction of blood flow unlikely (35, 71).

To conclude, the pathogenesis of severe malarial anemia involves a cascading interaction between parasite and erythrocyte membrane products, cytokines and endothelial receptors, leading to inflammation, activation of platelets, hemostasis, a procoagulant state, microcirculatory dysfunction and tissue hypoxia, resulting in various organ dysfunctions manifesting in severe malaria (47).

Transmission – the bottleneck of malaria life cycle

The most vulnerable stages of malaria parasites are the ookinete and sporozoite stages, both of which form and migrate within the mosquito vector (72, 73). These stages are essential within the malaria life cycle and required for transmission through the mosquito and to the human host. Ookinetes are products of zygote fertilization after bloodmeal consumption, and it is the only invasive stage that doesn't require replication so that the number of ookinetes produced is the same as the number of fertilization events. Moreover, the ookinete suffers from severe reduction in numbers caused by host protective mechanisms (72). As the parasite invade a cell, they not

only inflict physical damage but also secrete substances such as the GPI-anchored surface protein Pbs21 and the PbSub2 protease, which may facilitate the invasion process. Parasite invasion triggers a series of host defence mechanisms such as nitric oxide synthase (NOS) production and initiate pathways that lead to cell death. The abundance of NOS and blood-derived arginine at this time suggests that huge amounts of nitric oxide (NO) may be generated. Under this particular condition, the outcome of invasion would be determined by several factors such as the speed at which parasite crosses the cells, the rate of NO production, how long the cell can sustain this lethal condition and other defence responses as it leads to cell death. Ookinetes would have a limited time window to emerge unharmed from the cell. However, it has been discovered that more than 95% of parasites are able to escape unharmed. In contrast, it is more likely that the major losses of mature ookinetes occur before the parasites reach the cytoplasm of the invaded cell and/or after oocyst formation. As NO is very toxic and highly diffusible, the induction of NOS could have dramatic effects on the ookinetes present in the lumen of the midgut or early oocysts (reviewed in (72, 74)). The ookinetes that traverse through the midgut epithelial cell layer to the basal lamina side of outer wall and survive this journey and develop into oocysts, the only parasite developmental stage that grows extracellularly and this growth and development creates thousands of sporozoites. Once fully developed and egressed, these sporozoites are released into mosquito hemocoel and they migrate to the salivary gland ready to infect next mammalian host and continue their life cycle. Like ookinetes, sporozoites also suffer heavy losses during their journey to the salivary gland, thus these two stages are considered as the bottle-necks of the malaria life cycle (72, 75). However, much remains to be understood with respect to malaria transmission to humans by the vectors.

Malaria drug development

The use of artemisinin-based combination therapies (ACTs) to treat malaria dates back to the early 1990s, after chloroquine, sulfadoxine-pyrimethamine (SP) and mefloquine had been deployed sequentially in Southeast Asia and resistance to them had developed. In 1979, Chinese scientists had published the chemical structure of

artemisinin, a potent antimalarial derived from the sweet wormwood plant *Artemisia annua* (76, 77). ACTs were adopted fairly quickly in Southeast Asia with some produced locally, e.g. Vietnam has produced various dihydroartemisinin (DHA) piperazine combinations since 1995.

Meanwhile chloroquine and pyrimethamine resistant strains of *P. falciparum* had spread to Africa leading to an increase in child mortality (78-80). This crisis raised concern about controlling malaria and greater financial resources were brought to bear on this health problem. An important part of the response was the recognition that new drugs were needed and an innovative approach was required to stimulate their development. New product development partnerships (PDPs) were created, such as Medicines for Malaria Venture (MMV, 1999) and the Drugs for Neglected Diseases Initiative (2003). The PDPs harnessed the expertise of the pharmaceutical industry and malaria academics, with financial support coming from a variety of sources. They were given a head start by focusing on the data from Chinese scientists and pharma (reviewed in (81)). By 2006, ACTs had become the recommended treatment for malaria caused by *P. falciparum* worldwide. The first ACT produced to recognized international standards of good manufacturing practice (GMP) was artemether-lumefantrine, approved by the US FDA in April 2009 (82). Artemether-lumefantrine, DHA-piperazine, artesunate-amodiaquine, artesunate-mefloquine and artesunate-sulfadoxine-pyrimethamine are currently the most widely used combinations. Drugs that are also available include pyronaridine-artesunate, artemisinin-naphthoquine, and arterolane-piperazine. Arterolane is a newer synthetic peroxide developed in India resembling the artemisinin derivatives.

P. knowlesi malaria is treated in the same way as *P. falciparum* malaria (83). For uncomplicated disease caused by *P. vivax*, *ovale* or *malariae* chloroquine remains the first line treatment in most parts of the world. However, there is increasing evidence of CQ resistance in *P. vivax* (84). In Indonesia, where failure rates are high, ACTs, in particular, DHA-piperazine, has replaced chloroquine for treatment. Severe malaria continues to be treated by either parenteral artesunate (the drug of choice), or quinine

or quinidine (USA only) (85, 86). A number of adjuvant therapies have been evaluated over the years but none are shown to improve the outcome (87).

Antimalarial drug development

An ideal antimalarial treatment would be a potent inhibitor of parasite multiplication, short course or single dose, well tolerated in pregnant women and young children, have a good safety profile, be affordable and have a low propensity for resistance to develop. Antimalarials with a synthetic peroxide scaffold, e.g. arterolane, artefenomel, were prioritized for development initially because the advantageous properties of the artemisinin derivatives were replicated (rapid onset, strong parasitocidal effect), but with modifications to remove some of disadvantages (reliance on agricultural supply, short duration of action, reproductive toxicity in animals) (88). There is a paucity of new agents in development for chemoprevention alone. DSM-265, a *Plasmodium* dihydroorotate dehydrogenase inhibitor, shows potential as a prophylactic agent for travellers, although doses studied so far in a human challenge model have not conferred seven days of protection from malaria infection, which would be required for weekly dosing (89). Pregnant women and infants are typically excluded from studies of new antimalarials, despite bearing the brunt of most malaria-associated morbidity and mortality. As a result, they are the last to receive them since it takes time for sufficient efficacy and safety data to accumulate post-registration (90-92). However, ACTs are now approved for treatment of malaria in pregnancy and use in intermittent preventive treatment in pregnancy (IPTp) in Africa where resistance to SP treatment is high (93).

Like primaquine, tafenoquine is an 8-aminoquinoline active against asexual stages of *P. vivax*, but more importantly against hypnozoites which cause relapse. This long acting drug (elimination half-life 14-19 days) is being developed for single-dose relapse prevention for vivax malaria and a new drug application was submitted to US FDA and the Australian Therapeutic Goods Administration in late 2017. The development process has been slow and painstaking, necessitated by the known potential for hematological toxicity (acute hemolytic anemia) in persons with glucose-

6-phosphate dehydrogenase (G6PD) deficiency, an X-linked enzymopathy found in malaria endemic areas. Tafenoquine has the potential to revolutionize radical therapy of relapsing malaria by enhancing patient adherence compared to the standard 14 days of primaquine and may be a potent tool to accelerate vivax malaria elimination; however, it will need to be deployed with concurrent reliable point of care G6PD activity testing to screen out individuals at risk. Differences in metabolism of primaquine with a resulting impact on efficacy for relapse prevention have been observed in association with allelic variation in the cytochrome 2D6 gene (94). Preliminary investigation has indicated the same may not be true for tafenoquine, but more evidence is needed (95). Tafenoquine is now registered, however, there is a lack of options for safe use in populations where G6PD deficiency is prevalent, and this is an impediment for the elimination of vivax malaria (96). Recent progress in the development of methods to screen for hypnozoitocidal and transmission blocking compounds may lead to more agents being identified in the coming years.

The decreased efficacy of ACTs in Southeast Asia has led to a re-evaluation of combination therapy for malaria. Rather than only using triple combinations of existing drugs as a salvage strategy to try to improve cure rates in areas where resistance has already taken hold, there is an argument for introducing them in countries with no evidence of resistance, sooner rather than later, as well as ensuring all new combinations developed are composed of three or more drugs. The drawbacks of this approach include increased development time, increased costs and increased risk of toxicity; thus, any ambition to produce a novel triple combination that is efficacious against all strains of malaria in time to replace currently failing ACTs may have to be sacrificed in the interests of expediency. However, this could be the goal for future generations of novel antimalarial combination therapies.

Drugs in clinical development for transmission-blocking

Reducing malaria transmission substantially will accelerate malaria elimination. Drugs preventing malaria transmission target gametocytes, sporogony or the mosquito vector by reducing its survival (endectocides). Single low-dose (0.25 mg/kg)

primaquine is now recommended alongside antimalarial treatment in endemic countries targeting elimination for its activity against mature gametocytes; thus, the potential for drug-drug interactions with new blood schizonticides needs to be evaluated. Very few new agents are in development specifically for transmission blocking, although some have multistage activity, e.g. imidazolopiperazines (KAF 156) (97). *methylene blue* is an old parasitocidal agent with blood stage activity and the added benefit of activity against mature male and female *P. falciparum* gametocytes. It is being developed by the University of Heidelberg in combination with ACTs as a strategy to protect against artemisinin resistance emergence and reduce transmission (98).

Re-purposing of antimicrobial drugs for malaria prevention or treatment

Several antibiotics have weak to moderate antimalarial activity, e.g. doxycycline, clindamycin, azithromycin, and cotrimoxazole. Cotrimoxazole is given to patients with HIV to protect against *Pneumocystis jirovecii* pneumonia and has been shown to reduce malaria infections, and is a possible candidate for use as prevention in HIV-uninfected pregnant women or children (99). Azithromycin is amenable to once-daily dosing and has a good safety profile; therefore it could theoretically be combined as part of a short course once daily combination therapy or be used for prevention. Drawbacks include selection for resistance in other common important pathogens, e.g. *Salmonella enterica* serovar Typhi, *Streptococcus pneumoniae*. In the 1970s, the discovery of ivermectin, a common antiparasitic agent used to treat animals, led to the Nobel prize in Medicine being awarded to William C. Campbell and Satoshi Omura in 2015. The prize was shared with Tu Youyou in recognition of the discovery of qinghaosu (artemisinin) to treat malaria. Ivermectin has also been shown to reduce malaria transmission by reducing mosquito survival (100). It has other uses for human disease, e.g. mass treatment for lymphatic filariasis, thus has the potential to be used as a tool in integrated disease-control programmes (101).

Transmission blocking strategies

In 2015, RTSs/AS01 was recommended by the WHO as the first malaria vaccine that can provide partial protection against malaria in young children (102-104). The vaccine aims to trigger the immune system to defend against the first stages of malaria when *P. falciparum* sporozoites enter the human bloodstream. It was designed to prevent parasite from infecting liver and further develop and cause any disease symptoms. However, novel anti-malaria drugs and vaccines are needed in order to eliminate and eradicate malaria. A strategy to combat malaria would be to target multiple metabolic pathways with combination drug therapies combined with an efficacious vaccine that can reduce both morbidity and mortality. This make transmission blocking strategy a promising option since, once the sexual parasite development is disrupted, it can lessen burden of infection rate and reduce the chance of spreading drug resistance.

The candidates for transmission blocking vaccines can be divided into two categories; firstly, antigens that are expressed in gametocytes and gametes, the immunity against these antigens is naturally boosted by infection since they can be detected during their life cycle in mammalian host; and secondly, antigens that are only expressed in zygotes and ookinetes located in the mosquito vector.

Development of transmission blocking drugs, on the other hand, has been focused on parasite kinases identified in the study of parasite development during mosquito stages (105-107). Moreover, the fact that the structure of many malaria kinases are unique and do not have any obvious orthologues in humans host makes them attractive targets for antimalarial drug development. However, kinases have been considered to be difficult targets to drug and develop specificity.

Proteases have also been considered to be suitable candidates for transmission blocking drug development. Genomic studies reveal 92 proteases in *P. falciparum*, most of them playing important roles in events in the malaria parasite sexual stages such as egress of female gametocytes from infected erythrocytes, exflagellation of microgametocytes, and cell-cell interactions between male and female gamete prior

to cell fusion (108). While much research mainly focuses on proteases responsible for merozoite invasion and food digestion (109-111), some have been identified that are specific for parasite sexual stages such as cysteine protease metacaspase II, aspartyl protease plasmepsin VI, and serine protease *PF*Sub3 (108, 112). Cysteine/serine inhibitors TPCK and TLCK have been confirmed to block exflagellation in *P. falciparum*, whilst the cysteine protease falcipain 1 plays a role in oocyst formation but is not required for exflagellation (113-115).

The mosquito vector also holds interesting potential to malaria parasite transmission blocking strategies (116-118). Several studies have addressed the importance of the interaction between host and parasite, many molecular targets have been identified including xanthurenic acid which plays a significant role in order to trigger the gametogenesis process (119, 120). CpdAg1, a midgut carboxypeptidase gene, is also considered as another interesting target since, in *Anopheles gambiae*, it can be upregulated during parasite infection and antibodies against the protein B-Ag1, a downstream product of CpdAg1, can inhibit parasite development within the midgut of the mosquito (121, 122).

Aspartic proteases as drug targets

Some protease inhibitors used for HIV patients, including saquinavir and ritonavir, have been shown to inhibit parasite growth during the asexual stage (123). More studies have also shown that HIV protease inhibitors can disrupt the *P. berghei* life cycle in mice, late stage gametocytes, pre-erythrocytic liver stage parasite, and the serum taken from HIV patient treated with these inhibitors inhibits parasites growth *in vitro* (124-127). Unfortunately, despite the fact that HIV protease inhibitors may be able to decrease malaria incidence in children, the studies involving phase III clinical trials of lopinavir and ritonavir on women in sub-Saharan region haven't yielded any beneficial effect compared to nevirapine based therapy making them unlikely to be antimalarials in their own right (128, 129). However, since HIV protease inhibitors are effective against multiple stages of malaria parasite development, this makes them

and their targets in the parasite interesting areas to pursue to develop new antimalarials.

The aspartic acid proteases in *P. falciparum* and other *Plasmodium* spp. are called plasmepsins and they hold potential as important drug targets. In *P. falciparum* there are ten genes that encode aspartic acid proteases, each of them has a different role in parasite life cycle (130)(Fig4).

Food vacuole plasmepsins

Plasmepsin I, II, IV, and the histidine aspartic protease (HAP) are expressed in asexual stages and are localised to the parasite food vacuole (131). Previous studies show these proteins work cooperatively to degrade haemoglobin endocytosed from the parasite-infected erythrocyte cytoplasm to provide nutrients for parasite growth and maturation (132). However, gene knockout studies have shown that these plasmepsins are not essential for parasite survival indicating they are not the best choice as a drug target (133).

Plasmepsin V

Plasmepsin V is an aspartyl protease expressed by protozoan parasites of *Plasmodium* species. It is responsible for the recognizing and processing of effector proteins for export to infected erythrocytes (134, 135). During each round of asexual parasite replication, effector proteins exported to the host cells by these parasites are responsible for a remarkable process of cellular renovation that is induced in parasite infected erythrocytes (10, 136). Remodelling of the infected erythrocyte provides a means for the parasite to gain nutrients and remain hidden from the host's immune response in a relatively protected niche (53).

Plasmepsin V is a type I integral membrane-bound protease with the active domain located on the luminal side of the endoplasmic reticulum (ER) (134, 135, 137). Its main function is to recognize and cleave a pentameric sequence (RxLxE/Q/D) known as the

Plasmodium Export Element (PEXEL) (136), or vacuolar transport signal (138) on the carboxy side of the leucine residue (139, 140). This sequence motif is located 15-30 amino acid C terminal to a hydrophobic ER type signal sequence, and is required for export of hundreds of effector proteins (136, 138, 141). This processing step by plasmepsin V reveals the export signal (xE/Q/D) and directs these proteins to the plasma membrane and the parasitophorous vacuole membrane that surrounds the parasites (134, 135, 137, 140). At the parasitophorous vacuolar membrane, these exported proteins are recognized by the *Plasmodium* translocon, a protein machine that translocates them into parasite-infected erythrocytes (142, 143). Cleavage of the PEXEL motif by plasmepsin V is essential for protein export in *P. falciparum*-infected erythrocytes (134), (144). Expression of a dominant negative form of plasmepsin V in *P. falciparum* results in reduced export of proteins to host erythrocyte and impaired growth, indicating that plasmepsin V is required for this trafficking pathway (135, 137). A PEXEL mimetic compound containing statine, a molecule with a hydroxyl functionality that mimics the transition state of amide bond proteolysis, inhibits *in vitro* activity of *P. falciparum* and *P. vivax* plasmepsin V, blocks protein export and inhibits *P. falciparum* growth (137). This inhibition by a PEXEL mimetic has shown that plasmepsin V plays a crucial part in protein export and that this process is essential for parasite survival.

Plasmepsin VI

While the function of some plasmepsins is known and they are vital for parasite survival, others including those that function at the mosquito stage of transmission, are not well understood. Plasmepsin VI, according to the transcriptome expression profile, is highly expressed in late stage female gametocytes and oocysts (www.plasmodb.org). It was previously demonstrated in reverse genetic screening studies that this enzyme was essential for transmission in *P. berghei* (145). The results have shown that parasites lacking expression of plasmepsin VI are unable to produce sporozoites due to a defect in sporulation within the oocyst resulting in the absence of sporozoites in the salivary glands. Consequently, these mutant parasite lines fail to

infect mice (145). However, an in depth understanding of the plasmepsin VI function is lacking.

Plasmepsin VII

Based on the evidence of gene expression data plasmepsin VII is expressed in transmission stages (146, 147). It has been reported that ablation of plasmepsin VII expression had no apparent fitness loss with respect to progression through the life cycle. While the data indicated that plasmepsin VII is not essential in *Plasmodium* life cycle, a similar pattern of gene expression for plasmepsin VI and plasmepsin VII in the mosquito stages can be detected and further investigation is required to prove if any functional redundancy exists among mosquito stage expressed plasmepsins that may compensate loss of function (148).

Plasmepsin VIII

A reverse genetic screen in *P. berghei* has shown that lack of plasmepsin VIII did not affect growth and development of asexual blood stages (149). It has also been found that ablation of plasmepsin VIII did not affect the formation of ookinetes and subsequent transformation into oocysts. However, sporozoites from plasmepsin VIII knockout parasites did not show gliding motility, which is essential for sporozoite egress from the oocyst and subsequent invasion of salivary glands (149).

Plasmepsin IX and X

Within erythrocytic stages, plasmepsin IX and plasmepsin X are predominantly expressed at the schizont stage, suggesting they play important roles during egress and/or invasion (150). A reverse genetics study showed that, in the absence of plasmepsin IX, intracellular development occurred normally until the schizont stage. However, a significant reduction in the numbers of ring stage parasites was observed and the result was more pronounced when the parasites were treated with trypsin to remove non-invasive adherent merozoites from the host cell surface. Moreover, the

cysteine-rich protective antigen (CyRPA), a protein that essential for merozoite invasion, was hardly detected in plasmepsin IX knockdown parasites. Taken together, these data suggested that the replication defect in parasite lacking plasmepsin IX is caused by impaired egress and invasion (151).

Plasmepsin X, on the other hands, is expressed in the mature gametocytes and its function was demonstrated using compound 49c, a peptidomimetic competitive inhibitor that has been shown to target plasmepsin IX and X. Although 49c did not affect differentiation from asexual stages into microscopically mature gametocytes, it prevented further development into fertile gametes. The amount of processed serine protease (SUB1), one of the plasmepsin X substrates, was also strongly reduced with 49c treatment, indicating that plasmepsin X is required for parasite egress during gametocytogenesis, since SUB1 is critical for parasite egress from erythrocytes (152). Plasmepsin X can also be detected in ookinetes and, after treatment with 49c, the number of oocysts in infected midgut is significantly reduced (151). Designated cell-traversal protein for ookinetes and sporozoites (CelTOS), a protein that is crucial for ookinetes to traverse host cells into the site of oocyst development (153), is found to be cleaved by plasmepsin X. The level of processed CelTOS was inhibited by 49c suggesting that plasmepsin X is essential for oocyst formation and could be a potential target for transmission blocking strategies (151).

Characterization of remaining plasmepsins is required in order to identify that represent the best potential drug targets and to understand the function of these enzymes in malaria parasite biology. In this study, I aim to analyse the function of plasmepsin VI in *P. falciparum* in order to provide more information on its role during the *P. falciparum* life cycle in the mosquito. I will analyse the functional importance of plasmepsin X in the mosquito stage in order to determine if this enzyme holds potential as a drug target for transmission blocking strategies.

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PM I   NAGDSVTND.VANVMYYGSAQIGDNKCK.PAFIPDTGSANLWVPSAQNTIGC...KTKNLYDSNKKK.TYEK
PM II  SSNDNIELVD.FQNMIFYGDAEVDNQCP.FTFILDTGSANLWVPSVKCTTAGC...LTKHLYDSSKSR.TYEK
HAP    SEDNVEELKD.LANVLSFGAKLGDNGQK.PNPLHTASSNVVPSIKCTSES...ESKNHYDSSKSK.TYEK
PM IV  SENDSIELDD.VANLMFYGGQIGTNKCP.FMPFIDTGSANLWVPSVNDISIG...STKHLVDASASK.TYEK
PM V    DLVYKLYGDIIDYAYVLDIDIGKPSQR.ISLLIDTGSSSISFPCNGCK.DGCIHMEKPYNLNY..SK.TSSI
PM VI   LSYIQEDLN.FHNSQFIADIGVGNPPQV.FKVPFDTGSSNLAIPTKCIKGGC...ASHKKFNPNSKSR.ITK
PM VII  IKLKKQDIFS.KKLSTYYGEVQIGEQSENMMNLVFDTGSSQVILNDTKNSLC...NNIH.SKYKRTKSF
PM VIII IGISTIVLKGGYINRQFIEGINIGNPPQT.FKVLFDTGSTNLWIPSKNCFTRAC...YNKKRYKHKI..SKNY
PM IX   NDKVTLPLQQ.LEDSQVVGYYIQIGTPPQT.IRPFDTGSTNIWIVSTKCKDETC...LKVHRYNHKLSS.SFKY
PM X    ENIFLVLPHK.LRDSQFVGLLVGTTPQT.VYPIFDTGSTNVVVVTTA.EEESC...KKVRRYVFNKSK.TERR

PM I   .DGTK.....VEMNVVSGT.VSGFFSKDIVTIAN.LSPYKFIETDIN.....GFPAYALG
PM II  .DGTK.....VEMNVVSGT.VSGFFSKDLTVGN.LSLPYKFIETDIN.....GFPPTYTAS
HAP    .DDTP.....VKLTSKAST.ISGIFSKDLVTIGK.LSVPYKFIETDIN.....GFPFYSES
PM IV  .DGTK.....VEMNVVSGT.VSGFFSKDIVTIAN.LSPYKFIETDIN.....GFPAYALG
PM V    LYCNKSNCP-(11).....YLQSYCESSQIYGFSDIVTLPSYNNKNKISFEKLMQCH.....MHESLFLHQ
PM VI   NLKSNKNEII-(24)-EKTPTLTFLFYGTGT.SSLEQSYDQVYKGLYKIKHQICGLAIBG.....KESLHPFSDL
PM VII  VYKYDKKG.....LPSV.IFIFLISGKI.VAPEANVLNKCIIYTFILKN.....NSRHR
PM VIII KL.VKKKDP.....VSLFSTGE.IHIAVTDIHL.GDIKVKNEEGLASMI.....SDPFSDM
PM IX   YEPHTN.....LDIMPGTGI.IQVIGVETFKIGP.FEIKNQSEGLVKREKASDNKS..NVFRI
PM X    SPIEK.....LHIFVSGS.ISSVGTDTFMLEK.HLVNRQTEGLVSE.SNNNKGNGNIFDI

PM I   QFDGIVGL.....GWKDL.S.IGSVDVSVVEKNQNKIEQAVFTFYLFDDDKHK.GYLTIGGIDRF.YEG..
PM II  TFDGIVGL.....GWKDL.S.IGSVDVSVVEKNQNKIEQAVFTFYLFDDDKHK.GYLTIGGIDRF.YEG..
HAP    DVNGVFGI.....GWKDL.S.IGSIDVIVEKTONKIEQAVYSIYLPENKNK.GYLTIGGIDRF.FDG..
PM IV  EFDGIVGL.....GWKDL.S.IGSIDVSVVEKNQNKIDNALFTFYLFDDDKHV.GYLTIGGIDRF.YEG..
PM V    QATQVLFGLSLTKPNQVPTF.....VDLFKHTPSLKPIYSICVSEH...GCEIIGGYPDYFLSNQK-
PM VI   PFDGIVGL.....GFSR.....GLKRNISFYVE...NRS.GATIFGKANKYTVBQK..
PM VII  .DGIIGL.....GFQNGDSIKRGIK.LLDI.KDDKILTNKYNKQPGYYLSDKEGYILGGI.NRLKNTDPE-
PM VIII QFDGLFGL.....GISDK..K.KKQLIYDSIPKNILEKNM.AIYYKKNVDDD.GATIFGGYKKE..NS..
PM IX   NFDGIVGL.....APPEMLS.TG.KSTLYEN.MSSYKLOHNE.SIYIGKD.SKY.SALIFGGV.KNS.FEG..
PM X    SFDGIVGL.....GFPGLS.AG.NIFFDN.LKQNPVNDPQSFYISPY.DGK.SLIIIGGSKSF.YEG..

PM I   ....QLTYEKLNHDL.YWQVDDLDLHFGNLTVEKA.....TAIVDSGTSSITAFTEFLNK.F
PM II  ....PLTYEKLNHDL.YWQITLDAHVGNIMLEKA.....NCIVDSGTSAITVTDPLNKM.L
HAP    ....PLNYEKLNHDL.MWQVDDLDLHFGNVSSKA.....NVILDSATSVITVTEFFNQFV
PM IV  ....PLTYEKLNHDL.YWQIDLDLHFGKYVMQKA.....NAVDSGTSTITAFTEFLNK.F
PM V    (44)-EDIVVQAITRK.YYYYIKIYGLDLYGTNIMDKKE.....LDMLVDSGSTTHIFENIYNGIN
PM VI   ....SIEFPFVISLI.YWEINLLDIQLSHKNLFLCE.....SKKCRAAINTGSSLIIGFTPIQPL
PM VII  ....EIMVWTPVST.MGYWTIQIMGTIRKEYVNNHFEENKEEB-(11)-.GGKNS.IIDTQTYLYIAPKNTMEN.Y
PM VIII ....SIEFPDVTSSK.YWATQMKGLKINDVFLDVCCKNHQB.....FCQAVITGTSSTIAGTEKEGRERREY
PM IX   ....DIYMPFVVKXY.YWEIHFGLYIDHOKF.CCGVNSIV-(54)-KKKNYLFDGTSFNSVFKDEIBEYF
PM X    ....DIYMLPVVKES.YWEVKGLELYIGKERI.CC..DEES-.....YVIFDTGTSYNFMISQMKTL

PM I   EGLDVVKIPFLPLYIT....-TCN.NPKLPTLERSA.....TNVYTLFEEYLLQQIFBFG..ISLCM
PM II  QNLVDVIKVPFLPFYVT....-LCN.NSKLPTLERSA.....NGKYTLFEEYLLQHIHVBG..PGLCM
HAP    ESASVFKVPFLSLYVT....-TCG.NTKLPTLERSA.....NKVYTLFEEYLLQLEPLENIF..SALCM
PM IV  RDMNVIKVPFLPLYVT....-TCD.MDDLPTLERSA.....NNKYTLFEEYMDPLS.ID..PALCM
PM V    YYLDILCIHDMTNIYE-(44)-CWKSLLENLENLYITLS.....NNYKMIWKSSYLYKKESFW....CK
PM VI   EKN.LERDCSNKES.....LPIISVVLKN.....VEGKEITLDFMEDYIIEEGTENNNTLECM
PM VII  KDLKIN.NCDEKYN.....LPHIIFQIKSDEIKTIKGSATIEIVLTNDYVIEYVKKNNNTKECI
PM VIII E.....LTPKDYI.....VNSRIDP.....VLRSP.....CN
PM IX   RVVPSKKCDDSNIDQV.....VSSYPNTVIN.....KMPETITSSQYLVRKNM....CK
PM X    NLHSTACTEQNYKDI.....LKSYPILIKVVF.....EILIEHHEEYMLINDV.....CM

PM I   VSIFVVLNKN...TFILGD.PFMRKYFTVFDY...DNHTVGFALAKKL
PM II  LNIIGLDFVP...TFILGD.PFMRKYFTVFDY...DNHSGIALAKKNL
HAP    LNIIVFIDLEKN...TFVLDG.PFMRKYFTVFDY...DNHTVGFALAKNL
PM IV  LYILEVVIDDN...TFILGD.PFMRKYFTVFDY...EKESVGFALAKNL
PM V    GLBKQVNNKP.....ILGL.TFIFKNQVIFDL...QQNQIAFIESKCPSNLT-(67)
PM VI   FGIMPLDVPPRG.PIFIPGN.SFIRKYTIFDN...DHKLIGLIEANHNF
PM VII  LGIOFDEQSEEDNVGWTLGQ.VFLKAYTIFDK...DNLKIGFVRSKRVNLT
PM VIII FAFMFINISSNG.YLYILGQ.IFLOKYAIEEK...DNMKIGLAKSI
PM IX   PAFMEIEVSSEYG.HAVILGNATFMYYYTYVRRGNNNSSYVGIKAVHTEENE-(13)
PM X    PAYMOIDVPSERN.HAXLLGSLSPMNFETVFEVVGTSRSPMVGVARAKGKN

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TRENDS in Parasitology

Figure 4 Nomenclature and sequences of *P. falciparum* plasmepsins

Predicted sequences of aspartic proteases identified from the *P. falciparum* database. Plasmepsin VI has been previously named as plasmepsin III before being changed to Plasmepsin VI later (154). Plasmepsin III was later changed to histio-aspartic protease (HAP) to avoid confusion (155). Sequences of the plasmepsins and HAP are aligned in the regions approximately corresponding to the mature enzyme sequences and the number in the brackets indicated omitted sequences. Active sites with aspartic residues are marked with an asterisk while residues with similar properties are coloured as follows; negative residues in red, positive residues in blue. Serine and threonine residues in orange, aliphatic residues in yellow, aromatic residues in purple and hydrophobic residues in gray (reviewed in (130)).

Hypothesis and aims

1. Plasmepsin VI

We hypothesize that, based on previous study in *P. berghei* model, plasmepsin VI (PMVI) is an active aspartyl protease in *P. falciparum* plays an essential role in sexual parasites. In this study, we aim to use CRISPR-Cas9 to biologically characterize PMVI in *P. falciparum* NF54 parasites, and recombinantly express PMVI protein to elucidate PMVI function and whether it is required for sexual parasites. Together, these studies will aid in determining whether targeting PMVI is a viable approach to help overcome ART-resistance, or if PMVI itself is a novel transmission-blocking antimalarial target.

2. Plasmepsin IX and X

We hypothesize that plasmepsin IX and X (PMIX and X) are actively involved in the transmission of *P. falciparum* gametocyte to mosquitoes and plays a role across the mosquito stages of infection. This project aim to use WM4 and WM382, our antimalarial with novel mechanism of action, to assess the role of PMIX and X and to ultimately determine if these aspartyl proteases are transmission-blocking targets.

Chapter 2 – Materials and Methods

Ethics statement

Use of human blood was approved by the Walter and Eliza Hall Institute of Medical Research Human Ethics committee under approval number HREC86/17.

Isolation of plasmid DNA

High purity of plasmid DNA from small scaled culture was extracted using QIAprep miniprep kit (QIAGEN GmbH, Hilden, Germany). Isolation of plasmid DNA was performed according to the standard protocol by manufacturer. Principle of this method is based on alkaline extraction and selective adsorption of plasmid DNA onto silica membrane in the presence of high salt buffer while contaminants pass through the column. Plasmid DNA was then eluted by applying sterile distilled water to the membrane and the purified plasmid DNA was collected. The concentration of DNA was determined by measuring the absorbance at 260 nm. A OD unit of double stranded DNA is equal to 50 µg/ml DNA, the ration of $A_{260/280}$ of 1.8 was considered acceptable.

Sanger-sequenced plasmids were maxi-prepped prior to transfection into parasites or baculovirus (BV)/insect cells using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen) according to the manufacturer's instructions. Briefly, a 10 mL bacterial culture in S-broth with appropriate antibiotics was established from an existing miniprep culture (100 µL) or glycerol stock scraping. This was cultured at 37°C for ~8 hours (250 rpm) then added to a 300 mL flask of S-broth containing the appropriate antibiotics. The resulting culture was cultured overnight at 37°C with shaking (250 rpm). Plasmid DNA was then isolated from E. coli cells following kit instructions. Following purification plasmid DNA was ethanol precipitated, resuspended in TE buffer (Invitrogen) and stored at -20°C until use.

Agarose gel electrophoresis

Agarose gel (1%) was prepared by dissolving 1 g of ultrapure agarose (SEAGEM) in 100 ml of TAE buffer (45 mM Tris, 45 mM acetic acid, 1 mM EDTA, pH 8.0). Agarose suspension was melted in microwave oven until completely dissolved and allowed to be cooled, Ethidium bromide (0.5 µg/ml) was added prior to pouring into a casting chamber equipped with comb. After allowing the agarose to be harden at room temperature, it was placed in the electrophoresis chamber filled with TAE buffer. DNA samples were mixed with 1/5 volume of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) and loaded in the wells. DNA markers were also loaded parallel in the same gel as markers for DNA molecular size. Electrophoresis was carried out by applying constant voltage at 80 volts until bromophenol blue migrated to about 3/4 of the gel, at which time electrophoresis was stopped. DNA patterns were visualized under UV transilluminator and the size of DNA fragment was estimated by comparing with the size of DNA markers. The results were photographed using Gel Doc software (Bio-rad laboratories).

Plasmid DNA digestion with restriction enzyme

Plasmid DNA prepared as described previously was digested with desirable restriction endonucleases for characterization the desired transformants or sub cloning into the plasmid vectors. DNA was digested with restriction enzyme using 5 U of enzyme/µg DNA in 1x buffer and the total volume should be contained less than 5% glycerol. Buffers for specific restriction enzymes were followed to the commercial recommendation. The digestion reaction was incubated at suitable temperature of each enzyme for 2 hrs or overnight. Digested DNAs were then analyzed on agarose gel electrophoresis.

Purification of DNA fragments from agarose gels

In order to purify the DNA fragments or the PCR products, DNA was first analyzed by agarose gel electrophoresis. After the target bands were confirmed and visualized by

UV transilluminator, the target bands were excised from the gel using clean razor blade. DNA was then extracted using QIA quick gel extraction kit according to manufacturer's protocol.

Cloning of guides

The pUF_Cas9 vector used in this work was previously edited to remove the selectable marker and insert a single guided RNA (sgRNA) cassette. This pUF_Cas9 vector encodes both Cas9 and sgRNA expression in the same vector. Cas9 function continues in the absence of drug selection of transfected parasites, and the vector should be gradually lost by the parasite over time.

A pair of 20 bp guide oligonucleotides were designed for guided plasmid, flanked with the 5' and 3' sequences. Flanking sequences are homologous to regions within pUf_Cas9 vector and allow cloning of guides using the InFusion system (Takara) as previously described. Oligonucleotides were synthesized by integrated DNA technologies (IDT).

100 μ M of each guided oligonucleotide (10 μ l) was added to 10 μ l of NEB buffer 3.1 (NEB) in a final reaction volume of 100 μ l and heated at 95 °C for 5 minutes to allow guides to anneal. The internal heat block containing the reaction tube was removed from the hotplate and allowed to slowly cool to room temperature overnight. Annealed guides were then cloned into *Bt*zII-digested pUF_Cas9 using the infusion method, following the manufacturer's instructions. The infusion mixture was then used to transform NEB 5 α -competent *E. coli*, from which colonies were picked for overnight culture and plasmid DNA miniprep. Correct clones were identified via restriction digest and confirmed using Sanger sequencing.

Sequences of PCR primers used for cloning

Primer Name	Purpose	F/R	Sequence
KR13	PMVI K/O 5'HR	F	GATCCCGCGGATGCCGTACTTCCATATATTCCT
KR14	PMVI K/O 5'HR	R	GATCCTTAAGCTTGTTAGGATTAAATTTTTATGGG
KR16	PMVI K/O 3'HR	F	GATCCCATGGAGGACCTTTACCAAAAATTTAAAAAG
KR18	PMVI K/O 3'HR	R	GATCCCTAGGGGCTAATCCTATACATTGATGTT
KR3	Guided RNA	F	TAAGTATATAATATTAATTTAATCCTAACAAGTCTGTTTTAGAG CTAGAA
KR4	Guided RNA	R	TTCTAGCTCTAAAACAGACTTGTTAGGATTAAATTAATATTATA TACTTA

The grey sequences on KR13, KR14, KR16, KR18 show the restriction site for enzyme SacII, AflII, NcoI, and AvrII respectively. The grey sequences on guided RNA (KR3 and KR4) represent the sequence that are complementary matched with those within parasite genome and allow the homologous flank to be integrated properly.

Construction of oligonucleotide duplexes

The synthetic oligonucleotides were designed and synthesized by chemical synthesis in form of a single-strand oligonucleotide chain. The oligonucleotide duplexes were obtained by annealing the complementary strand of 2 synthetic oligonucleotides. 200 pM of each oligonucleotide fragment in a total volume of 200 µl were heated at 65 °C

for 5 mins, allowed to cool slowly to 37 °C for 2 hr and then 4 °C overnight. The annealed fragments were kept frozen at -20 °C until use.

Ligation of DNA fragments

After digestion with appropriate restriction enzymes, digested vector and insert DNA fragment were ligated using Electro ligase. The standard ligation reaction composed of digested vector backbone and insert DNA fragment in 1:4 molar ratio, 1 U of Electro ligase (NEB, 3 U/μl), ligation buffer (30 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM DTT, and 1 mM ATP) and sterile distilled water in final volume of 11 μl. The ligation reactions were incubated at room temperature for 30 mins. Ligation reactions were then incubated at 65 °C for 15 mins in order to inactivate ligase enzyme, ligated vectors were then put on ice if to be used within a few hours or stored at -20 °C.

Preparation of *E. coli* competent cells

The competent *E. coli* cells were prepared by MgCl₂-CaCl₂ treatment. A single colony of appropriate *E. coli* strain was inoculated into 5 ml. LB with desirable antibiotics at 37 °C overnight (14 – 16 hrs). The overnight culture was used as inoculum (1% inoculum size) to inoculate 250 ml LB with appropriate antibiotics. The culture was maintained in shaker incubator at 37 °C until A₆₀₀ reached about 0.4, at which time the cell culture was harvested by centrifugation at 3,000 rpm for 5 min at 4 °C. After removing the supernatant, cell pellet was gently resuspended in 1/5 original culture volume of ice-cold 100 mM MgCl₂ and was allowed to be on ice for 1 hr. Sterile glycerol was added to the cell suspension at 15% final concentration with gentle swirling. The resulting competent cells were stored in aliquots in pre-chilled sterile microcentrifuge tubes, followed by freezing at -80 °C until use. The known concentration of standard plasmid DNA was used to test the transformation efficiency of the competent cells.

Transformation

Electrocompetent cells were thawed on ice prior to transfection. After completely

thawed, ligation reactions were added into electrocompetent cells, the volume of ligation reaction used should not exceed 5 µl reaction per 50 µl cells. Electrocompetent cells were then mixed by finger flicking before transferred into electroporation cuvette and followed by the manufacturers recommendations for electroporation (2,500 V, 200 Ω, 25 µF, 2 mm gap cuvette). Super optimal broth (SOB) media was used as recovery media in order to transferred transformed cells to culture tube and incubated for 1 hr in 37 °C shaker incubator (200-250 rpm). Following incubation, transformed cells were spread onto appropriate antibiotic selection plates and incubated overnight at 37 °C.

Characterization of recombinant clones

Isolated bacterial colonies appeared on the agar plate supplemented with antibiotics were randomly picked and grown in 5 ml of media containing appropriate antibiotics. Plasmid DNA was extracted by alkaline lysis method as described previously. Putative recombinant DNAs were characterized by digestion with suitable restriction enzymes before analyzed by agarose gel electrophoresis.

Parasite culture

Asexual blood stage parasite cultures of NF54 wild-type and transfected *P. falciparum* parasites (NF54/PM6K/O, NF54/PM6HA, NF54/PM6Knock-in) were grown in *in vitro* culture (Trager and Jensen (156)). All parasites were supplied with O+ erythrocyte (Australian red-cross bloodbank, South Melbourne, Australia) at 4% hematocrit in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 26 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 50 µg/ml hypoxanthine, 20 µg/ml gentamicin, 0.18% NaHCO₃ and 0.43% (w/v) Albumax II™ (Gibco). NF54/PM6K/O and NF54/PM6HA transfected parasite lines have human *dihydrofolate reductase* gene that provides resistance to WR99210 drug while NF54/PM6Knock-in transfected parasite has *Blasticidin-S deaminase* gene that provides resistance to Blasticidin. These parasite lines were cultured in the presence of 5nM of WR99210 and Blasticidin in order to select for resistance marker. Cultures were incubated at 37 °C in a gaseous mix of 94% N₂, 1% O₂ and 5% CO₂.

Saponin lysis of whole parasite

Parasites with approximately 5-6% parasitemia were harvested and spun down at 1500 rpm for 5 mins. Parasite pellets were resuspended in 2X pellet volume of 0.15% saponin in PBS and incubated on ice for 10 mins. This mixture was then centrifuged at 4000 rpm for 5 mins and the pellets were extracted for genomic DNA (gDNA) by using commercial kit (QIAGEN GmbH, Hilden, Germany).

Synchronization of Parasite culture

Sorbitol synchronization was used in order to select for ring stage parasite Lambros and Vanderberg (157). Parasite pellets were resuspended and incubated with 5% D-sorbitol at 37 °C for 5 mins which causes 20 hr or older parasites to lyse. After the incubation, parasites were centrifuged at 1500 rpm for 5 mins to remove sorbitol solution, parasites were resuspended with RPMI media with 2% haematocrit and returned to culture.

Magnet purification

Parasites within erythrocytes digest hemoglobin as their food source. This process ended up with hemazoin crystal, which has high affinity for magnet, as an end product. Parasites in Trophozoite and schizont stages have an accumulation of these hemazoin crystals which, in turn, make them separable from ring stage parasites and uninfected erythrocytes. Parasites are passed through commercially available MACS CS magnet column (MACS Miltenyi Biotec) to select for late stage parasites. Following a 30 ml RPMI-HEPES wash step, the column is removed and parasites are eluted in culture media and returned to standard culture condition.

Parasitophorous vacuole enclosed merozoite structures (PEMS) purification

Late stage segmented schizonts (approximately 44-48 hrs) were incubated in culture medium contained 10 mM of N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E64, Sigma) for 4 hrs. E64 is a cysteine protease inhibitor that prevents schizont rupture (Salmon, Oksman (158), Soni, Dhawan (159)). Following incubation, PEMS were harvested by centrifugation at 3000 rpm for 5 mins and PEMS were washed again with RPMI media to get rid of remaining E64. After washing, the pellets were collected for transfection.

PEMS transfection

PEMS pellets were resuspended by commercially available nucleofector solution and transfected via nucleofection kit (Lonza). Once transfected, RPMI media were used to transfer the transfection mix to uninfected red blood cell pellet. This transfection mix was then put into incubator shaker at 37 °C for 20 mins at 1100 rpm. After 20 mins mark, transfection mix was resuspended with RPMI media with 3.5% haematocrit and returned to standard culture condition.

Percoll purification of late stage infected erythrocytes

13.3% sorbitol was prepared by dissolving 3.72 g of sorbitol in 28 ml of PBS. The 13.3% sorbitol was then mixed together with 10 ml 10X RPMI and 90 ml percoll solution in order to make 70% percoll solution. 70% percoll solution then filtered and made 4 ml aliquots and stored at -20 °C. The 70% percoll aliquot was thawed and prewarmed in water bath before use. Once completely thawed, percoll solution was mixed using vortex mixture to ensure homogenous solution. Fully synchronized parasites were transferred to 50 ml falcon tube before spun down at 1900 rpm for 5 mins. The supernatant was then removed and the pellet was resuspended with 1 or 2 ml of culture media. Resuspended pellet was carefully layered on top of 70% percoll solution using transfer pipette, the resuspended pellet can be layered up to 2 ml per 4 ml 70% percoll solution. The falcon tube was then centrifuged for 10 min at 3500 rpm

with 5 acceleration and 0 break. Once finished, 3 distinct layers of grainy top layer (infected erythrocytes), pink middle layer (percoll solution), and dark red/brown layer (uninfected erythrocytes and ring stage parasites) were expected. Top layer was then transferred to new tube and washed with initial volume of fresh medium if needed for further culturing or washed with PBS if needed to be harvested for further experiments Kariuki, Kiaira (160), Knight and Sinden (161).

Gametocyte culturing

Parasite culture was synchronized and maintained as described earlier at 5-10% parasitemia at 4% hematocrit. Once parasitemia reached 8-10% rings, parasites were diluted to 0.65 % parasitemia in desired volume for gametocyte culture (10 ml for petri dish or 6 x 5 ml for 6-well plate). Gametocyte media was changed every day without adding more red blood cells. On day 4, the smear was made from parasite culture in order to observe gametocyte development, crash of asexual stages and early gametocytes should be able to be observed during this period and medium volume was increased (e.g., from 5 ml culture to 7.5 ml). Gametocyte media was continued to be changed every day at 37 °C on a slide warmer within the biosafety cabinet until day 16 or 17. With this method, at least 3% gametocytemia should be able to be obtained.

Preparation of feeding apparatus

Parafilm was stretched by hand in both the x and y planes and stretched across glass water-jacket feeders of appropriate size. Where large cages of mosquitoes were fed, 2 medium size feeders, as opposed to one large feeder were used to maintain 37°C throughout the bloodmeal. Glass feeders were connected to a circulating waterbath set to 38°C via plastic tubing of appropriate diameter, using step-up/step-down connectors where necessary. Feeders were positioned on the tops of cages and held in place with adhesive tape. The waterbath was turned on at least 10 minutes prior to feeding to attain the correct temperature.

Gametocyte bloodmeal preparation

Stage V gametocytemia was determined by light microscopy examination of methanol fixed, Giemsa stained thin smear under 1,000 magnification. During the process of blood meal preparation, all reagents used were maintained at 37 °C slide warmer, surplus media was aspirated from day 17, 6-well gametocyte cultures and discarded. Remaining of culture was then resuspended and transferred to eppendorf, and pellet at 2,200 g for 5 mins at 37 °C. Supernatants were discarded and an equal volume of heat-inactivated human serum (HIHS) was added. Thoroughly mixed bloodmeals were then introduced to blood-feeders.

Midgut dissection

On day 7 after feeding with gametocyte bloodmeal, infected mosquitoes were aspirated into a cup and anesthetized by subjecting at 4 °C until immobilized. The mosquitoes were then placed in a petri dish that is kept on ice in order to keep mosquitoes anesthetized throughout the whole process. Glass slide was prepared by dropping 1X PBS on followed by transferring mosquitoes into this drop of PBS. Mosquitoes were stabbed at the thorax by needle tip and forceps were used to grabbed the last abdominal segment before pulled off the mosquito abdomen. After abdomen was pulled off, midgut was detached from mosquito thorax by forceps. Collected midguts were stained with mercurochrome for 20 mins before placed on glass slide and counted under microscope.

Hemolymph extraction

Between day 10 – 18 after feeding with gametocyte bloodmeal, infected mosquitoes were aspirated into a cup and anesthetized by subjecting at 4 °C before kept in it on ice. 29G U-100 insulin syringe was prepared by filling up with 1 ml of 1X PBS following by placed it on resting station. Mosquitoes were transferred to a glass slide under microscope before cut at the 2 segments at the bottom of abdomen with dissection scissor. PBS filled syringe was carefully inserted into the soft cuticle of mosquito thorax

until the bevel was entirely in thorax. Syringe was gently moved with mosquito attached to vertically aligned with Eppendorf tube. PBS was slowly dispensed into mosquito body in order to flushed out hemolymph (around 5 drops of PBS was enough to completely flushed out for one mosquito). Hemolymph was then centrifuged at 11,000 g for 6 mins without brake in order to concentrated sporozoite. Sporozoite was resuspended with PBS and loaded into hemocytometer, the hemocytometer was then left for at least 6 mins before counting under microscope.

Salivary gland dissection

On day 17 after feeding with gametocyte bloodmeal, infected mosquitoes were aspirated into a cup and anesthetized by subjecting at 4 °C before kept in it on ice. Mosquito was then moved to the PBS on glass slide, following by pinned by needle tip at the thorax. Mosquito's head was gently pulled off using forceps. Complete salivary glands that comprised of 2 lateral lobes and a medial lobe were located and isolated by forceps. Isolated salivary glands were collected in PBS and centrifuged at 2,000 g for 3 mins, following by slowly crushing salivary gland pellet by pestle. Sporozoite was isolated by filtered through glass wool and centrifuged at 1,200 g for 2 mins. Isolated sporozoites were then concentrated by centrifuged at 3,000 g for 2 mins and counted by hemocytometer.

Transmission blocking assay

Gametocyte culture was prepared as described earlier. The culture was supplied with gametocyte media every day without adding more red blood cells and maintained in 6-wells plate. On day 4, the smear was made to observe gametocyte development. Once the crash of asexual stages and early gametocytes are observed, the total medium volume was increased (e.g., from 5 ml culture to 7.5 ml). Gametocyte media was continued to changed every day at 37 °C on a slide warmer within the biosafety cabinet until day 13. Antimalarial compounds were prepared by dissolving in DMSO before diluted in culture media in order to obtain the concentration of choice (10 nM for compound 49c, 10/40/80/100 nM for compound 1, and 0.5/1/2.5/25/50 nM for

compound 2). On day 13, if gametocytes were develop properly, supplied with the gametocyte media included with anti-malarial compounds. The gametocyte media with anti-malarial compound was then continued to change every day until day 16 or 17. At least 2 - 3 % gametocytemia should be able to obtained on the feeding day. Gametocyte culture was harvested and the % stage V gametocyte was determined. % stage V gametocyte was adjusted to 0.2 % with fresh uninfected erythrocyte and heat-inactivated human serum as described earlier.

Immunofluorescence assays (IFAs) and imaging

Oocysts were isolated from infected mosquito's midgut before spun down and resuspended in fixative (4% paraformaldehyde in PBS, 0.01% glutaraldehyde), and fixed for 30 minutes at room temperature with rolling. Cells were spun down and fixative removed and replaced with permeabilization buffer (0.1% Triton X-100 in PBS), and incubated for 20 minutes at room temperature with rolling. Cells were spun down, washed once in 2% bovine serum albumin (BSA, in 1X PBS) to remove triton X-100, and blocked in 2% BSA for either 1 hr at room temperature or overnight at 4 °C with rocking. Blocking buffer was removed and primary antibodies (in blocking buffer) applied for either 2 hr at room temperature or overnight at 4 °C. Cells were spun down, washed in blocking buffer and secondary antibodies (in BSA) applied for 1-2 hrs at room temperature with rocking. Stained oocysts were then spun down, washed 3 times in 1X PBS and resuspended in PBS for mounting. Primary plasmeptin VI specific antibodies used for IFA was synthesized by Genscript (1:1,000). Secondary Alexa fluorophore-conjugated antibodies were used (goat anti-rabbit at 1: 1,000 dilution).

Coverslips were prepared for parasite mounting by cleaning using an open flame to remove dust, and a thin layer of 1% polyethyleneimine (in water) applied and allowed to dry for longer 1 hr. Resuspended stained oocysts were applied to cover slips and allowed to settle for at least 30 mins, before draining excess PBS. Coverslips were mounted on slides onto a drop of Vectorshield (Vector Laboratories) mixed with ~ 0.1

ng/ μ l 4', 6'-diamidino-2-phenylindole, DAPI (Invitrogen). Coverslip edges were sealed with nail polish and allowed to dry before imaging.

Slides were imaged using a Deltavision Elite microscope and images collected with a Coolsnap HQ2 CCD camera through an Olympus 100X objective with Softworx software. Images were assembled and analyzed utilizing imageJ/Fiji software, and Adobe Photoshop and Illustrator.

Cloning constructs for expression of plasmepsin VI protein

The pTriex 2 backbone vector was obtained from Tony Hodder and used for constructs wild-type FL *Pf* plasmepsin VI, mutated *Pf* plasmepsin VI, *Pb* plasmepsin VI, and *Pv* plasmepsin VI. The vector contains a GP67 signal sequence immediately upstream of a sequence containing Kpn I and Xho I restriction sites. It also contains N-Terminal fusion tag. The tag is comprised of (1) a Flag peptide, to enable affinity purification, (2) a Sumo domain (~ 10kDa), for solubility and increased expression levels and (3) a Tobacco etch virus (TEV) enzyme cleavage site, for removal of the fusion tag for structural studies.

Synthetic genes encoding codon-optimized full-length *Pf* plasmepsin VI sequences (with appropriate additional base insertions to render them in frame in the backbone vector) flanked by either *Kpn* I and *Xho* I sites were synthesized (Genscript) and inserts isolated from donor pUC57_Amp vectors via gel electrophoresis (ISOLATE II PCR and Gel Kit, Bioline).

The synthetic genes were inserted into the multiple cloning sites of the pTriex2 vector using 5' *Kpn* I/3' *Xho* I restriction sites. The vector was transformed into (*E. coli* HST08 strain) and the incorporation of the *plasmepsin VI* genes verified by restriction digestion and confirmed to be in frame by Sanger sequencing of the 5' and 3' ends of the coding region. Both *WT* and *Mut* genes of plasmepsin VI were independently transfected into baculovirus using the Oxford FlashBAC system as per the manufacturers instructions.

Culture of Sf21 insect cells

Sf21 insect cells were routinely cultured in insect-XPRESS Protein-free insect cell medium with L-glutamine (Lonza) and sub-cultured at least twice weekly to a minimum of 0.3×10^6 cells/ml. Both baculovirus-infected and uninfected cells were culture in either 500 ml, 1 litre or 2 litres schott bottles with caps loosened and covered in foil in a 28 °C shaking incubator (130 rpm).

Insect cell transfection and baculovirus stock preparation

The flashback system was used to generate recombinant baculoviruses for use in this study. Prior to transfection, Sf21 cells (0.75×10^6 or 1×10^6 cells in 1 ml of media) were plated in 6 well plates and left to adhere. Sterile-filtered plasmid (250 ng) in 2.5 μ l was mixed with 100 μ l of sterile water, 2.5 μ l of flashback baculovirus DNA (~ 20 ng/ μ l, Mirus Bio) and 8 μ l of Cellfectin II (Invitrogen) and incubated at room temperature for 15 minutes. This mixture was added to one well of Sf21 cells at $\sim 50\%$ confluency and incubated overnight at 37 °C. The following day, 2 ml of media was added to each well and plates incubated for a further 4 days. This transfection step begins passage 1 (P1). At day 5, supernatant containing recombinant baculovirus was filtered (0.22 μ m), added to 50 ml. of fresh Sf21 cells (0.5×10^6 cells/ml) and incubated for a further 3 days, beginning passage 2 (P2). Following this, cells were spun down (3,000 g for 5 minutes), supernatant filtered, and 10 ml used to infect 100 ml of fresh Sf21 cells (0.5×10^6 cells/ml). 3 days later, this passage 3 (P3); or passage 2b (P2b) if amplification of virus is needed, supernatant was isolated as per P2. At all steps, remaining baculovirus-containing supernatants was stored in foli-wrapped tubes at 4 °C.

At P2, P2b, and P3 samples of supernatants were taken to confirm protein expression via western blot. Samples were run in both reducing and non-reducing sample buffers (NUPage LDS Sample buffer (4X), Invitrogen, with 2-mercaptoethanol, 2ME added for reducing buffer) as per general western blot protocol and detected using an anti-FLAG antibody and HRP-linked secondary antibody.

Western blot analysis

In this study, all proteins were separated using 4-12% Bis-Tris reducing polyacrylamide gels (Life Technologies, 1 hr at 165 V) and transferred to nitrocellulose membranes (1 hr at 105V). Membranes were blocked with 5% skim milk in 1X phosphate-buffered saline with 0.1% tween 20 (PBST) for either 1 hr at room temperature or overnight at 4 °C with rolling. Primary antibodies were diluted in blocking buffer (1 x 15 min). Horseradish peroxidase (HRP)-linked secondary antibodies were diluted in blocking buffer and incubated with membranes for 30 minutes at 4 °C with rolling. Membranes were then washed either 3 x 10 min at room temperature or overnight at 4 °C in PBST with rolling. Antibody binding was detected after incubation of membranes with chemiluminescent substrate (Supersignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) and exposure to autoradiography film. Primary antibodies used include rabbit anti-FLAG (Sigma-aldrich; 1: 1,000). Precision Plus Protein Dual Color standards (Bio-rad) was used as a standard marker.

Large scale production and purification of recombinant *P. falciparum* plasmepsin VI

Sterile filtered P3 baculovirus-infected supernatant (15 ml) was used to infect 1 litre of Sf21 cells, and cells incubated for 3 days as above. Passage 4 (P4) supernatants were centrifuged at 6,000 g for 15 minutes, and sterile-filtered. Samples were incubated with affinity Anti-FLAG M2 resin bead (sigma-aldrich) overnight at 4 °C prior to column purification. Protein-bound beads were added to a column and washed extensively with 40 column volume of Tris buffered saline (TBS) (20 mM Tris pH 7.5, 150 mM NaCl). The column was then filled with 3 – 5 column volume of competitive elution buffer (FLAG peptide (Sigma-aldrich) diluted 1/100 in TBS) and left for 30 mins. After 30 mins mark, eluate was collected in Eppendorf and stored at 4 °C. The column was then washed with 10 column volume of TBS, 5 column volume of Glycine (0.1 M glycine HCl, pH 3.5 in 1 M Tris, pH 8.0), and 10 column volume of TBS, respectively. The anti-FLAG M2 resin was resuspended with TBS before transferred to breakthrough and kept at 4 °C while column was recycled by filling with TBS and

stored at 4 °C until next round of purification. Presence of protein in fractions were confirmed using non-reducing and reducing SDS-PAGE as mentioned above.

Time of drug killing

3D7 parasite culture was synchronised using 5% sorbitol (Sigma) twice at 46 hour intervals then again when the culture was a mix of late schizonts and early rings. Triplicate 10ml cultures containing either 80nM WM4, 5nM WM382 or DMSO (Sigma) vehicle control were set up at 3% rings and 4% hematocrit. The parasitemia of each culture was quantitated every 8 hr for 48 hr by collecting 50 µl samples for counting by flow cytometry. The developmental stage of the parasites was confirmed at each time point by microscopic examination of Giemsa stained thin blood films. Media and compound were replaced at the 32 hr time point.

Parasitemia determination by flow cytometry

50 µl of parasite culture was fixed at room temperature for 30 min with 50 µl of 0.25% glutaraldehyde (ProSciTech) diluted in PBS. Following centrifugation at 1200 rpm for 1 minute, supernatants were discarded and parasites were stained with 50 µl of 5X SYBR Green (Invitrogen) diluted in PBS. 50,000 cells were counted by flow cytometry using a Cell Lab Quanta SC – MPL Flow Cytometer (Beckman Coulter). Growth was expressed as a percentage of the parasitemia and all samples were tested in triplicate.

***P. falciparum* schizont egress inhibition assay**

Synchronized trophozoites (3D7) were treated with compounds WM4 or WM382 at various concentrations, or vehicle control (DMSO) and allowed to develop until late schizonts and early ring stage parasites were observed in the control plates. Parasites purified by magnet separation (MACS; Miltenyi Biotec) were eluted into complete RPMI containing 1 nM Compound 1 (C1) to prevent further egress and synchronize egress to within a 5 min window (151). When schizont preparations were fully mature, the C1 was washed out with warm complete RPMI-Hepes medium and schizonts

transferred to a microscopy chamber for live video time lapse (1 frame per 0.5 sec) imaging under DIC conditions using a Zeiss Live Cell Axio observer microscope with x63 oil immersion objective and Zen Blue software used for image capture. Total parasite numbers and egress events were calculated over 5 min window and expressed as average $\pm$ SD of 3 replicate experiments.

Peters' 4-day suppressive test against *P. berghei* infection in mice

Male Swiss mice were infected intraperitoneally (IP) with 1×10^6 *Plasmodium berghei* ANKA parasitized red blood cells withdrawn from a previously infected donor mouse. Test compounds were prepared in a vehicle consisting of 10% DMSO/90% Solutol (5% Solutol. HS-15 in 0.9% saline). Two hours post infection, mice were treated on 4 consecutive days (q.d. regimen, once a day) with an IP dose of test compounds (WM4, WM5: 20 mpk) or chloroquine (Sigma) (10 mpk), or received an IP injection of vehicle as a control. Peripheral blood samples were taken 24 hr after administration of the last dose, and parasitemia was measured by microscopic analysis of Giemsa-stained blood smears. Parasitemia values were averages for 6 mice per group and are expressed as percent parasitemia.

Peters' 4-day suppressive test and dose ranging test against *P. berghei* infection in mice

'Donor' female Swiss mice were infected intraperitoneally (IP) with blood stage *P. berghei* parasites constitutively expressing GFP (*P. berghei* ANKA GFPcon 259cl2 (162)). 3 days later, groups of 4 'acceptor' Swiss mice were infected intravenously (IV) with 1×10^7 parasitized erythrocytes from the 'donor' mice. Two hours post infection, experimental mice (4 per cohort) were left untreated (control mice) or treated on 4 consecutive days with oral doses of the WM382 or chloroquine (10 mpk).

For the Peters' 4-day suppressive test (Fig.5.5), mice were treated orally with WM4 or WM382 for 4 days by a b.i.d. dosing regimen (twice a day) at 1) 20, 100 mpk for WM4 and 2) 1, 3, 20 mpk for WM382 per day, with the first dose given 2 hours after infection.

Peripheral blood samples were taken 12 hours after the last treatment, and parasitemia measured by flow cytometry (proportion of GFP-positive cells in 100,000 recorded events using FACSCalibur, BD) and microscopic analysis of Giemsa-stained blood smears. Parasitemia values were averages for 4 mice per group and are expressed as percent parasitemia.

Chapter 3 – *P. falciparum* plasmepsin VI is required for parasite development in the mosquito stage

Introduction

The aspartic acid protease family of Plasmepsins (PM) are responsible for many crucial processes throughout the parasite life cycle and there have been many studies on the role of these enzymes in the biology of *Plasmodium* spp. (130). The plasmepsins in the asexual stage have been identified and shown to play an important role in parasite development. The function of plasmepsins in the sexual stage, on the other hand, still remains largely unknown mostly due to the complex life cycle within *Anopheles* mosquitoes and limited experimental tools.

Plasmepsin VI (PMVI) is one of the plasmepsins that are expressed only in the sexual stage life cycle. It is expected to have a crucial role during mosquito stage and should be considered as a potential target for transmission blocking strategies for malaria treatment. The function of PMVI has been previously studied in *P. berghei* using reverse genetic engineering (145). With PMVI knockout parasites, they found that there is no defect during asexual stage and gametocyte development. The PMVI knockout parasites can infect mosquitoes and form oocysts at the outer layer of infected midguts. However, the sporulation process which gives birth to thousands of sporozoites is completely absent in midguts infected with PMVI knockout parasites. Moreover, instead of normal oocyst morphology, oocysts in midguts infected with PMVI knockout parasites appear to be immature or degenerated oocysts. According to this defect, no sporozoites were detectable in the salivary glands of infected mosquitoes and, therefore, the transmission of *P. berghei* is blocked. There is little information on the function of PMVI in *P. falciparum* as yet.

In this study, we generated *P. falciparum* parasites that lack expression of PMVI by generating a line in which the *PMVI* gene has been disrupted using CRISPR-Cas9. This has allowed us to determine if PMVI is essential and to investigate potential for transmission blocking strategies in *P. falciparum*.

Results

Generation of *P. falciparum* parasites that lack expression of plasmepsin VI

In order to investigate the possible role of PMVI in the malaria parasite life cycle, we generated *P. falciparum* NF54 parasites in which *PMVI* gene was disrupted by double-crossover homologous recombination so that the protease was not expressed in either sexual and asexual stages (Fig 3.1). The double-crossover homologous recombination was introduced into the parasite genome using CRISPR-Cas9 (163, 164).

From genomic DNA of *P. falciparum*, we detected a proper PAM (protospacer adjacent motif), a short DNA sequence required for Cas nuclease to cut, at the end of the first intron. We then designed 2 pairs of primers in order to generate 2 homologous flanks used to replace the original *PMVI* gene during the repair process. Both homologous flanks were amplified from parasite genomic DNA template which was extracted from NF54 parasite culture. Both homologous flanks were analyzed by agarose gel electrophoresis (Fig 3.2A). Expected DNA bands of 633 bp and 622 bp were detected and cut out from the gel before digestion with restriction enzymes. In order to generate PMVI knockout construct (NF54 Δ PMVI), 5' and 3' homologous flanks were inserted at the N terminus and C terminus of the *dihydrofolate reductase* (*dhfr*) resistant gene cassette respectively (Fig 3.1). This provides antibiotic marker for transfectant selection and results in target gene disruption after transfection. This plasmid also contains the *ampicillin* resistant gene cassette at the end of 3' homologous flank which will serve as another antibiotic marker for transformation. Once transformed, ampicillin-resistant colonies were picked and plasmid containing NF54 Δ PMVI gene was then extracted and digested with *Sac* II and *Avr* II (Fig 3.2B). Linearized donor plasmid with NF54 Δ PMVI gene was analyzed by agarose gel electrophoresis and the band of 3,412 bp size was detected. This DNA band was then cut and purified from agarose gel directly before transfection into parasitophorous vacuole enclosed merozoite structures (PEMS) of NF54 wild-type parasite along with pUF-Cas9 plasmid, a plasmid with oligonucleotides encoding the guide sequence (gRNA) and

Cas 9 nuclease sequence included. Once transfected, guided RNA directed Cas9 nuclease to native *PMVI* gene location. Cas9 then induced double strand breaks which, later on, get repaired by homology-directed repair (HDR) pathway. NF54 Δ *PMVI* gene cassette was then used as a repair template to replace *native PMVI* gene in NF54 parasites and, therefore, disrupt *PMVI* gene function. Two *P. falciparum* NF54 Δ *PMVI* clones with the expected genotype were identified by southern blot analysis (Fig 3.2C). Genomic DNA from the parasite clones was analysed by Southern blots by digestion with *Avr* II and *Pvu* I, and a band of 5.4 kb was observed whereas the parental NF54 line showed a band of 3.3 kb. This confirmed that both homologous flanks were successfully integrated into the parasite genome.

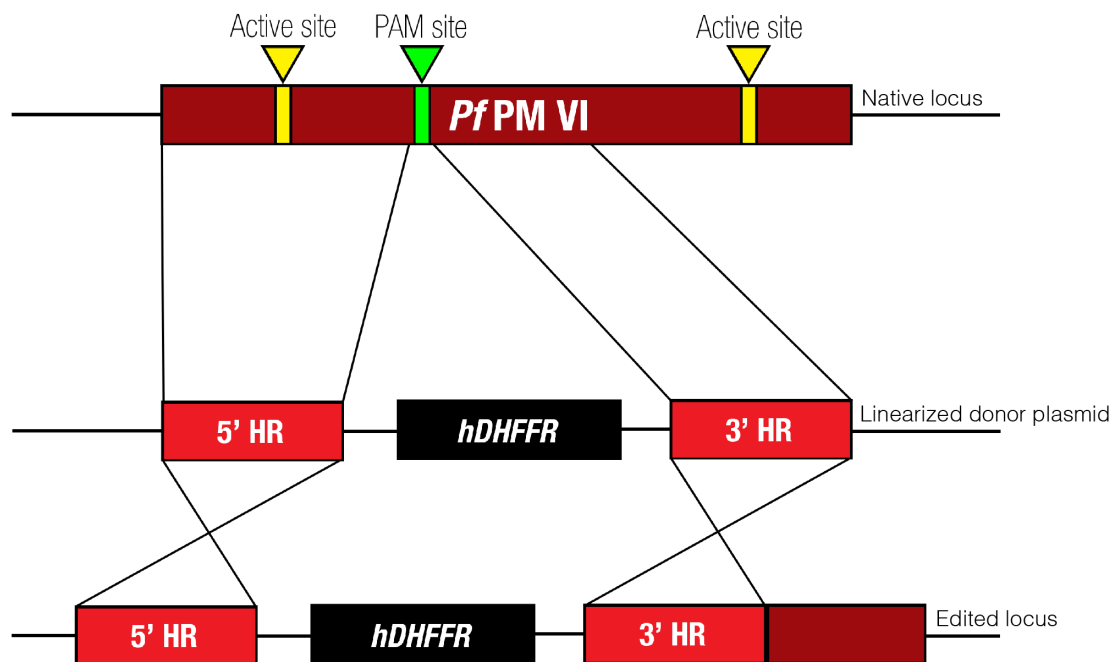


Fig. 3.1 Schematic representation of allelic exchange to genetically disrupt the *PMVI* gene in *P. falciparum*

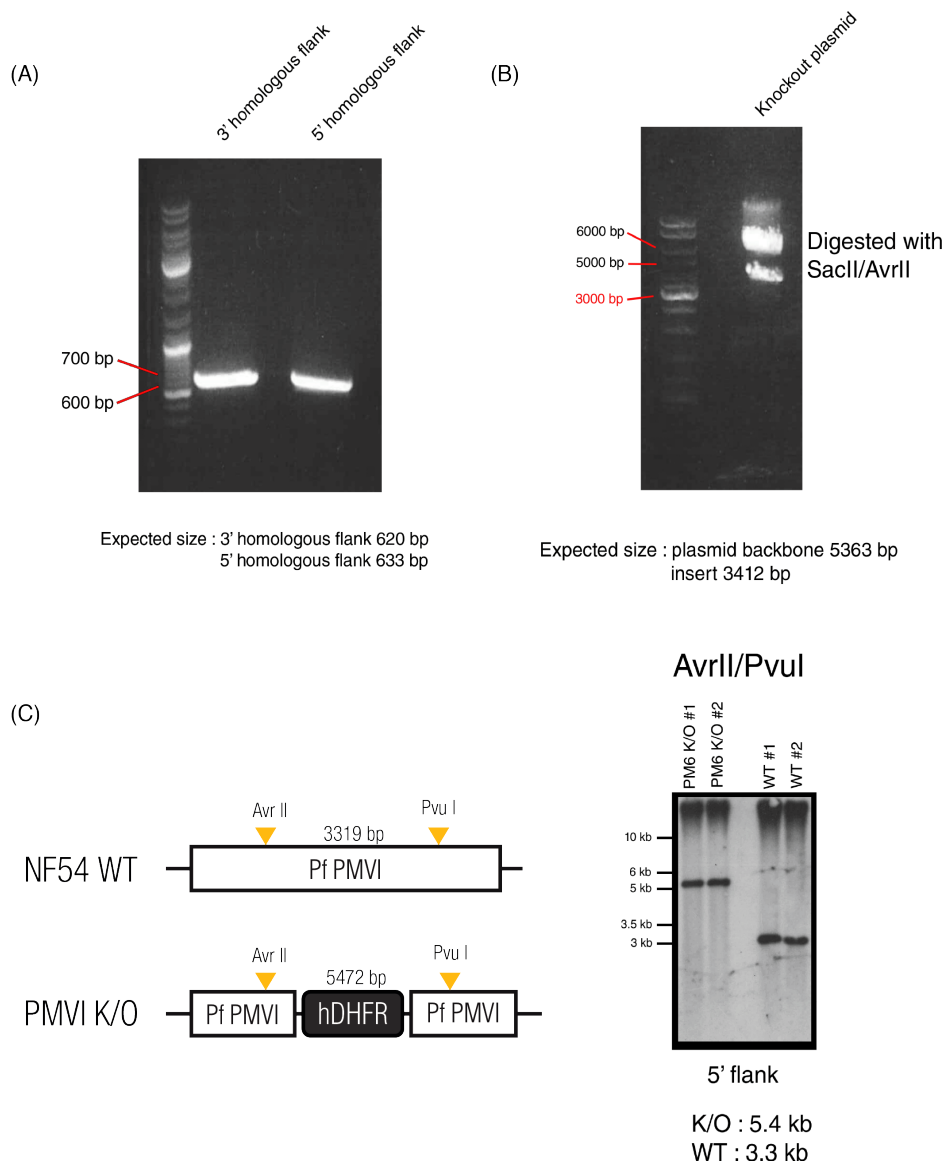


Fig. 3.2 *Pf* PMVI K/O construct (NF54 Δ PMVI) was successfully generated and integrated into *P. falciparum* genome

(A) 620 bp and 633 bp PCR product were run on 1% agarose gel in 1% TAE buffer. Gel was stained with ethidium bromide and visualized under UV light. Lane 1 represents DNA ladder while lane 2 and 3 represent PCR product of 3' homologous flank and 5' homologous flank, respectively. (B) 3,412 bp of digested product from *Pf* PMVI K/O plasmid was run on 1% agarose gel in 1% TAE buffer and visualized under UV light. (C) Genomic DNA from NF54 wild-type and *Pf* PMVI K/O parasites were digested with *Avr* II and *Pvu* I before hybridized with 5' probe.

Characterization of NF54 Δ PMVI 1 and 2 *P. falciparum* lines that lack expression of PMVI

Once NF54 Δ PMVI parasites were confirmed by southern blot to have the PMVI gene disrupted, we compared its growth rate to that of the parental NF54 line. There was no significant difference in asexual intraerythrocytic growth rate for NF54 Δ PMVI compared to NF54 wild-type consistent with this protease not being functional in the blood stage (Fig 3.3A).

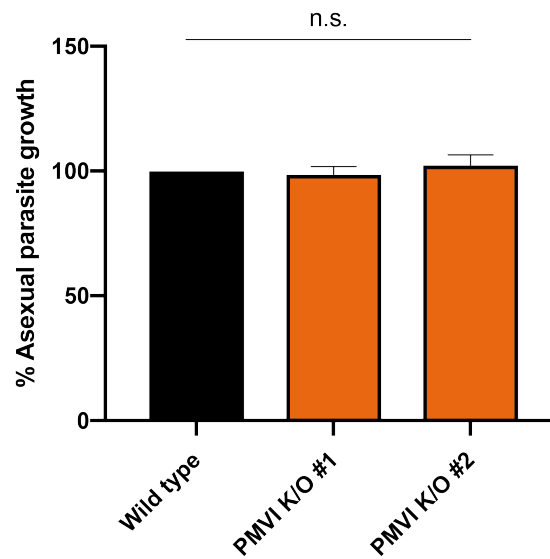
The ability of NF54 Δ PMVI parasites were then tested for their ability to develop to gametocyte stages. NF54 Δ PMVI parasites were synchronized and grown under conditions for development of gametocytes. After 17 days blood smears were analyzed in order to determine % parasitemia of stage V gametocytes. No significant difference in the number of stage V gametocytes was observed between NF54 Δ PMVI and NF54 wild-type parasites (Fig 3.3B). Therefore, it can be concluded that the function of PMVI was not required for the development of *P. falciparum* gametocytes to stage V.

We next tested the transmissibility of the NF54 Δ PMVI gametocytes to mosquitoes. Mature gametocytes from NF54 Δ PMVI and wild-type parasites were harvested and adjusted with fresh uninfected erythrocytes and heat-inactivated human serum (HIHS) to 0.2% parasitemia before being fed to *A. stephensi* mosquitoes. On day 7 after bloodmeal feeding, female mosquitoes were aspirated and midguts dissected, stained with mercurochrome and the number of oocysts counted by microscopy. Consistent with previous report in *P. berghei* (145), no significant difference in oocyst numbers relative to NF54 wild-type was observed (Fig 3.4A). Therefore, PMVI function was not required for transmission to mosquitoes and the development of oocysts in the mosquito midgut.

To determine if PMVI function was required for sporozoite egress from oocysts and traversal and invasion of salivary glands the female mosquitoes infected with NF54 Δ PMVI and wild-type parasites were grown until day 17 post feeding, aspirated

and dissected for salivary gland isolation. Salivary glands were crushed and resuspended in PBS and the number of sporozoite determined using the hemocytometer to assess transmission. Interestingly, the number of sporozoites present in the salivary glands were significantly decreased compare to NF54 wild-type (Fig 3.4B). However, there were a small number of sporozoite in the NF54 Δ PMVI salivary glands, in contrast to previous studies with the murine malaria species, *P. berghei* in which they observed no parasites (145). We also analyzed the hemolymph from infected mosquitoes every 2 days from day 10 to 18 post-feeding in order to determine if the defect was in sporozoite development process or its motility. The results showed that the number of sporozoites in hemolymph was significantly decreased in NF54 Δ PMVI compared to NF54 wild-type parasites (Fig 3.5). This indicated that PMVI function was required in *P. falciparum* for sporozoite development.

(A)



(B)

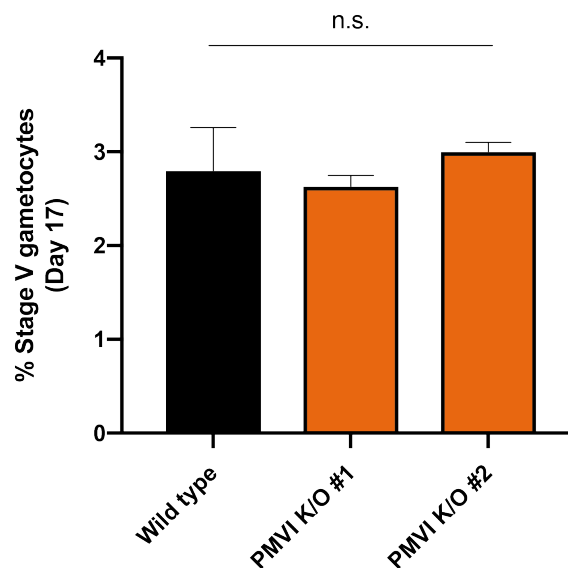


Fig. 3.3 Asexual stage and gametocyte development are not affected by the absence of PMVI function

(A) The asexual growth rate of NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites after 48 hr was not significantly different. (B) Percentage of stage V gametocyte observed in NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites on day 17 after initiation of gametogenesis was not significantly different. Experiment were done in triplicate, all data are the mean and standard error of the mean. Mann-Whitney U test was used to determine the significance of this data. KO#1 and #2 are independently derived KOs.

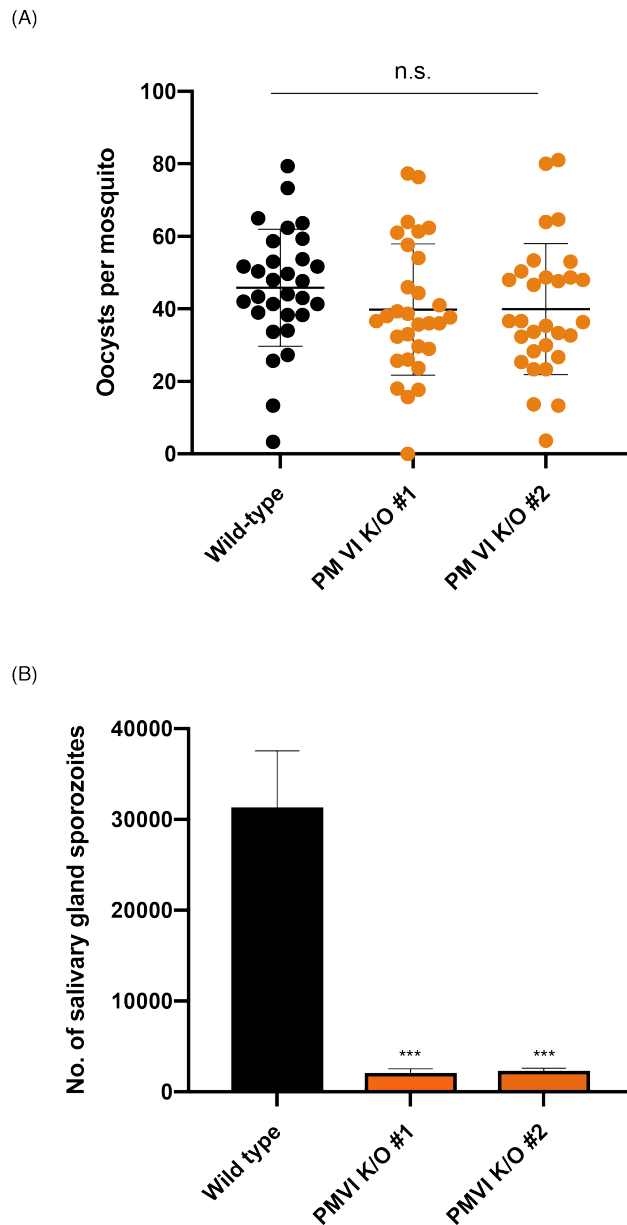


Fig. 3.4 PMVI plays an important role in transmission of *P. falciparum* to mosquitoes

(A) Number of mosquito midgut oocysts produced by NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites on day 7 post bloodmeal was not significantly different. (B) Number of salivary gland sporozoites per mosquito isolated on day 17 post bloodmeal in NF54 Δ PMVI (PMVI K/O) parasites was significantly decreased compared to NF54 wild-type parasites (** $P < 0.0001$). Experiment were done in triplicate, all data are the mean and standard error of the mean. Mann-Whitney U test was used to determine the significance of this data. KO#1 and #2 are independently derived KOs.

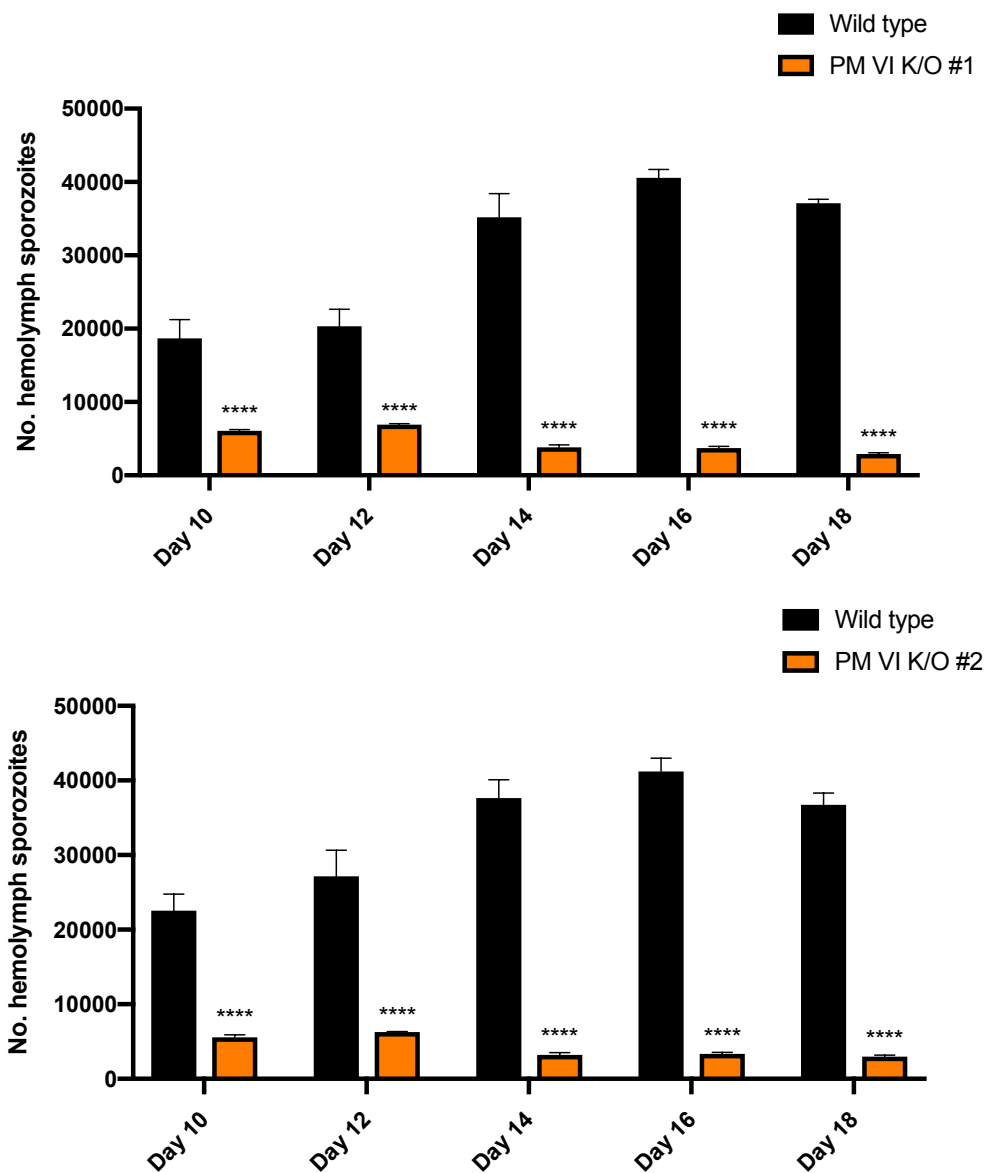


Fig. 3.5 Absence of PMVI directly effects sporozoite development within oocyst

Number of hemolymph sporozoites per mosquito of NF54 Δ PMVI (PMVI K/O) parasites isolated on day 10, 12, 14, 16, 18 post blood meal are significantly different compared to NF54 wild-type parasites (**** $P < 0.0001$). Experiment were done in triplicate, all data are the mean and standard error of the mean. Mann-Whitney U test was used to determine the significance of this data. KO#1 and #2 are independently derived KOs.

Complementation of PMVI function in the NF54 Δ PMVI parasites

In order to confirm that PMVI function is required for *P. falciparum* development in the mosquito, we designed a Crispr/Cas9 vector that when integrated complement the loss-of-function mutation. This was done by designing a plasmid construct that contained the *PMVI* gene sequence with all introns deleted (Fig 3.6). 5' and 3' homologous flanks were transferred from previous PMVI knockout construct (NF54 Δ PMVI) as these flanks were used as repair template to introduce synthetic PMVI into NF54 Δ PMVI (PMVI K/O) parasites.

Additionally, the codon sequence of the gene sequence region, not used for homologous recombination, were changed so that they were not homologous to the endogenous sequence but still encoded the same protein. This was flanked by a 5' and 3' homologous sequence (Fig 3.7). The synthetic *PMVI* recodoned sequence was ligated into plasmid as previously described before transformed into Stellar competent cells. The positive colonies were screened with ampicillin resistance trait and picked randomly for digestion check before transfected into *Pf* PMVI K/O parasite. The linearized plasmid was transfected into the NF54 Δ PMVI strain of *P. falciparum* together with the pUF-Cas 9 plasmid. After homology-directed repair (HDR) process, synthetic *PMVI* recodoned sequence was replaced between 5' and 3' homologous flanks along with blasticidin resistant cassette (Fig 3.6). Plasmepsin VI knock-in parasites (NF54 Δ PMVI/comp) were selected with blasticidin and analysed by Southern blots. This confirmed that the expected homologous recombination event had occurred to integrate the construct into the disrupted *PMVI* gene (Fig 3.8). Digestion with *Avr* II and *Ava* I, a band of 6.8 kb in the NF54 Δ PMVI/comp cloned line was replaced by a 3.3 kb band in the NF54 Δ PMVI parental parasite line. This confirmed that the synthetic *PMVI* gene was successfully integrated into the parasite genome in a way that should allow it to be transcribed and expressed.

The NF54 Δ PMVI/comp (PMVI knock-in) parasites line was compared to NF54 to determine if blood stages and gametocytes had a growth or development defect. As

expected, there was no significant difference in the asexual intraerythrocytic growth rate and gametocytogenesis compared to NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites (Fig 3.9A, B). The stage V gametocytes from each parasite line were then fed to female *A. stephensi* mosquitoes and the number of oocysts within midguts was determined using microscopy on day 7 post-bloodmeal. The result was consistent with that observed for the NF54 Δ PMVI (PMVI K/O) and NF54 parasites (Fig 3.10A). However, for salivary glands isolated from mosquitoes on day 17 post-feeding, we detected an increased level of sporozoites in NF54 Δ PMVI/comp (PMVI knock-in) parasites compared to NF54 Δ PMVI (PMVI K/O) (Fig 3.10B). Additionally, we determined the number of sporozoites in hemolymph from day 10 to 18 after feeding and this showed that the number of sporozoites released from oocysts was fully restored to wild type levels in the NF54 Δ PMVI/comp (PMVI knock-in) parasites (Fig 3.11). These data suggest that we have complemented the function of PMVI in the NF54 Δ PMVI/comp (PMVI knock-in) parasites line and that PMVI is essential for transmission of *P. falciparum* through mosquitoes.

In order to confirm expression of *Pf* PMVI in oocysts we used specific antibodies to determine its localization and expression in oocysts. According to the PMVI transcription profile (PlasmoDB), we prepared gametocyte cultures from NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites. Gametocytes were fed to female mosquitoes and the midgut isolated for immunofluorescence antibody assays (IFA). Oocysts were isolated from infected mosquito's midgut before spun down and resuspended in fixative before permeabilized with Triton X-100. Expression of PMVI was then stained with primary PM VI specific antibodies. We detected a diffuse intracellular expression pattern using the PMVI monoclonal antibody within oocysts of NF54 wild-type parasites (Fig 3.12). In contrast, no immunofluorescence was observed in NF54 Δ PMVI (PMVI K/O) parasites consistent with lack of expression of the protein due to disruption of the gene_(Fig 3.12). This confirms that PMVI is expressed in *P. falciparum* oocysts and this was consistent with previous observations in *P. berghei* (165).

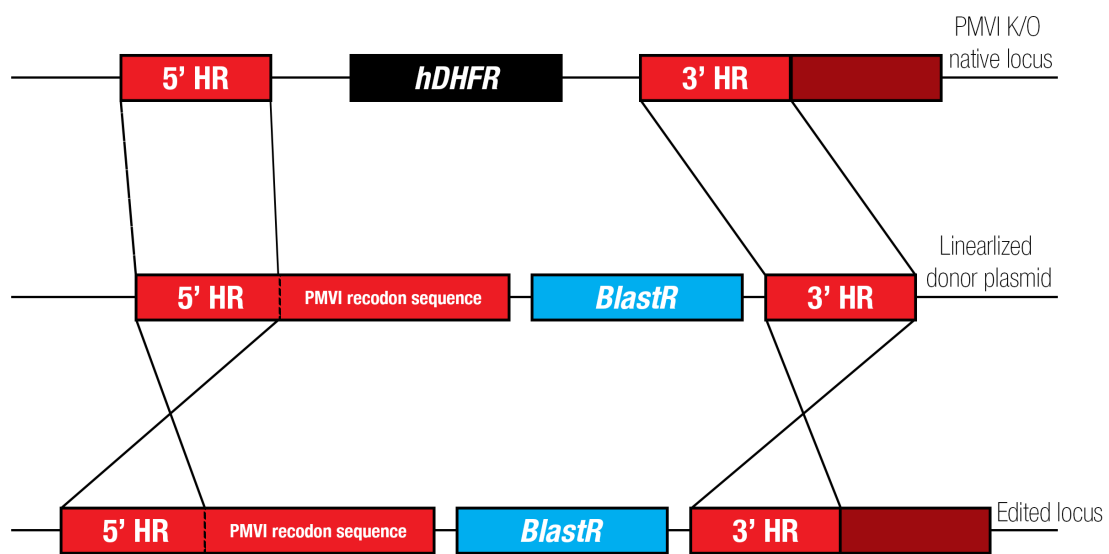


Fig. 3.6 Schematic representation of allelic exchange to genetically compensate plasmepsin VI function

ATGCCGTA CTTCATATATTCCTATACATTTTAAATTTTTTGTGTTCTTGTTTCATATATGTCC
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 TGTATTATCATATATCCAAGAAGATTTATTAAATTTTCATAATAGTCAATTTATTGCTGATA
 TAGGGGTAGGAAATCCACCTCAAGTATTTAAGGTGGTTTTTGATACAGGGTCCAGTAATCTA
 GCAATCCCATCAACGAAATGTATCAAGTAAAAAATATAAAATATGGGAGAATGATATAAGCT
 CATTTCTCAATTTCTTGCACTTCATAAAAAACATTTATTGGAAGGATGTTTATATGTATATA
 CATCTTGTTGTTACATATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACATATACATATATAA
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 CAACCCGAACAAAAGCCGTACCTTTACCAAAAACCTGAAAAACAATCAAGAGAGCGTGTACA
 CCTATATTCAGTATGGCACCGGCACCAGCATTTCTGGAACAGAGCTATGATGATGTTTATCTG
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 TAGCGATCTGCCGTTTGATGGTATTGTTGGTCTGGGTTTTAGTGATCCGGATTTTCGTAGCC
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 GATTATATCATCGAAGAAGGCGATACCGAAAACAATACCCTGGAATGTGTTATTGGTATTAT
 GCCGCTGGATGTTCCGCCTCCGCGTGGTCCGATTTTTATCTTTGGTAATAGCTTCATCCGCA
 AATACTACACCATCTTCGATAACGATCATAAACTGATTGGTCTGATCGAAGCCAACCACAAC
 TTTTAA

Fig. 3.7 Sequence of synthetic Plasmepsin VI

All introns except the one included in the 5' homologous flank (highlighted in grey) were removed. The remaining exons were replaced with a recodoned sequence (highlighted in cyan).

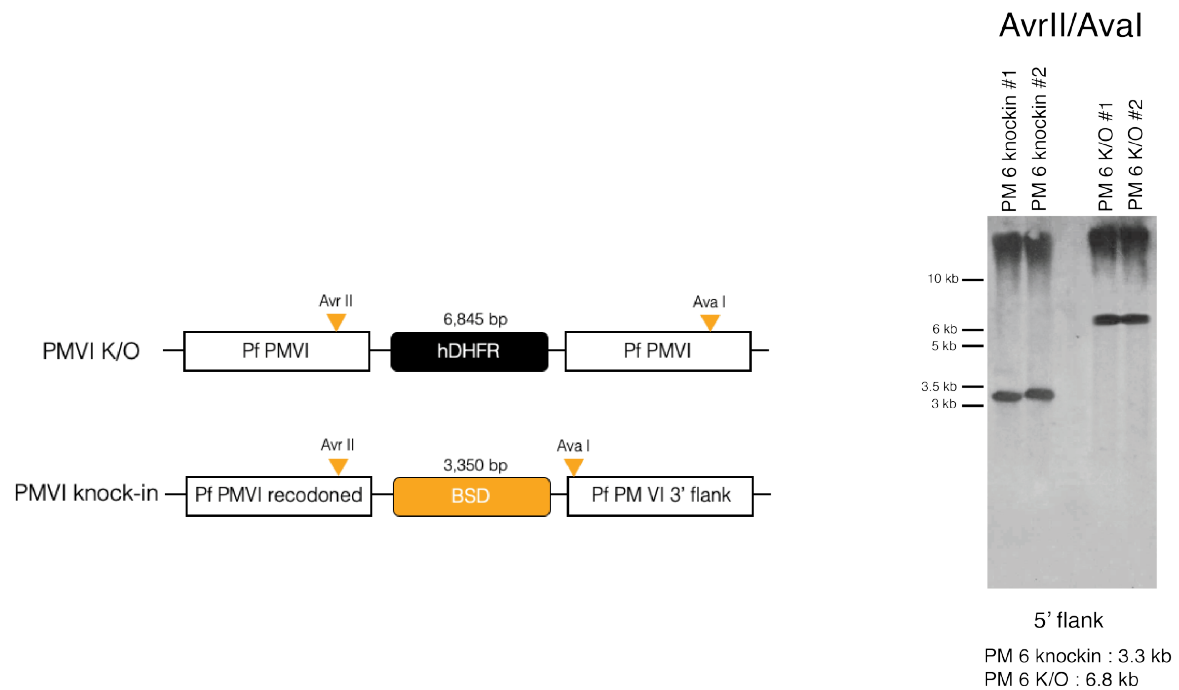


Fig. 3.8 NF54 Δ PMVI/comp (PMVI knock-in) construct was successfully generated and integrated into NF54 Δ PMVI (PMVI K/O) parasite genome

Genomic DNA from NF54 Δ PMVI/comp (PMVI knock-in) and NF54 Δ PMVI (PMVI K/O) parasites were digested with *Avr* II and *Ava* I before hybridized with 5' probe.

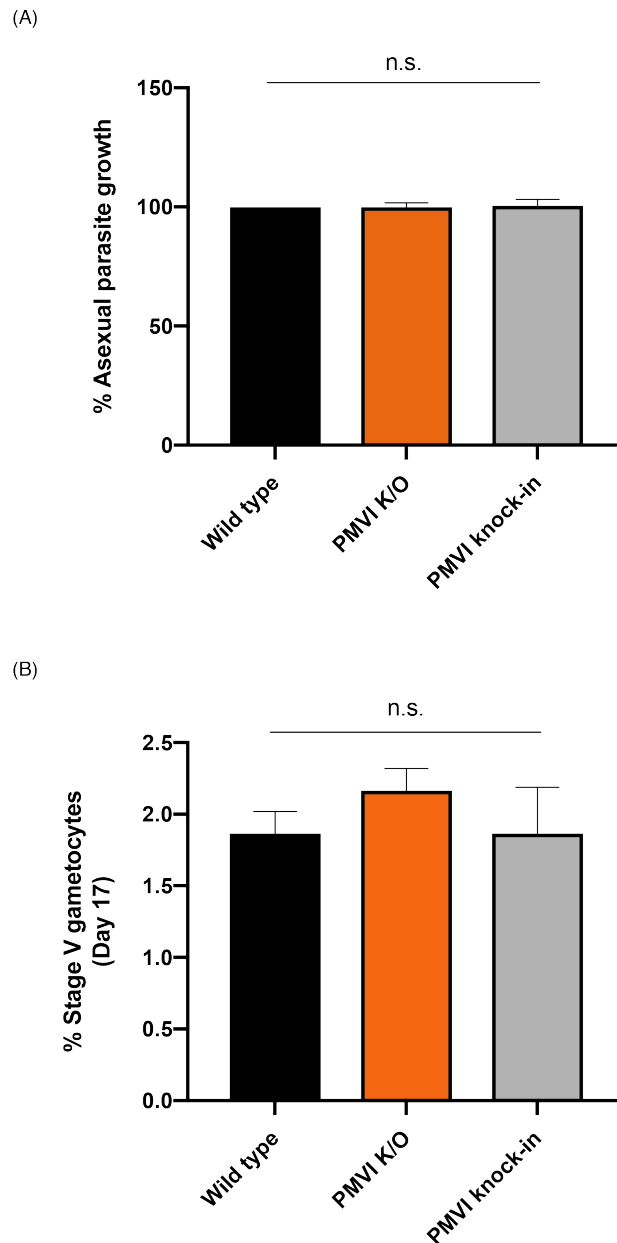


Fig. 3.9 NF54 Δ PMVI/comp (PMVI knock-in) asexual and gametocyte parasites develop normally

(A) The asexual growth rate of NF54 wild-type, NF54 Δ PMVI (PMVI K/O), and NF54 Δ PMVI/comp (PMVI knock-in) parasites after 48 hr is not significantly different. (B) Percentage of stage V gametocyte observed in NF54 wild-type, NF54 Δ PMVI, and NF54 Δ PMVI/comp parasites on day 17 after initiation of gametogenesis is not significantly different. Experiment were done in triplicate, all data are the mean and standard error of the mean.

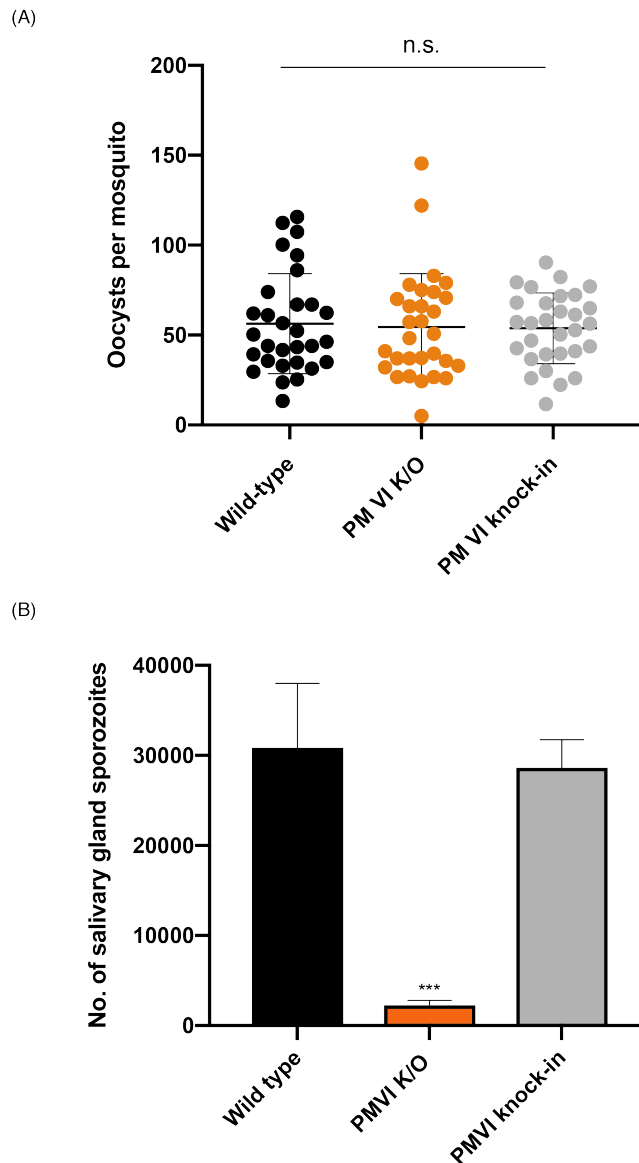


Fig. 3.10 Complementation of NF54 Δ PMVI (PMVI K/O) parasites with functional PMVI gene restores transmission through mosquitoes

(A) Number of mosquito midgut oocysts produced by NF54 wild-type, NF54 Δ PMVI (PMVI K/O), and NF54 Δ PMVI/comp (PMVI knock-in) on day 7 post bloodmeal was not significantly different. (B) Number of salivary gland sporozoites per mosquito isolated on day 17 post bloodmeal in NF54 Δ PMVI/comp (PMVI knock-in) parasites was significantly increased compared to NF54 Δ PMVI (***) $P < 0.0001$). Experiment were done in triplicate, all data are the mean and standard error of the mean.

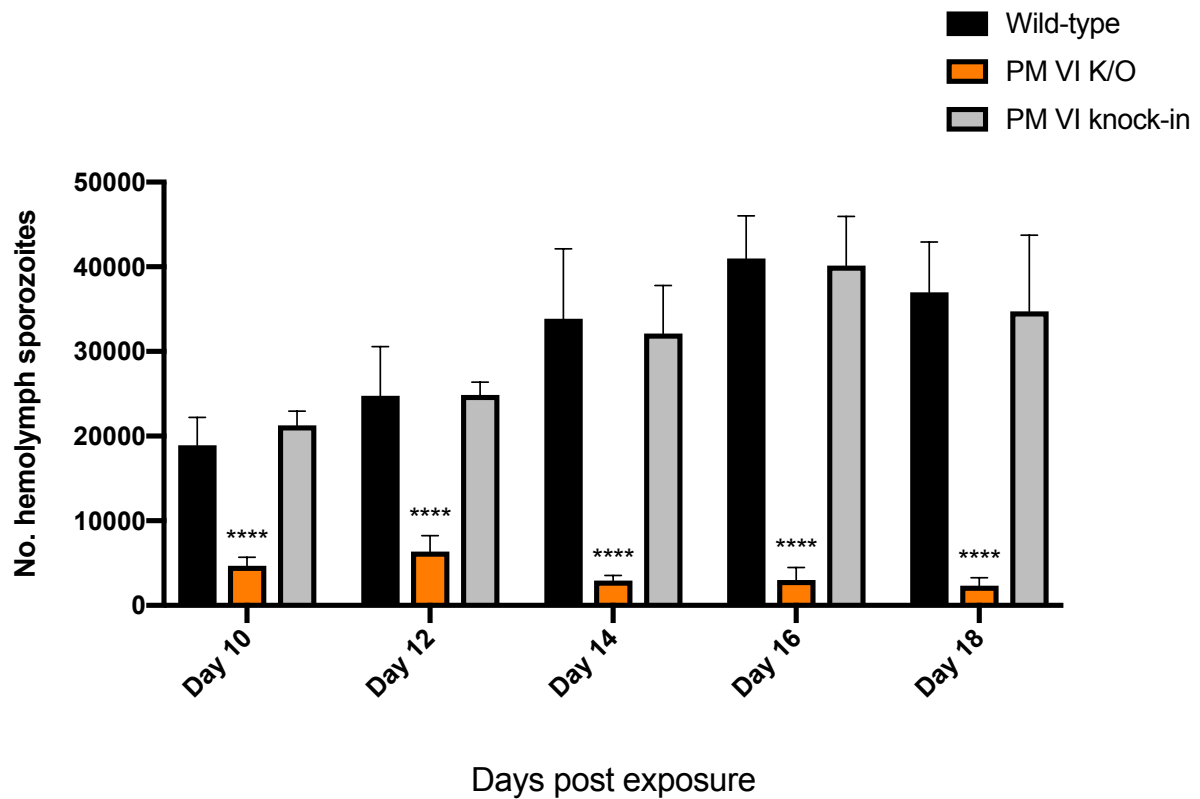


Fig. 3.11 Complementation of NF54 Δ PMVI (PMVI K/O) parasites with a functional *PMVI* gene restores normal sporozoite development

Number of hemolymph sporozoites per mosquito of NF54 Δ PMVI/comp (PMVI knock-in) parasites isolated on day 10, 12, 14, 16, 18 post blood meal are also significantly increased compared to NF54 Δ PMVI (PMVI K/O) parasites (**** $P < 0.0001$). Experiment were done in triplicate, all data are the mean and standard error of the mean.

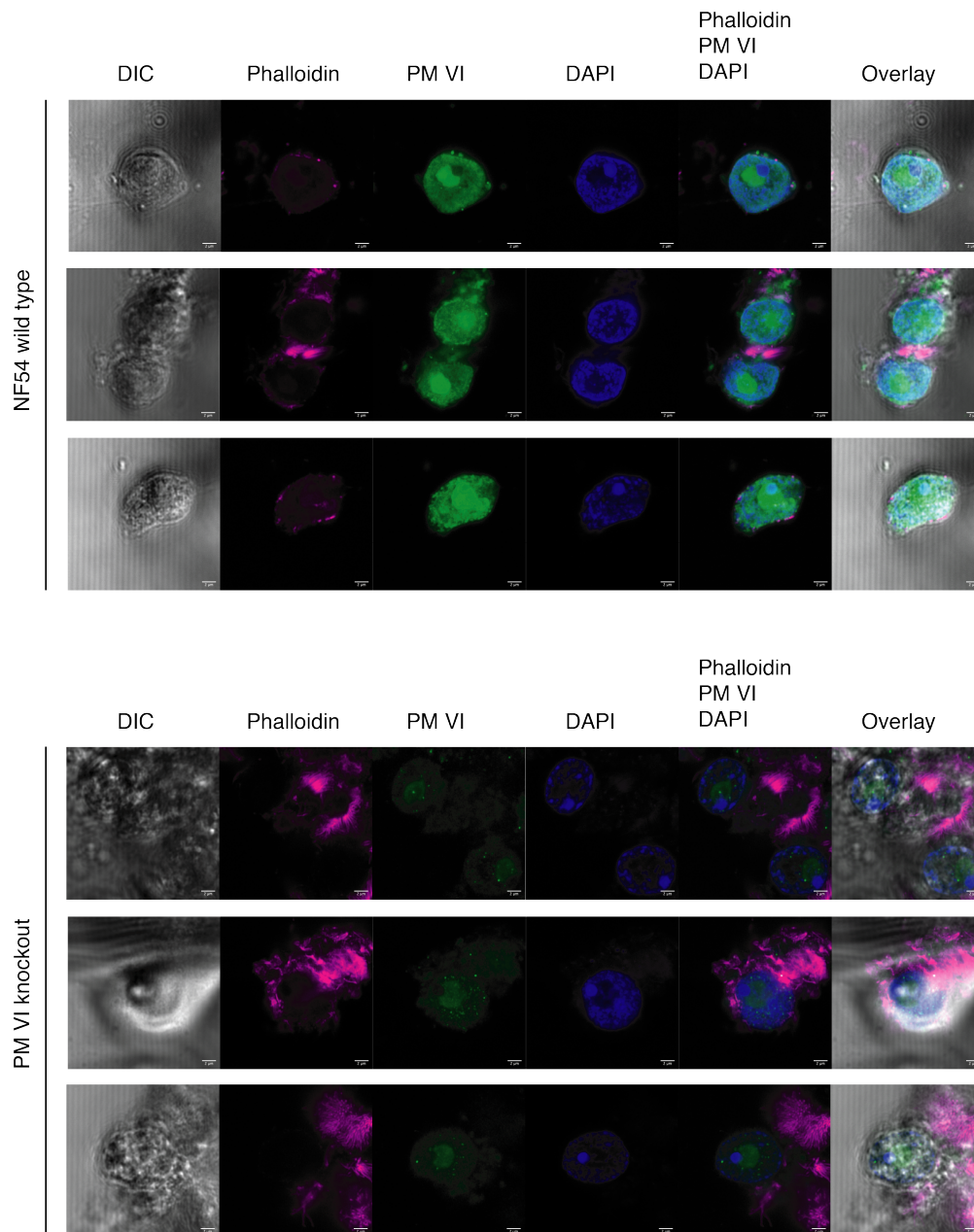


Fig. 3.12 Localization of PMVI in mosquito derived oocyst

IFAs were performed on mosquito midguts infected with NF54 wild-type and NF54 Δ PMVI (PMVI K/O) parasites. All samples are obtained on day 7 post bloodmeal. F-actin were labelled with phalloidin (magenta), the expression of PMVI were shown with GFP in green while nuclear were stained with DAPI in blue. The merged image of the three separate channels is shown in the last panel. Scale bars = 2 μ m.

Discussion

There are several plasmepsins that are crucial for parasite development. Those that are expressed during mosquito stages are potentially targets for antimalarial drug development. The reasons for this are: firstly, parasite development in mosquito stage is believed to be the only bottleneck in the malaria life cycle since it is the only invasive stage that doesn't require replication and secondly, it is the only development stage where parasite grow extracellularly, so that they are more easily exposed to drugs or host responses raised by a vaccine (72, 75). PMVI is one of the enzymes that plays an essential role for parasite transmission. Although the exact function that PMVI plays in transmission remains undefined disruption of the gene in *P. berghei* caused a defect in sporozoite development (145). However, the description and characterization of this enzyme has been incomplete and the functional role of PMVI has not been fully evaluated. Moreover, no studies have been performed in *P. falciparum* as yet. In this study, we have shown that, in *P. falciparum*, lack of PMVI expression causes a defect in sporozoite development which blocks transmission.

In order to evaluate the function of PMVI in *P. falciparum*, we generated a parasite line lacking expression of this protease using CRIPR-Cas9. As in previous reports for *P. berghei*, parasites lacking functional PMVI are able to develop and mature normally in asexual and gametocyte stages. The number of oocyst in infected midgut was also consistent with the data obtained from the previous study in *P. berghei* (145). The same defect was also observed in *P. falciparum* as for *P. berghei* with respect to the absence of sporozoites in the salivary gland of infected mosquito. However, unlike *P. berghei*, for *P. falciparum* there were a small number of sporozoites present in the salivary gland. The decreased number of sporozoites in *P. falciparum*-infected mosquito hemolymph also suggest there are defects in sporozoite development rather than sporozoite motility or invasion.

The phenotype observed for the NF54 Δ PMVI parasites was confirmed by complementation using a functional PMVI gene. The complemented parasites showed full restoration of sporozoite development and migration of numbers to the salivary

gland. Moreover, subcellular localization using a monoclonal antibody to *Pf* PMVI exhibited a diffuse intracellular staining pattern on oocyst while the same expression pattern was not detected for NF54 Δ PMVI parasites. These results are consistent with PMVI being important for sporozoite development or sporozoite release from oocysts. Although more work has to be done to address the specific function of PMVI and determine its interacting partners that are potentially involved in sporozoite development, this data presents PMVI as a potential target for transmission blocking strategies in *P. falciparum*.

It has been previously argued that defects in sporulation could be caused by the absence of expression of the male genome during the very early phase of parasite development in the mosquito. Without de novo gene transcription from male gametocytes, parasite development might depend on molecules inherited from female gametocytes as translationally repressed RNA or protein (166). This speculation is consistent with the data from genetic crosses which has found that mutant phenotype of Δ PMVI is maternally inherited. According to previous studies, the aspartic proteases are one of the best characterized members of a family of genes that are specifically transcribed but translationally repressed in the female gametocyte, are thus thought to function during meiosis in the zygote (165, 167).

Early development of most animal species is characterized by a transcriptionally silent phase that is regulated exclusively by maternally inherited components and that lasts until zygote gene activation, when a major change in gene expression pattern must occur to allow further development (168, 169). It appears that a similar switch from maternal to zygotic control of development occurs during ookinete formation. While 2 factors controlling transcription and translational repression in the female gametocyte have recently been identified (167). However, it is still unclear which triggers and factors control the specific de novo expression of proteins required in the mature ookinete.

Despite clear evidence for expression during ookinete formation (165), a lethal phenotype due to lack of PMVI can only be observed during sporulation. Sporulation

represents the end-point of several complex development cascades and would likely to be the point where earlier defects in many diverse processes would first become morphologically apparent (165). According to this, precise cellular function of PMVI still remains enigmatic as lack of PMVI could cause any upstream defects before it becomes morphologically visible in sporulation. Furthermore, it is still unclear, whether substrates of PMVI are of parasite or mosquito origin.

In contrast, PMVI homologues in other species do not have a role related to sporulation. Eimepsin, the PMVI homologue in *Eimeria tenella*, are found to be localized to the refractory body before relocalization to the apical end of sporozoites and merozoites during invasion (170). Eimepsin is suggested to be GPI-anchored, although the GPI-anchor addition sequence is missing from the gene models of the plasmodium orthologues (171). This feature suggests that Eimepsin could be involved during invasion of the sporozoite and merozoite as GPI-anchored secretory aspartic proteases in *Candida albicans* have been reported to be involved in maintenance of cell surface integrity, cell separation during buffing and host-pathogen interactions, particularly adhesion (172).

Unlike the phenotype observed from an experiment with *P. berghei* model (145), our phenotype caused by lack of PMVI in *P. falciparum* is more consistent with the idea that PMVI might be involved with a function other than sporozoite development alone. According to our findings with NF54 Δ PMVI parasites, a small amount of sporozoite can still be detected within hemolymph and salivary gland indicated that sporulation is likely to be unaffected by lack of PMVI. This data was supported by previous studies with other plasmepsins. Plasmepsin X, for example, has been demonstrated to process several downstream proteins such as PfSUB1 and MTRAP in order to participate in parasite egress process within mosquito (151). Taken together, it could be that, in our NF54 Δ PMVI parasites, parasites were able to proceed through sporulation normally and gave birth to thousands of sporozoite. However, due to the defect in egress caused by lack of PMVI, majority of sporozoites were unable to egress from oocyst and enter mosquito hemolymph which leads to significantly decreased number of sporozoite in salivary gland and failure in transmission later on.

Chapter 4 – An expression of *Pf* plasmepsin VI recombinant protein.

Introduction

In the past 10 years, malaria control and prevention have been substantially intensified with the implementation of both new vector control strategies and chemotherapies. However, the disease still remains a worldwide public health issue with *Plasmodium falciparum* causing more than 200 million cases and over 400,000 deaths per year. However, the complete elimination of malaria cannot be achieved by current methods where resistance to insecticides and drug therapies is increasing alarmingly, especially in areas of high transmission. New informed approaches for future drug development involve therapies based on molecular targets within biochemical pathways essential for parasite survival. Enzymes regulating such pathways are key targets for such approaches.

In chapter 3, we previously demonstrated that plasmepsin VI (PMVI) is essential for parasite development during the sexual stage of the parasite's lifecycle as PMVI knockout parasites (NF54 Δ PMVI) had significantly decreased number of sporozoite in hemolymph and salivary gland which results in failure in transmission. Further confirmation of the involvement of PMVI during mosquito stage was evaluated using PMVI knock-in parasites (NF54 Δ PMV//comp). As previously described, the number of sporozoite released from oocysts fully complemented in PMVI knock-in parasites (NF54 Δ PMV//comp) (Fig 3.10, 3.11) indicating that this aspartyl protease is a valuable target for transmission blocking drug development.

In this study, we attempted to express PMVI recombinant protein as it can be used for catalytic-activity testing and crystallization. These data are essential as it is required in order to determine PMVI structure and its potential inhibitors. Taken together, these data should be able to provide more in-depth information on the mechanism of action of PMVI with sexual stage of *P. falciparum* life cycle.

Results

Expression and characterization of recombinant plasmepsin VI protein

The biochemical and enzymatic properties of *Pf* plasmepsin VI were investigated using recombinant forms of the protein expressed in baculovirus infected insect cells. Synthetic genes (Geneart) encoding the expected full length mature form and a mutant with both active site Asp (amino acid residue # 118 and 315) residues replaced with Asn were cloned into a modified pTriex2 vector (Novagen). This vector enables the inserted gene product to be expressed with an N-Terminal fusion tag. The tag is comprised of (1) a Flag peptide, to enable affinity purification, (2) a Sumo domain (~10kDa), for solubility and increased expression levels and (3) a Tobacco etch virus (TEV) enzyme cleavage site, for removal of the fusion tag for structural studies. The synthetic genes were inserted into the MCS of the pTRIEX2 vector using 5' KPN1/3' XHO1 restriction sites. The vector was transformed into (*E. coli* HST08 strain) and the incorporation of the *plasmepsin VI* genes verified by restriction digestion and confirmed to be in frame by Sanger sequencing of the 5' and 3' ends of the coding region. Both *WT* and *Mut* genes of plasmepsin VI were independently transfected into baculovirus using the Oxford FlashBAC system as per the manufacturers instructions. Infective virus was cultivated in Sf21 cells (ThermoFisher) and passaged until a titer suitable for protein expression was obtained (usually passage 3, P3). This stock was titrated and levels of protein expression determined by western transfer (see methods) to establish the required infective dose for maximal protein expression.

The initial virus amplification steps (P1 and 2) are conducted in 2ml cultures containing 1×10^6 sf21 cells. This enables an infective dose of virus to be determined for virus amplification in each passage. After successfully transfected, Sf21 cell culture was harvested and centrifuged in order to obtain cell supernatants, these cell supernatants are considered as passage 1 (P1) of virus. 500 μ l of P1 virus was then used as a starter to generate passage 2 of virus which is expected to yield better protein expression. P1 infected Sf21 cells was incubated in 28 °C incubator for 4 days. After incubation, cell supernatants, indicated as P2, were harvested and the level of protein

expression was determined by western blot using anti-Flag antibody (Fig 2A). Cell supernatants taken at P2 of virus from wild-type *Pf* plasmepsin VI indicated clean expression of full-length monomers under both reducing and non-reducing conditions when detected with anti-Flag antibody (Fig 2A). Usually virus titer/protein expression levels are quite low for the P1 and P2 steps, but plasmepsin VI WT is detected as early as the P2 stage (fig 2a). However, the plasmepsin VI MUT protein could not be detected in any significant amounts throughout the virus amplification process, even though microscopic analysis of insect cell monolayer cultures infected with each recombinant baculovirus had similar levels of plaque and cell rupture associated with virus activity. This indicates that the mutated protein may not be expressed as a stable product.

At this point, all downstream work utilized wild-type full-length *Pf* plasmepsin VI protein only. We then attempted to collect P2 cell supernatants and use it as starter to generate another passage of virus (P2b) as doing this should amplify the amount of protein expressed from virus infected Sf21 cells and increase the yield of protein production. Once P2b virus was obtained, various amount of P2b were then used to make a titration of P3 in both reducing and non-reducing buffer. P3 titration was run on western blot to confirm the level of protein expression. According to the titration of 3, intensity of the band is shown to be significantly increased starting from the lane that was induced by 50 μ l of P2b virus per 1×10^6 Sf21 cells (Fig 2B). This concentration is considered as the amount that, after used for induction, will maximize protein expression while not kill infected cells rapidly.

Once a high virus titer and level of protein expression has been reached then the virus volume is scaled up in suspension culture to enable larger-scale production of the required recombinant protein (P3). The infective virus dose of P3 for cultures used for protein production was again determined in 2ml cultures containing 1×10^6 Sf21 cells. The titration from passage induced by P3 was then determined using western blot (Fig 2C). According to the titration with P3, intensity of the band is shown to be increased starting from the lane that was induced by 5 μ l of P3 virus per 1×10^6 Sf21 cells indicated that this is the proper amount for induction of 1 litre large scale *Pf* plasmepsin

VI recombinant protein expression (Fig 2C). Once identified, 5 μ l of P3 virus per 1×10^6 Sf21 cells was then used to infect 1 litre of Sf21 cell culture to generate large-scale protein expression.

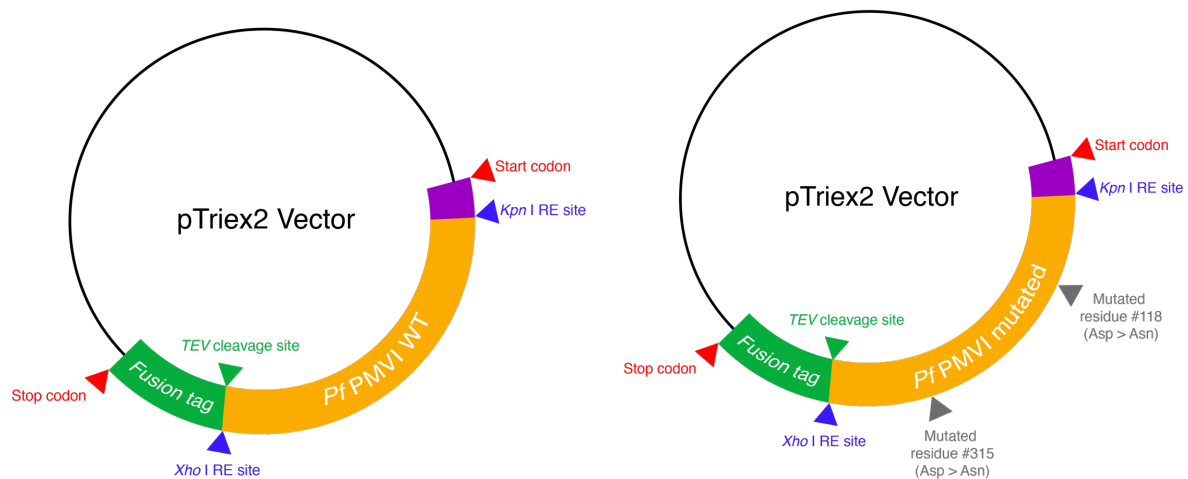


Fig. 1 Constructs used in this study.

Schematic of protein constructs to be expressed using the insect cell/baculovirus expression system. Orange depicts sequence matching that of plasmepsin VI in *P. falciparum*, whereas, in mutated construct, sequence in catalytic sites were modified (highlighted in grey) so it cannot function properly. Both wild-type and mutated sequences were integrated into pTriex2 vector via *Kpn I* and *Xho I* restriction sites (highlighted in blue). Fusion tag domain (with FLAG peptide and SUMO domain included) is highlighted in green. These sequences were used for affinity purification and improvement of protein solubility and expression. TEV cleavage sites are highlighted in green located at the end of plasmepsin VI sequence as it was used to remove fusion tag.

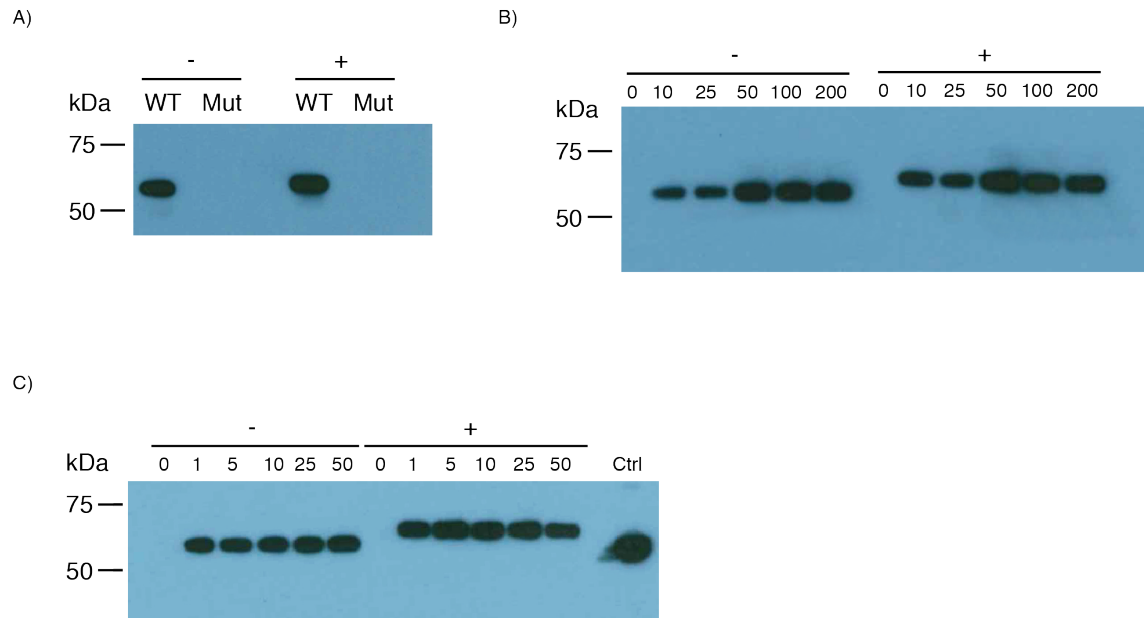


Fig. 2 Expression of *Pf* plasmepsin VI recombinant protein in baculovirus supernatant.

(A) P2 supernatants from Sf21 cells culture infected with P1 virus from wild-type *Pf* plasmepsin VI and mutated *Pf* plasmepsin VI were analyzed by western blot in order to determine the level of protein expression. (B) Titration of P3 demonstrated expression level induced by P2b virus. (C) Infective viral dose of P3 for large-scale expression was determined by western blot. +/- indicate reducing and non-reducing conditions.

Purification of *Pf* plasmepsin VI recombinant protein

Following large-scale infection of Sf21 cells with virus and amplification over several days, cell supernatants were centrifuged and concentrated to remove precipitate and cell debris. Purification of full-length *Pf* plasmepsin VI was attempted using an affinity M2 resin, as our protein contains a FLAG tag which then binds to M2 monoclonal antibody attached on each resin bead. Cell supernatants with M2 resin included were filtered by gravity flow column (Bio-rad). Due to interaction with M2 via FLAG tag, our proteins were trapped together with M2 resin on top of membrane, breakthrough was collected to be used as negative control. FLAG peptide was diluted 100 times with Tris-buffered saline (TBS) before used as competitive elution buffer. Our target proteins were eluted and fractionated to around 250 µl per fraction. Each fraction was then loaded on SDS-PAGE before analyzed using Coomassie blue staining (Fig 3A, 3B). Breakthroughs (B/T) and unpurified supernatants were also run and analyzed together with eluted samples and were considered as negative control (Fig 3A, 3B). The tiny band of proteins were detected at the correct size based on molecular sequence suggesting that this might be our target protein. However, the detected band was found to appear as doublet at slightly different molecular weights. This result is uncommon for protein express using baculovirus system, however, we are convinced that this should be caused by glycosylation of our target protein instead of expression of non-related proteins. The purification process was repeated and the eluted samples were monitored at the end of each purification step. The M2 purification was repeated until none of protein band was detected on the SDS-PAGE indicating that almost all of our proteins are already purified and isolated (Fig 3C).

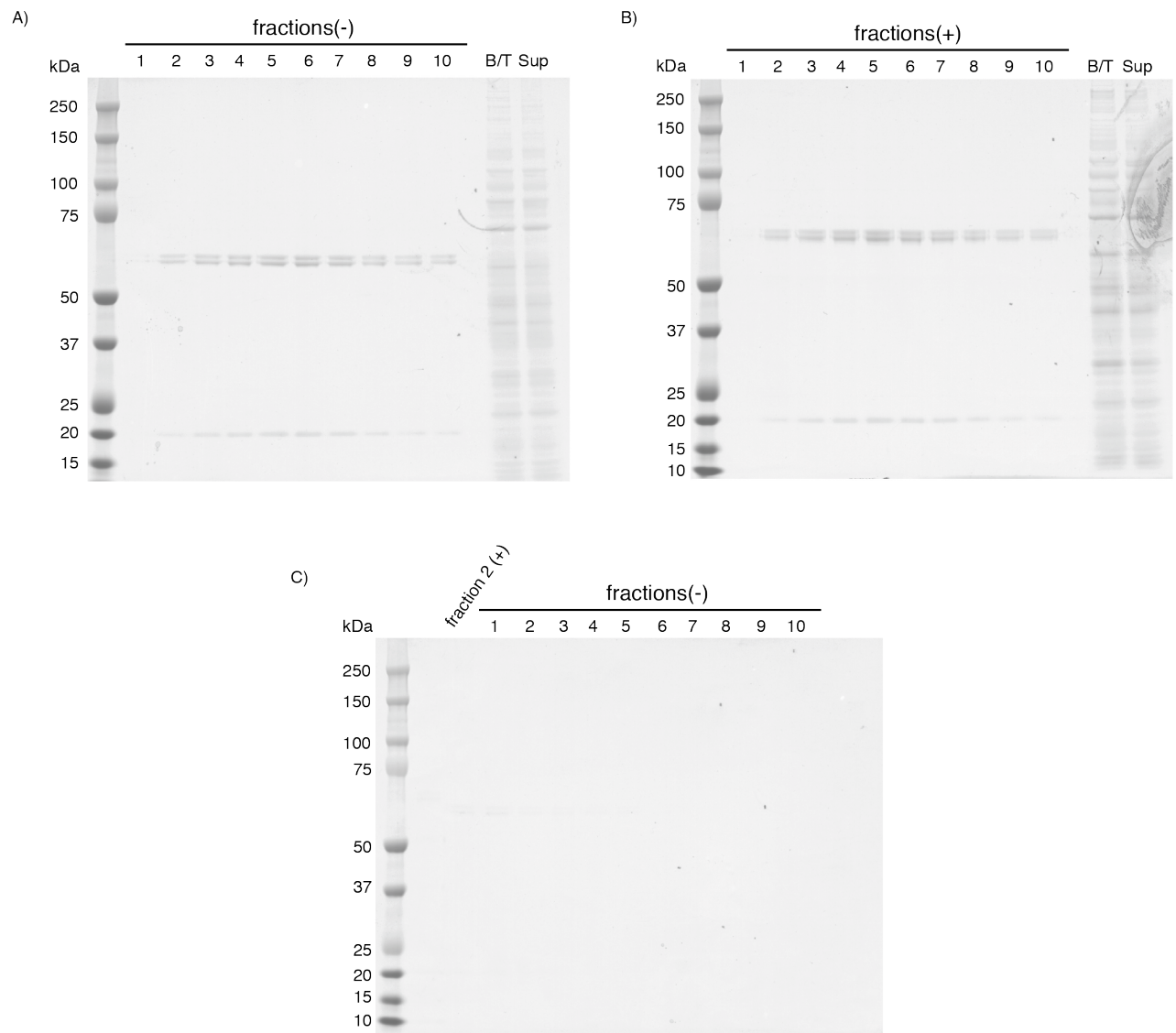


Fig. 3 Purification of wild-type *Pf* plasmepsin VI from baculovirus supernatant.

(A) Fractionated *Pf* plasmepsin VI from first round of purification analyzed in non-reducing buffer. (B) Fractionated *Pf* plasmepsin VI from first round of purification analyzed in reducing buffer. (C) Fractionated *Pf* plasmepsin VI from final purification analyzed in non-reducing buffer (only fraction 2 was analyzed in both non-reducing and reducing buffer).

Concentration of purified *Pf* plasmepsin VI recombinant protein

In order to characterize catalytic function of *Pf* plasmepsin VI recombinant protein, we attempted to concentrate purified proteins using vivaspin centrifugal column with polyethersulfone (PES) membrane (molecular weight cutoff 30 kDa). After centrifuged, according to size differentiation, our target proteins were trapped on membrane until eluted using elution buffer as previously described in chapter 2: materials and methods. Concentrated proteins were then determined for its level of expression by SDS-PAGE (Fig 4A). Unfortunately, instead of clean expression of full-length monomers like we previously observed in our western blot results, our concentrated protein ended up with multiple bands at lower size than what we expected (Fig 4A). This data suggests that it could be caused by precipitation of our protein instead of our protein becomes solubly aggregated. However, we wanted to make sure that this issue is caused by our protein itself not the centrifugal column membrane. In order to answer this question, we attempted to change from PES centrifugal column to nitrocellulose centrifugal column and repeated the concentration process again. Protein concentrated using nitrocellulose membrane was determined for its level of expression by SDS-PAGE (Fig 4B). Surprisingly, with nitrocellulose membrane, proteins were concentrated and shown at the correct size based on molecular sequence (Fig 4B). This data was consistent with what we observed earlier after purification and it was significantly improved compared to what we obtained from PES membrane (Fig 3, 4A). Amount of protein recovered from concentration process was measured by nanodrop at 280 nm Abs and we were able to obtain around 0.67 mg of protein from 1 litre expression volume. Taken together, these data suggest that our proteins were trapped and stuck with PES membrane and result in the elution of precipitation form. This event was reversed after concentrated with nitrocellulose membrane which leads to the elution of the monomeric form of our protein while its precipitation form might be stuck with the nitrocellulose membrane. Also, it is important to mention that we were able to observe a tiny smear above 150 kDa suggesting that some of our proteins could transformed into its soluble aggregated form (Fig 4B).

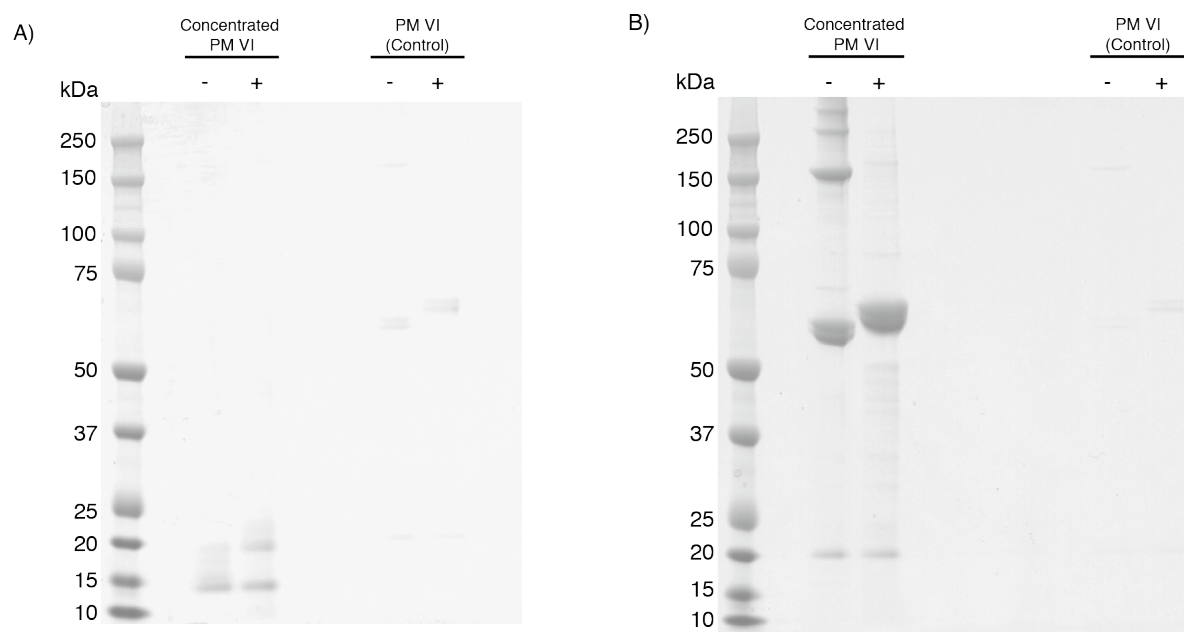


Fig. 4 Concentration of wild-type *Pf* plasmepsin VI.

(A) Wild-type *Pf* plasmepsin VI concentrated by polyethersulfone (PES) membrane.
 (B) Wild-type *Pf* plasmepsin VI concentrated by nitrocellulose membrane. +/- indicate reducing and non-reducing conditions.

Study of catalytic function of *Pf* plasmepsin VI recombinant protein

In order to characterize function of *Pf* plasmepsin VI, we attempted to identify a condition that maximizes catalytic activity of our recombinant protein. Concentrated proteins were then incubated at room temperature with acid buffer with various pH including 4, 5, 6, 7, 8 as aspartyl proteases generally prefer acidic conditions. After 24 hr. incubation, the cleavage of recombinant protein was run on SDS-PAGE before visualized using Coomassie blue staining. The intensity of protein bands were consistently detected from pH 6 to 8 (Fig 5A). However, the intensity of *Pf* plasmepsin VI was slightly decreased in sample incubated with pH 5 buffer indicating that our recombinant proteins were processed (Fig 5A). The noticeable differences in protein intensity were observed from sample incubated with pH 4 buffer as the band was barely visible suggesting that it was completely processed (Fig 5A).

Once we able to identify appropriate pH for catalytic activity, we then attempted to determine the amount of incubation time that maximize the cleavage. Concentrated proteins were incubated with acid buffer pH 4 at room temperature during the time courses of 2 hr, 4 hr, 6 hr, 8 hr, and overnight (24 hr.). Each sample was then loaded on SDS-PAGE and analyzed using Coomassie blue staining as described previously. The intensity of *Pf* plasmepsin VI was slightly decreased starting from the sample incubated for 8 hr. (Fig 5B). After 24 hr. incubation, the intensity of loaded proteins were significantly decreased compared to other samples suggesting that our proteins were processed starting from 8 hr. before reach their peak at 24 hr (Fig 5B). This pattern of cleavage is consistent to what we expect from aspartic proteases, especially plasmepsin X as its band intensity is noticeably decreased on pH 4 as well (Janni Christensen, unpublished data).

Given that the positive results obtained earlier during time courses, we are convinced that we can use this recombinant protein to study the structure and function of *Pf* plasmepsin VI. In order to obtain sample for crystallization and activity testing, we performed size-exclusion column chromatography to purify *Pf* plasmepsin VI. Based

on size calculated from molecular sequence, we expect the peak of purified proteins between fraction 41 to 49. However, the peak was detected from protein fraction 16 to 20 instead suggesting our proteins were fractioned faster than what we expect and the size of purified proteins was bigger than its original size (Fig 6A). Each protein fraction from each peak was then collected and loaded on SDS-PAGE to analyze its contents. The band of *Pf* plasmepsin VI was detected starting from fraction 19 and starting to disappear from fraction 21 (Fig 6B, 6C). In contrast, we were unable to detect our recombinant proteins between fraction 43 to 45 (Fig 6D). It also important to mention that this band of *Pf* plasmepsin VI was also found in fraction 53 but the intensity was significantly lower than those in fraction 19 to 21 (Fig 6D). Taken together, these data indicate that majority of our protein materials were bigger than their original size and fractionated faster than what we expected. We were convinced that this should be our protein as its catalytic activity was shown in previous experiment (Fig 5). The amount of protein from fraction 16 to 21 and 41 to 49 were quantified using nanodrop at 280 nm Abs. We were able to obtain around 0.27 mg of protein from fraction 16 to 21 while the amount of protein obtained from fraction 41 to 49 was unable to read from the machine suggesting that none of the protein was fractionated in sample number 41 to 49. According to this, our protein materials could transform into soluble aggregated form leading to bigger size which, later on, can be detected in fraction 19 to 21. We strongly believed that this transformation into aggregated form was caused by the interaction between the SUMO tag and our protein.

In order to overcome this aggregation issue, we attempted to determine protein stability once SUMO was removed. If our protein can maintain its stability without SUMO tag, we should be able to obtain *Pf* plasmepsin VI in monomeric form and we should be able to use it for crystallization to study its structure. Concentrated *Pf* plasmepsin VI was incubated with TEV protease and loaded on SDS-PAGE before analyzed with Coomassie blue staining. Unfortunately, instead of monomeric band of *Pf* plasmepsin VI, multiple bands were detected after incubated with TEV protease (Fig 7A). These multiple bands indicate protein breakdown that might be caused by self-digestion after SUMO tag was removed. Taken together, this data implies that our

protein unable to maintain its stability without SUMO tag and alternative option to overcome aggregation issue is needed.

We also attempted to express full-length plasmepsin VI from *P. berghei* and *P. vivax* in order to overcome this issue as it has been shown that expression from different species could ended up in proper protein folding and leads to monomeric protein expression (Tham lab, unpublished data). However, after P2 virus was obtained, we were unable to detect any protein expression from Sf21 cell culture infected with full-length *Pb/Pv* plasmepsin VI (Fig 7B). The transfection process was repeat several times and, so far, no expression can be detected from *Pb* and *Pv* constructs. Due to time constraints, no further experiments were attempted to solve this issue yet.

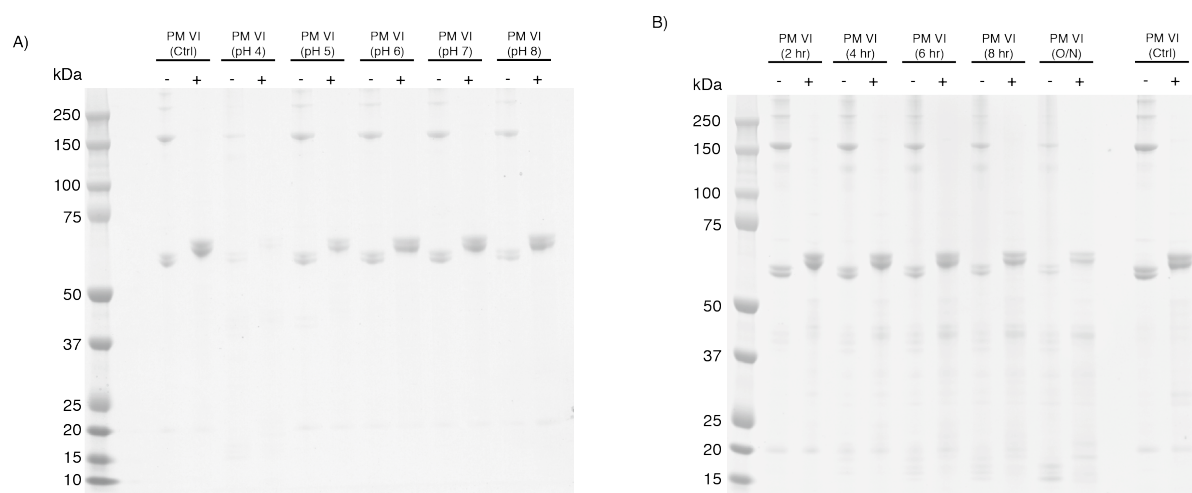
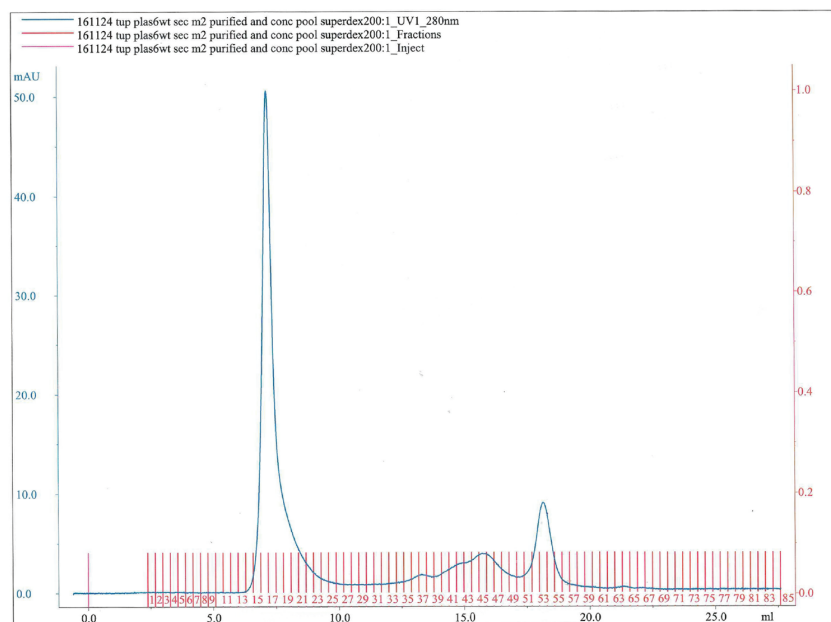


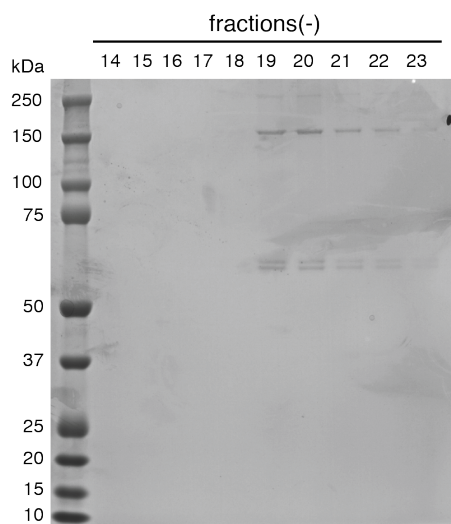
Fig. 5 Optimization for the appropriate pH and time for *Pf* plasmepsin VI activity.

(A) Catalytic activity of concentrated *Pf* plasmepsin VI in various acidic conditions. (B) Catalytic activity of concentrated *Pf* plasmepsin VI incubated in pH 4 acid buffer at different time-point. +/- indicate reducing and non-reducing conditions.

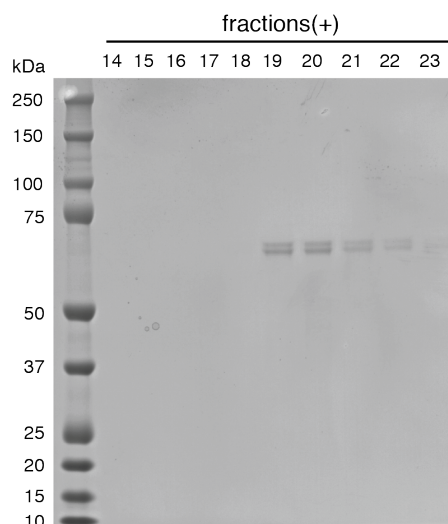
A)



B)



C)



D)

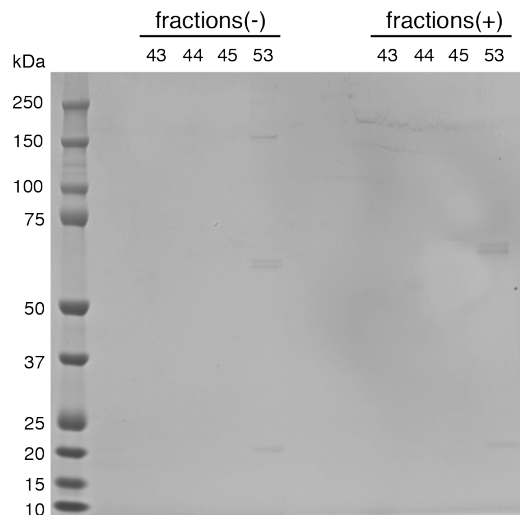


Fig. 6 Size exclusion chromatography of concentrated wild-type *Pf* plasmepsin VI.

(A) Concentrated protein purified using size exclusion chromatography, peaks were analyzed with 280 nm UV. (B) Purified fractions number 14 to 23 analyzed in non-reducing buffer. (C) Purified fractions number 14 to 23 analyzed in reducing buffer. (D) Purified fractions number 43 to 45 and 53 analyzed in both non-reducing and reducing buffer. +/- indicate reducing and non-reducing conditions.

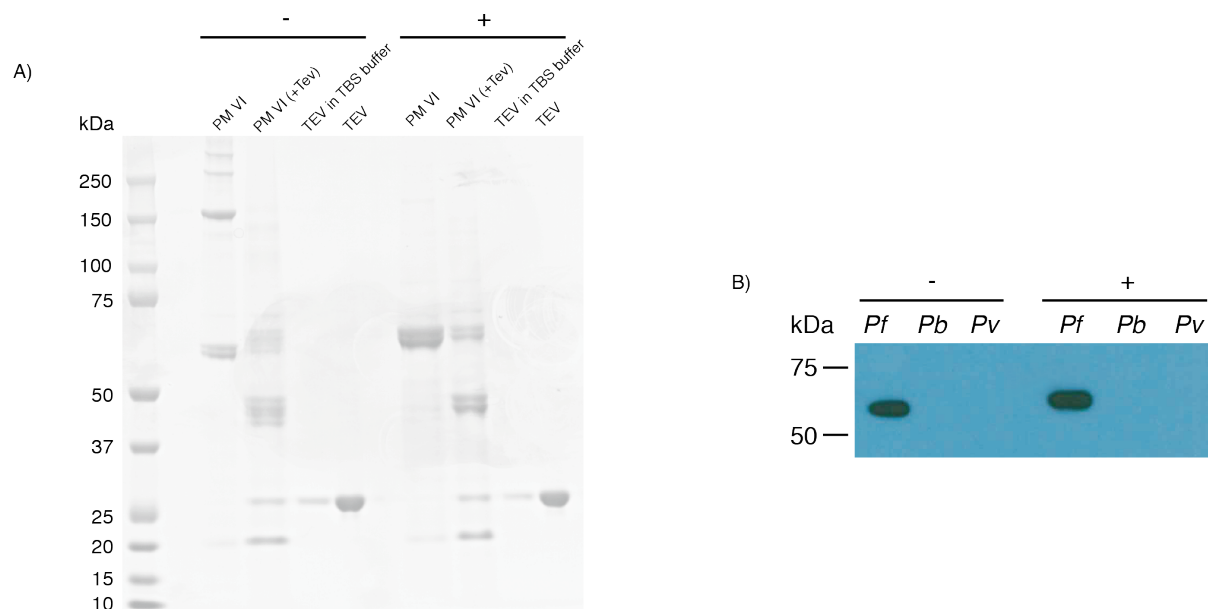


Fig. 7 Removal of fusion tag using TEV protease and the expression of *Pb* and *Pv* plasmepsin VI

(A) *Pf* plasmepsin VI stability test before and after removal of fusion tag in both non-reducing and reducing buffer. (B) P2 supernatants from Sf21 cells culture infected with P1 virus from wild-type *Pf*, *Pb*, or *Pv* plasmepsin VI were analyzed by western blot in order to determine the level of protein expression.

Discussion

As previously demonstrated in chapter 3 of this thesis, plasmepsin VI is found to be essential during mosquito stage of *P. falciparum*. It also required for transmission from mosquito to human host as experiment with gene disruption demonstrated that the number of sporozoite in salivary gland was significantly reduced in mosquito infected with plasmepsin VI knock-out parasites. Due to this importance, we attempted to explore the optimization of expressing plasmepsin VI of *P. falciparum* so that further studies can one day reveals the mysteries of this enzyme function and structure.

We firstly attempted to express not only wild-type full-length *Pf* plasmepsin VI but also mutated *Pf* plasmepsin VI as we want to use it as negative control for activity testing. However, as shown in Fig 2, only protein from wild-type full-length *Pf* plasmepsin VI construct was detected indicating that the mutated residues at the catalytic sites in mutated construct might, somehow, interfere with protein folding which results in disrupted protein expression (Fig 2A).

As we progressed with wild-type full-length *Pf* plasmepsin VI, we were able to obtain reasonable amount of protein expression with P3 virus. This level of expression allowed us to scaled-up our *Pf* plasmepsin VI expression up to 1 litre of Sf 21 cells culture. Expressed proteins were analyzed and the size seems to match perfectly with its molecular weight based on molecular sequences. We also encountered problems during concentration process as, with PES, the precipitation form of plasmepsin VI was detected instead of monomeric form (Fig 4A). This issue was overcome by changing centrifugal column to one with nitrocellulose membrane suggesting that monomeric form of plasmepsin VI can directly interact with PES membrane.

We also noticed that band intensity from plasmepsin VI was noticeably decreased depend on acid addition particularly on pH 4 with 24 hr incubation. This data is consistent with what we expected from aspartic proteases as it usually prefers acidic condition and plasmepsin X also shown to prefer pH 4 in order to function properly (Janni Christensen, unpublished data). Once catalytic activity was confirmed, we

attempted to purify our protein using size-exclusion chromatography as purified protein is required for further study especially for its structure. Data from size-exclusion chromatography demonstrates that our recombinant proteins tend to be bigger than its original size as it was shown to fractionated with early fractions (fraction 19 to 21) (Fig 7B, 7C). However, according to catalytic activity testing, we are convinced that there is active plasmepsin VI enveloped by soluble aggregation. This idea was supported by our data from activity testing as it took almost 24 hr before band intensity completely disappeared (Fig 5B).

We then attempted to break this soluble aggregation in order to expose the active form of plasmepsin VI so we can use it for crystallization. Since we believed that the soluble aggregation was caused by interaction between SUMO tag and the plasmepsin VI protein itself, we determined if plasmepsin VI can maintain its stability after SUMO tag was removed. Unfortunately, our protein was unable to maintain its stability after SUMO tag was removed as multiple bands can be observed after incubation with TEV protease (Fig 7A). Due to this, alternative method to overcome this issue is required. We also attempted to express plasmepsin VI from *P. berghei* and *P. vivax* construct but, so far, we were unable to obtain monomeric form of plasmepsin VI yet. Due to time constraints, no further experiments were attempted to solve this issue yet.

Although this project is at its infantile stage, the continuation will bring about beneficial results. The completion of this project will increase the expression of plasmepsin VI and open the doors to new experiments that will unravel the mysteries of this enzyme. Future projects will include sufficient yield of protein purification, which will then be used in kinetic analysis with different inhibitors to test for the inhibition constants. Inhibitors that exhibit a strong affinity for the active site will then be used in x-ray crystallography. The kinetic analysis and structural data obtained from x-ray crystallography can direct the way to a new drug target against malaria that may have potential of saving millions of lives.

Chapter 5 – A multi-stage antimalarial compound targeting *P. falciparum* plasmepsin IX and X blocks oocyst formation.

Introduction

In the past 10 years, malaria control and prevention has been substantially increased with implementation of new vector control strategies and chemotherapies. However, the disease still remains a worldwide public health issue with *Plasmodium falciparum* causing more than 200 million cases and over 630,000 deaths per year (WHO, 2018). Malaria eradication is now being discussed as a realistic goal, but new tools are needed in order to achieve this goal, especially for malaria elimination in areas of high transmission. It will be difficult to achieve eradication of malaria unless drugs and/or vaccines that interrupt malaria transmission can be developed and deployed (173). Consequently, identification of proteins required for transmission of *Plasmodium* is important so that potential novel drugs can be developed that would be of use in elimination programs through drug administration of infected patients or asymptomatic carriers.

Artemisinin-based combination therapies (ACTs) have been recommended as first line treatment for uncomplicated *falciparum* malaria since 2001. Although ACTs rapidly remove asexual blood stages and early gametocytes from the patients, their effectiveness against transmission stage V gametocytes remains unclear (174-176). Currently, there are no antimalarial drugs that would be effective for blocking transmission of the sexual stages to mosquitoes. However, an option available for interruption of transmission at the liver stage is low dose primaquine which is recommended for use in areas of low transmission as a single administration following ACT in patients with *P. falciparum* malaria. Higher doses may be more efficacious, but the use of primaquine for reduction of transmission is limited because of the potential for hemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a relatively common genetic variation found in malaria endemic areas (177). Whilst there are good options for treating and controlling malaria, the decreasing efficacy of artemisinin and the partner drugs in ACTs reinforces the need for the discovery of new drugs with novel modes of action for malaria treatment, prevention and interruption of transmission (178-180).

Aspartyl proteases are known to be a valuable drug target for combating various diseases including HIV infection and those caused by other pathogenic microorganisms such as *Aspergillus* and *Candida* (181). Some protease inhibitors have been shown to block *P. falciparum* parasite growth during asexual stages (Skinner, McCarthy 2004) while others can disrupt the parasite life cycle in mice, including late stage gametocytes and the pre-erythrocytic liver stage (124-127). Compound 49c, a hydroxyl ethyl amine based scaffold compound, is an aspartyl protease inhibitor that has multi-stage anti-malarial activity although it does not have the pharmacokinetic properties of a drug and can be classed as a chemical tool compound (151). This protease inhibitor targets plasmepsin IX and X (PMIX and X), resulting in inhibition of *P. falciparum* egress during asexual stage development and parasite invasion. Moreover, the midguts isolated from mosquito fed with the blood of mice infected with *P. berghei* treated with 49c were also free of oocysts compared with controls, demonstrating transmission blocking activity of 49C. Whilst the identification of PMIX and X as drug targets has exciting potential, drug-like compounds need to be discovered for their development into novel antimalarials. Such compounds would be of great interest because they would have a novel mechanism of action.

A novel class of antimalarial compounds has been identified that target the aspartyl proteases PMIX and PMX through a high throughput screen of *P. falciparum* using a library of compounds at WEHI in collaboration with Merck. These hit compounds have been developed to lead stage through a concerted medicinal chemistry program.

The aim of the work in this chapter is to determine if the PMIX and PMX inhibitors block transmission to mosquitoes. This is important information because new antimalarials that block blood stage development and growth as well as transmission to mosquitoes are an unmet need for programs to eliminate malaria.

Results

WM4 and WM382 block parasite egress in *P. falciparum*

An aspartic protease inhibitor library was screened previously for potential hit compounds that target *P. falciparum*. This resulted in the identification of a novel class of compounds that potently blocked *P. falciparum* growth and included WM4. During optimization studies an orally bioavailable lead compound with potent *in vitro* and *in vivo* activity against malaria in *P. berghei* and *P. falciparum* mouse models of infection was discovered and named WM382.

RS-WEHI/Merck4 (WM4) from the imino pyrimidinone class of compounds was a mixture of two stereoisomers. Consequently, the enantiomers were purified to determine activity and it revealed that *R*-WM4 was the active enantiomer whereas the *S*-enantiomer were significantly less active. The Peters' 4-day suppressive test against *P. berghei* infection in mice was performed in order to determine if *RS*-WM4 had *in vivo* antimalarial activity (182). Additionally, *RS*-WM4 administered by intraperitoneal injection for 4 consecutive days, showed some *in vivo* activity when compared to chloroquine and suppressed *P. berghei* parasitemia significantly compared to vehicle control. Whilst WM4 had *in vivo* activity, it did not have sufficient drug-like properties and, consequently, a structure-guided medicinal chemistry program was mounted to derive other imino pyrimidinone compounds with properties required for antimalarial drugs. This identified WM382, which retained potency against *P. falciparum* growth with an IC_{50} of 0.7 nM and an IC_{50} of 0.18 nM against *P. knowlesi*.

In order to confirm that antimalarial compounds WM4 and WM382 inhibit the asexual stage of *P. falciparum*, WM4 and WM382 were added independently to early ring stages to follow growth and development. Both parasite development and growth proceeded normally in the presence of the DMSO vehicle control. However, WM4 and WM382 treated parasites were arrested at late schizont stage and no increase in parasitemia occurred (Fig 5.1A, 5.1C). Previously, it has been shown that compounds inhibiting PMX block egress of the developed merozoites from infected erythrocytes

(151, 183). Both WM4 and WM 382 potently inhibited egress of merozoites from the erythrocyte compared with vehicle control (Fig 5.1B). According to this, we believe that the defect in egress process observed in WM4 and WM382 treated parasites were caused by inhibition of PMX by these compounds.

WM382 has dual activity against PMIX and PMX whilst WM4 is specific for PMX

The ability of WM4 and WM382 to block egress suggested that they could target PMIX and/or PMX (151, 183). In order to address this possibility we constructed *P. falciparum* parasites in which we had placed the *PMX* and *PMIX* genes under conditional control so that we could knock down the level of protease expression (184). The gene *Pf* PMX was HA-glmS tagged using the 1.2 HLG3 vector. The 3' homology region was amplified from 3D7 genomic DNA and cloned using the *EcoR* I / *Pst* I restriction sites. Oligonucleotides encoding the guide sequence were cloned into pUF1-Cas9G by InFusion technology. The plasmids for homology-directed repair (HDR) and the guide plasmid were transfected simultaneously into *P. falciparum* PEMS obtained by E64 treatment following a previously published method (Volz et al, 2016). HA-glmS-tagged *P. falciparum* PM IX was generated following the same strategy. The level of PMIX-HA and PMX-HA expression were determined in the absence and presence of glucosamine, confirming expression of each protease was decreased in 3D7 PMIX HA and 3D7 PMX-HA parasites (Fig 5.2A)

Ring stage *P. falciparum* 3D7, PMX_HA-glmS and PMIX_HA-glmS parasites were cultivated with increasing concentrations of GlcN (Sigma). After 72 hr incubation at 37°C, trophozoite-infected erythrocytes were lysed in 0.06% saponin, pellets were solubilized in 2X reducing SDS-PAGE sample buffer and analyzed by anti-HA immune-detection. IC₅₀ of inhibition for WM4 and WM382 against *P. falciparum* 3D7, PMIX_HA-glmS and PMX_HA-glmS parasites, were determined in the absence and presence of 2.5nM GlcN (normal and reduced protein expression of HA-tagged protein, respectively).

The IC₅₀ of WM4 and WM382 against the different parasite lines was determined and this revealed that when PMX expression was decreased, the parasites were significantly more sensitive to WM4 (IC₅₀ 3.5 nM for 3D7-PMX_HA compared to 1.7 nM in the presence of glucosamine, 48.6% reduction) (Fig. 5.2 B). However, there was no significant change in IC₅₀ for WM4 when expression of PMIX was decreased (IC₅₀ 10.5 nM for 3D7-PMIX_HA compared to 11.9 nM in the presence of glucosamine). In contrast, both PMX_HA and PMIX_HA parasites were significantly more sensitive to WM382 when expression of PMX (IC₅₀ 0.82 nM for 3D7-PMX_HA compared to 0.49 nM in the presence of glucosamine, 59.7% reduction) or PMIX (IC₅₀ 0.84 nM for 3D7-PMIX_HA compared to 0.46 nM in the presence of glucosamine, 54.8% reduction) was decreased (Fig. 5.2 C). These data suggest WM4 targets PMX whilst WM382 acts as a dual inhibitor of both PMIX and X.

Here in this study, we aim to assess the ability WM4 and WM382 to block *P. falciparum* transmission to mosquitoes. Considering that these compounds target either PM X or both PM IX and X, they may be potential candidates for multi-stage anti-malarial drug development.

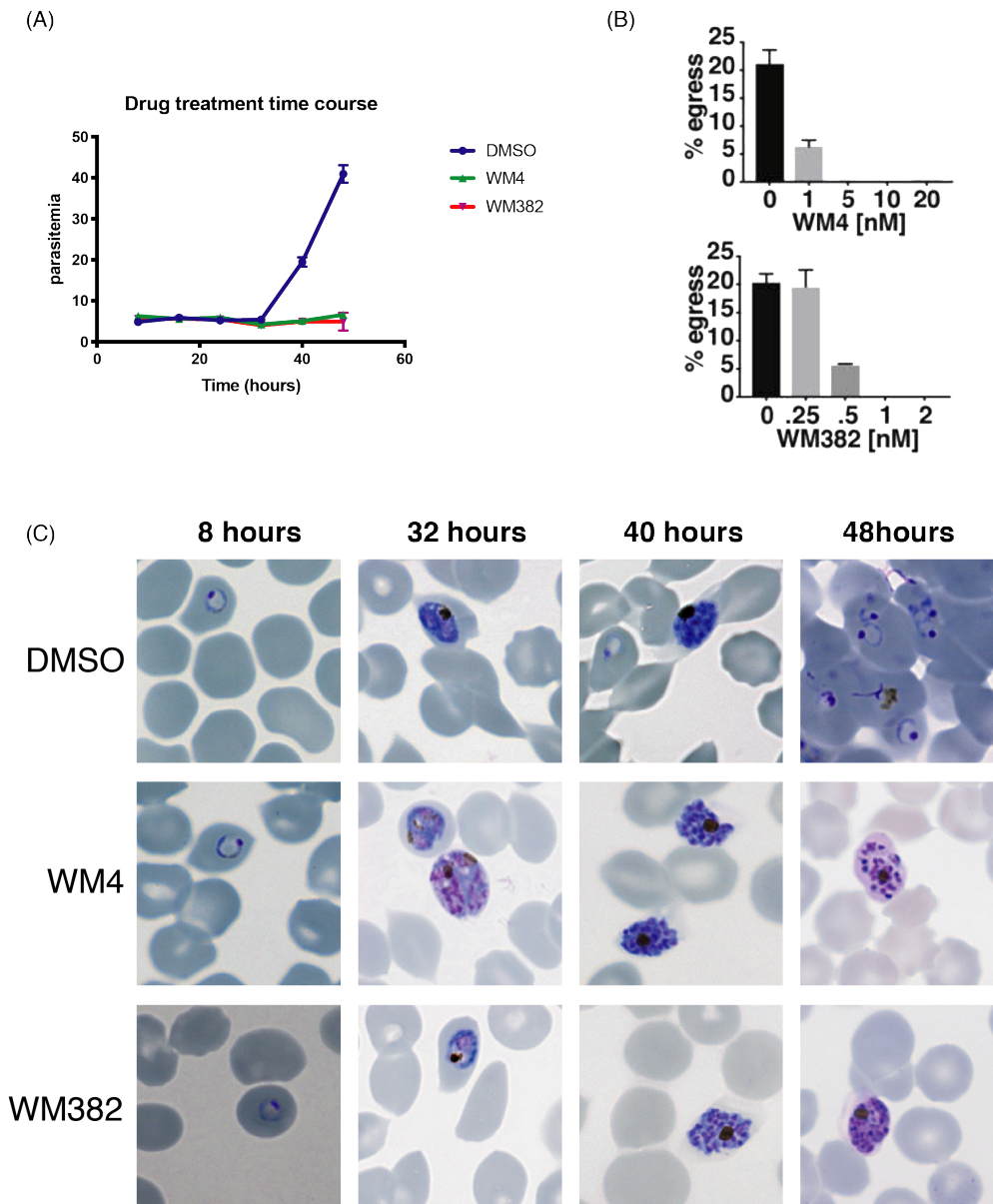


Fig. 5.1 Antimalarial WM4 and WM382 can disrupt parasite development in *P. falciparum* asexual stages by blocking parasite egress from schizont-infected erythrocytes.

(A) Time of parasite killing for WM4 and WM382 in the asexual stage life cycle. DMSO shown as control. Parasitemia was monitored in the presence of ~ 10 fold the IC_{50} of each drug. (B) WM4 and WM382 block egress of *P. falciparum* merozoites from the schizont. Parasites were grown in the presence of increasing concentrations of WM4 or WM382 as shown and the % egress was determined. (C) Giemsa stain smears of *P. falciparum*-infected erythrocytes incubated with DMSO, WM4, or WM382 with time shown in hr.

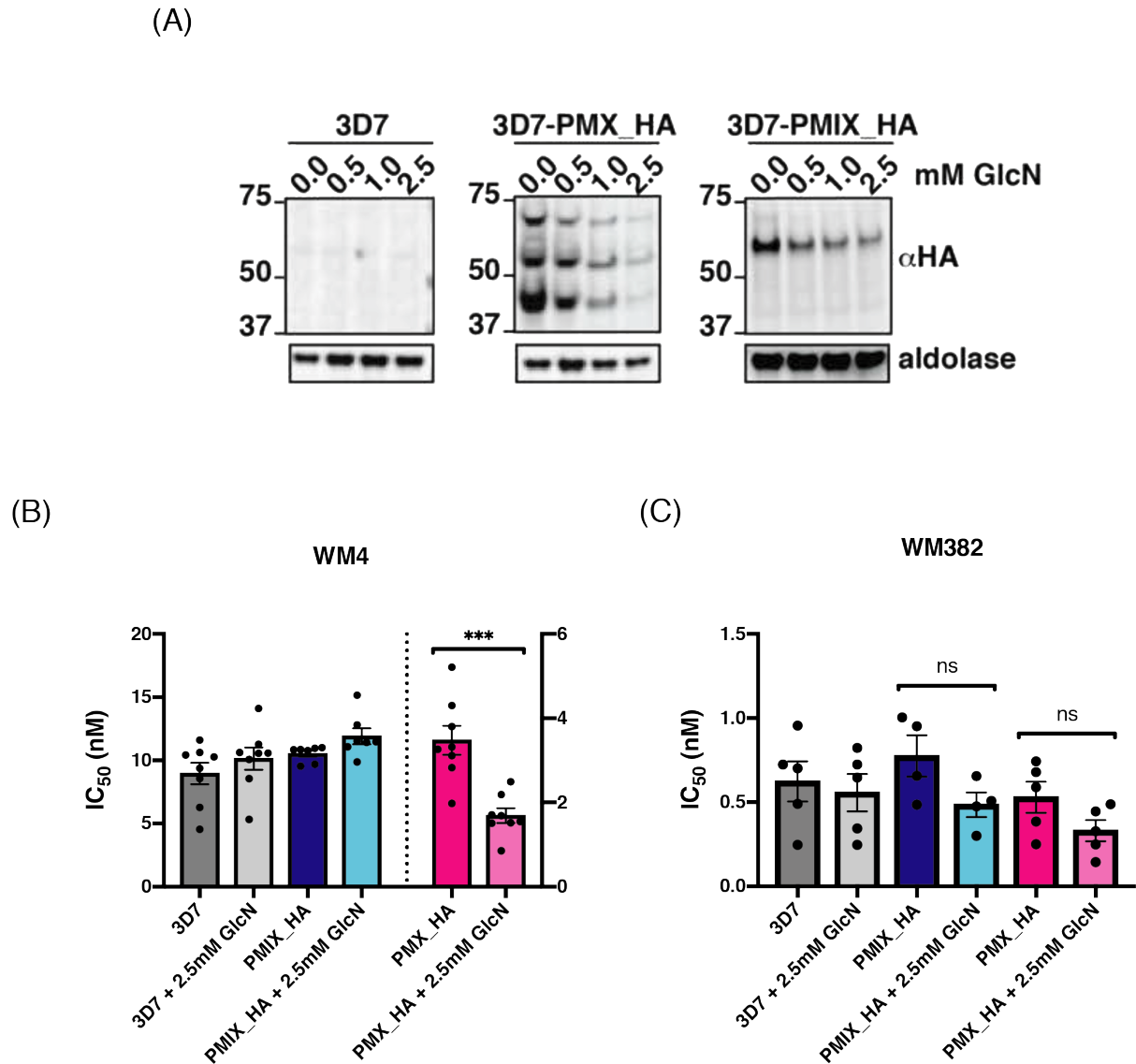


Fig. 5.2 Antimalarial WM4 targets PMX while WM382 targets both PMIX and X

(A) Knockdown of PMIX and X expression in *P. falciparum* using *glms* ribozyme and glucosamine (GlcN). Aldolase is shown as a loading control. 3D7 is the parental line and does not contain a HA tag. 3D7, 3D7 PMX-HA, and 3D7 PMIX-HA parasites were treated with different concentrations of glucosamine and probed with anti-HA antibodies. (B) Determination of WM4 IC₅₀ for *P. falciparum* in the presence (light colours) and absence (dark colours) of glucosamine. Shown are means with SD of eight experiments. (C) Determination of WM382 IC₅₀ for *P. falciparum* in the presence (light colours) and absence (dark colours) of glucosamine. Representative data from one of five independent experiments; shown are means with SEM.

WM4 and WM382 block transmission of *P. falciparum* to mosquitoes

To determine if WM4 and WM382 block transmission of *P. falciparum* to mosquitoes their activity was measured using Standard Membrane Feeding Assays (SMFA) compared to the active control 49c (151). Gametocytes were prepared from NF54 parasites as described in Materials and Methods and WM4, WM382 (10 nM, 40 nM, 80 nM, and 100 nM) or 49c (10 nM) were included in the media. Gametocytes were grown as per normal protocol but with the addition of drug to the culture medium for each day 4 days prior to blood feed or added directly into the blood meal before feeding. On day 17 after initiation of parasite culture, blood smears were made from gametocyte cultures and the % stage V gametocytemia determined (Fig. 5.3A). There was no difference in the ability of gametocytes to develop and mature to stage V gametocytes when treated with either WM4 or 49C confirming that neither compound has any effect on gametocyte development or maturation.

These gametocytes were then harvested and adjusted with fresh uninfected RBCs and heat-inactivated human serum (HIHS) to 0.2% parasitemia before feeding to *A. stephensi* mosquitoes. On day 7 after bloodmeal feeding, female mosquitoes were harvested, midguts dissected and stained with mercurochrome to count the oocysts within the midgut by microscopy. There was no significant difference in the number of oocysts in the infected midguts of mosquitoes fed on blood treated with WM4 compared to untreated or DMSO-treated gametocytes showing that this compound has no effect on oocyst development. This was in contrast to 49c which at 10 nM blocked oocyst formation. The same pattern was observed for parasites treated with WM4 4 days prior to blood feeding compared to those treated immediately prior to the blood feed (Fig 5.3B and 5.3C).

To determine if WM4 blocked sporozoite development, remaining female mosquitoes were maintained by feeding with sugar cubes and deionized water until day 17 post blood feeding when they were aspirated and dissected for salivary gland isolation. Salivary glands were isolated from 30 infected mosquitoes before crushed and resuspended in PBS, and the number of sporozoites determined by counting in a

hemocytometer to evaluate the effect of WM4 on sporozoite development. No significant difference was observed in the number of sporozoites in salivary gland from NF54 wild-type, DMSO treated, or WM4-treated gametocytes (Fig 5.3D). Therefore, WM4 had no significant effect on sporozoite development and migration to the salivary gland.

WM382 was also evaluated for its transmission-blocking activity in the same way as that used for WM4. As expected, no significant difference in gametocyte development was observed between NF54-untreated, 49c-treated, and WM382-treated parasites (Fig 5.4A). Inspection of infected midguts isolated and stained with mercurochrome, showed that oocyst formation in WM382-treated parasites was significantly decreased in a dose dependent manner (Fig 5.4B). Oocyst numbers in mosquitoes fed with gametocytes treated with 0.5 nM or 1 nM concentrations of WM382 were not significantly lower than controls, however, there was significantly lower oocyst numbers with gametocytes treated with WM382 at 2.5 nM, 25 nM, and 50 nM. Similarly, 49c-treatment of gametocytes blocked oocyst formation at 10 nM. Therefore, WM382 blocked transmission of NF54 gametocytes to mosquitoes.

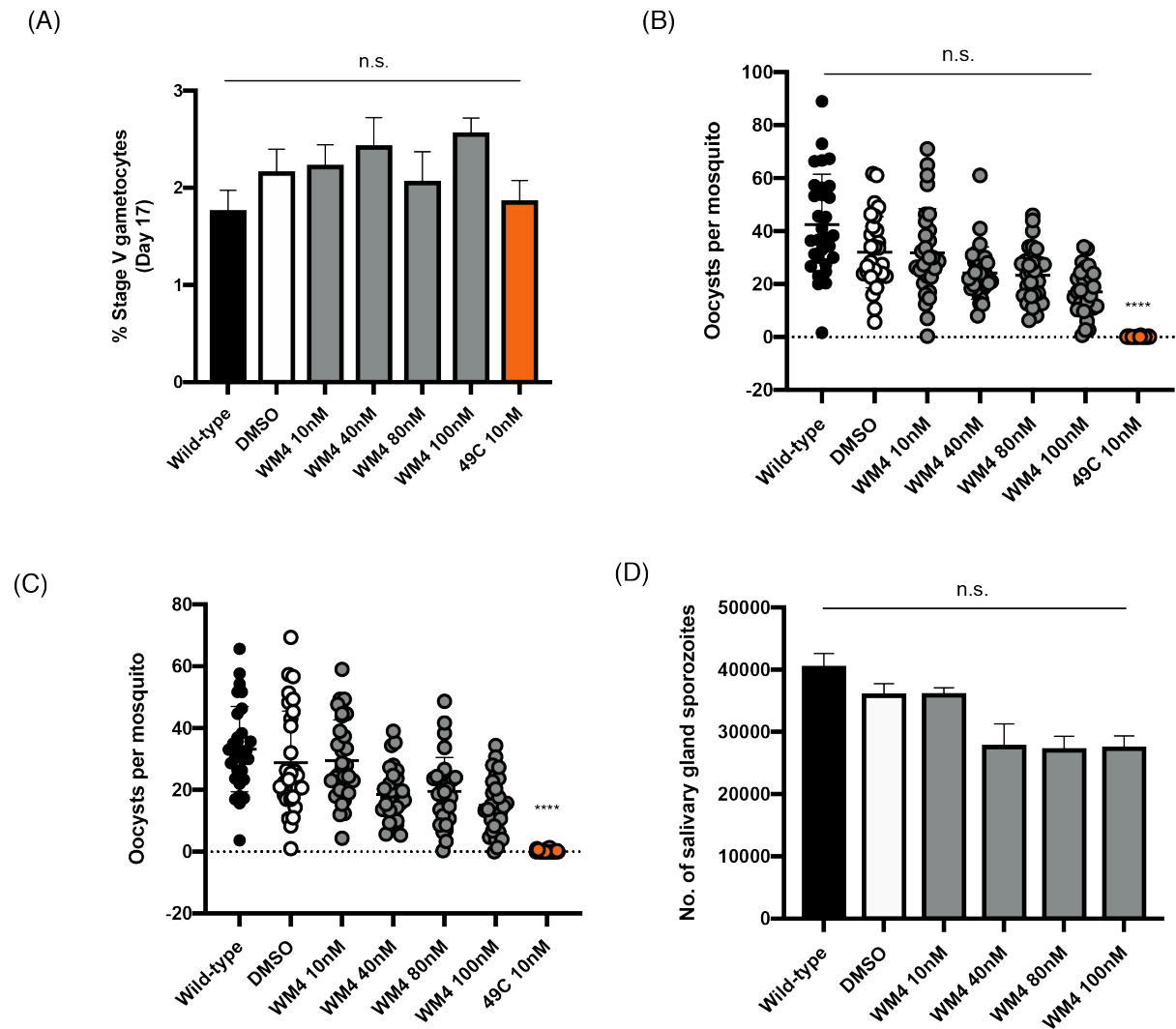


Fig. 5.3 WM4 does not block transmission of *P. falciparum* to the mosquito

(A) Percentage of stage V gametocyte observed in NF54 parasites treated with WM4 or 49c on day 17 after initiation of gametogenesis. (B) Number of mosquito midgut oocysts produced by NF54 treated with different concentrations of WM4 compared to 49c-treated parasites (**** $P < 0.0001$). (C) Number of oocysts detected in mosquito midgut after treatment with WM4 or 49c directly prior to blood feeding. (D) Number of sporozoites counted from 30 dissected salivary glands of NF54 parasites treated with WM4 or DMSO treated parasites. Experiment were done in triplicate, bars show mean values of 30 dissected midguts/salivary glands.

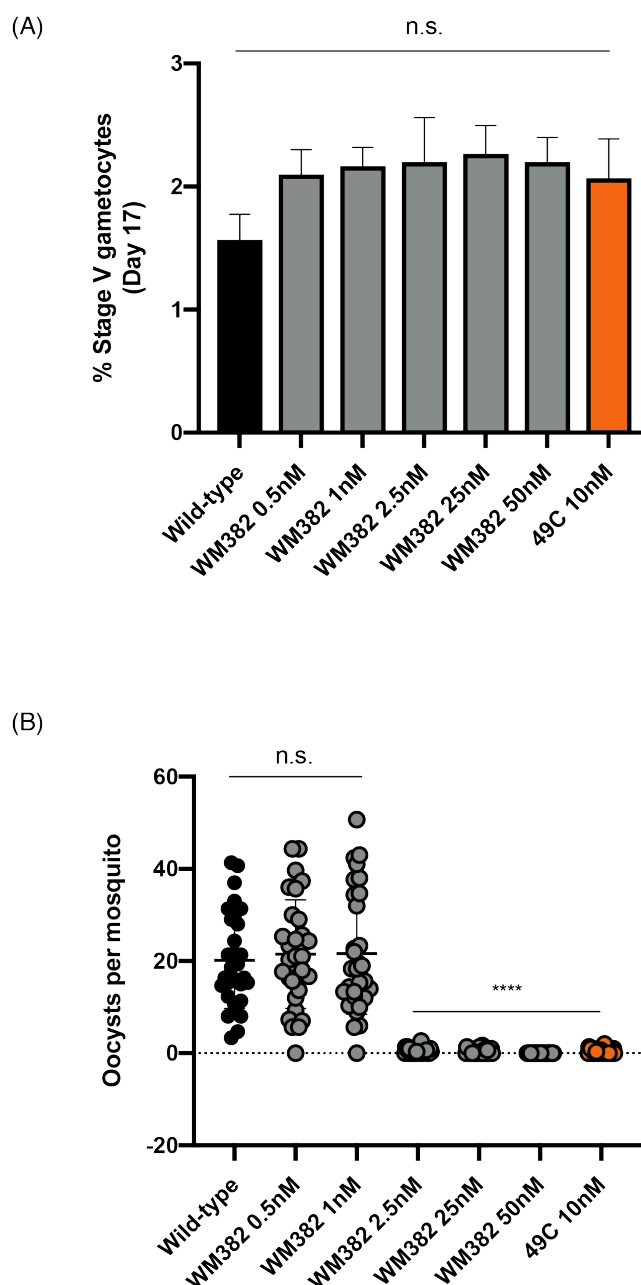


Fig. 5.4 Antimalarial WM382 can block oocyst formation in *P. falciparum* infected midgut

(A) Percentage of stage V gametocytes observed in NF54 parasites treated with WM382 and 49c on day 17 after initiation of gametocyte culture (B) Number of mosquito midgut oocysts produced by NF54 treated with WM382 compared to 49c treated parasites (**** $P < 0.0001$). Experiment were done in triplicate, bars show mean values of 30 dissected midguts.

WM4 and WM382 block transmission of *P. berghei* in vivo from mouse to mosquito

As shown above WM382 is an antiparasitic compound that potently blocks transmission of *P. falciparum* to mosquitoes using standard membrane feeding assay (SMFA). The *in vivo* efficacy of orally delivered WM4 and WM382 were evaluated in mice infected with *P. berghei*.

Mice were infected with *P. berghei* ANKA GFPcon and two doses of WM4 or WM382 were given to these infected mice via oral administration two days before feeding to *A. stephensi* mosquitoes. Midguts from mosquitoes fed on the infected mice were dissected on day 13 to count the number of oocysts. As expected, WM382 displayed significant *in vivo* efficacy when dosed at 1 mg/kg (mpk) compared to no treatment (wild type), suggesting that WM382 can block transmission from mouse to mosquitoes *in vivo* (Fig 5.5). However, in contrast with results observed in *P. falciparum*, WM4 also delivered the same level of transmission blocking effect as WM382 when dosed at 20 mpk (Fig 5.5).

We are unable to determine the reason or mechanism behind the differences between WM4 and WM382 treated parasites yet but we believe that it could be caused by specific differences between gametocyte development in *P. falciparum* and *P. berghei*. As *P. berghei* gametocyte development progress much more rapidly compared to *P. falciparum*, this could offer WM4 a chance to block PMX if it is early-stage gametocyte specific protein as the gap between early and late stage is much shorter in *P. berghei*.

(A)

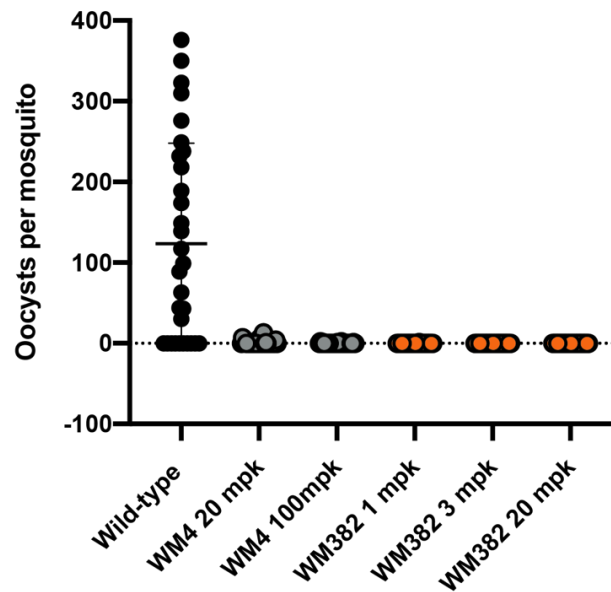


Fig. 5.5 Antimalarial WM4 and WM382 can block oocyst formation in *P. berghei* infected midgut

Number of mosquito midgut oocysts produced by NF54 parasites treated with WM4 or WM382. The intensity of oocyst infection is significantly decreased in mice treated with WM4 at 20 mpk and 100 mpk. The number of oocyst is also significantly decreased in mouse treated with WM382 at 1 mpk, 3 mpk, and 20 mpk.

Discussion

There are several plasmepsins that are crucial for parasite development. Those that are expressed during the mosquito stage of the life cycle are currently considered as promising targets for anti-malaria drug development. Drugs that block transmission are attractive for several reasons, firstly, parasite development in the mosquito stage is a bottleneck in the malaria life cycle since it is the only invasive stage that doesn't require replication and secondly, it is the only development stage where parasite grow extracellularly, thus their proteins are more readily exposed to drugs or host immune responses such as antibodies (72, 75).

PM IX and X are expressed in the asexual stage of *P. falciparum* and genetic disruption studies have demonstrated that they are essential for parasite development validating them as candidates for anti-malarial drug development (151). Compound 49c, a peptidomimetic competitive inhibitor, has been shown to be effective against *P. falciparum* *in vitro* and the rodent parasite *P. berghei* *in vivo* (185, 186). 49c does not affect intraerythrocytic development and allows the production of microscopically normal schizonts that do not egress from the host cell. More importantly, 49c was shown to be a valuable candidate for transmission blocking strategies by targeting both PM IX and X. Although 49c does not affect differentiation from asexual stages into microscopically mature gametocytes, it prevented oocyst formation within the mosquito midgut (151). Whilst 49c is a useful chemical tool it does not have the properties of a drug needed for its development as a novel antimalarial.

A phenotypic high throughput screen of a library of small molecules that inhibit aspartic proteases, performed against asexual blood stages of *P. falciparum*, has identified a novel class of antimalarial compounds. During optimization studies WM382, an orally available lead compound that has potent activity *in vitro* against *P. falciparum* and can cure *P. berghei* infections in mice, has been identified. This compound has dual activity against both PMIX and X and is active against blood and mosquito stages of the life cycle. Furthermore, its activity against 2 essential targets creates a high threshold

against the development of resistance. Therefore, efforts to identify an efficacious and safe antimalarial drug from this series of compounds are currently ongoing.

With our antimalarial compounds that specifically target and interrupt PMIX and X function, we are able to study the function of these proteases in *Plasmodium* biology. The inducible knock-down using glmS ribozyme system was then used to evaluate the dual activity of WM382 against PMIX and X. Inducible knock-down lines of PMIX or X were both more sensitive than parental 3D7 to WM382 (Fig 5.2). WM4, on the other hand, is a specific inhibitor of PMX alone, as functional knockdown rendered parasites more sensitive to WM4 while PMIX functional knockdown had no effect.

PMIX has been found to be localized in the rhoptries while PMX is present in the exonemes suggesting that substrates for each would be in these subcellular localizations (183). However, this is not consistent with the subcellular localization of known PMIX and PMX substrates that are already identified, including the *Pf*Rh and EBL invasion ligand which are localized in the rhoptries and micronemes respectively (187, 188). This suggests that the exposure of different proteins to PMIX and X before and during merozoite invasion is complex involving a cascade of events that release essential proteins at distinct times.

In this study, we found that our antimalarial compound WM4 is a specific inhibitor of PMX and WM382 is a dual inhibitor blocking both PMX and PMIX function (Fig 5.2). PMIX has been shown to be required for development of merozoites that can invade red blood cells whilst PMX was required for merozoite egress and invasion by proteolytically processing subtilisin 1 (*Pf*SUB1), apical membrane antigen 1 (*Pf*AMA1) and the pseudoproteases SERA5 and SERA6. As expected, our antimalarial compounds WM4 and WM382 are shown to have an inhibitory effect during asexual stage of *P. falciparum* with the same phenotype (Fig 5.1), (151).

According to our data, blocking of PMX activity results in a defect during erythrocyte egress (Fig 5.1). This protease is responsible for processing members of the EBL and Rh protein families required for invasion of erythrocytes. We currently don't know

how these PMX mediated processing events affect the function of these proteins but we believe that it could play an important role in maturation of the merozoite surface proteins as well as their ability to interact with their receptors. The individual Rh proteins are processed by PMX at different positions along their length and this is likely critical for their function. Rh5 is a well-known leading malaria vaccine candidate as it can form a complex with CyRPA and Ripr (189, 190) which, later on, required for binding to the erythrocyte surface protein basigin (191). It has been previously reported that N-terminal of Rh5 binds the GPI-anchored protein Pf113, tethering the Rh5/CyRPA/Ripr complex to the parasite plasma membrane, which may be critical for its function in the merozoite-erythrocyte interaction and invasion (192). Cleavage of Rh5 by PMX at the N-terminus would release the Rh5/CyRPA/Ripr complex from Pf113 at the merozoite surface. Insertion of processed Rh5/CyRPA/Ripr complex into the erythrocyte membrane occurs during invasion (189) and PMX-mediated processing of Rh5 may be crucial for this step.

PMIX and X are not only responsible for processing of key proteins involved in egress and invasion, they also process proteins important for ring stage development after invasion including RAP1 (193) and RON3 (194). During invasion, RAP1 is released into the parasitophorous vacuole and might play a role in establishing this compartment together with its interacting partners RAP2 and RAP3. RON3 is also deposited within the parasitophorous vacuole and is believed to be essential for development beyond the ring stage due to its function with the PTEX translocon during the export of parasite proteins into cytosol of erythrocyte. It is also required for transport of glucose from the host cell into the parasitophorous vacuole (194). However, unlike RAP1 and RON3 that are already identified as substrates of PMIX and function during ring stage development, there are likely to be others that are not yet identified. Together, PMIX and X have a broad, both direct and indirect, function in the activation of many proteins involved in egress and invasion as well as early parasite development, emphasizing their importance as drug targets (151, 183).

In *P. berghei*, PMX is confirmed to be expressed in mature gametocyte (151) and the inhibitor 49c blocked gamete maturation from gametocytes suggesting that PMX is

required for transmission. In this study, we determined the effect of the PMX specific inhibitor WM4 on *P. falciparum* transmission and found that there was no effect on the development of gametocytes or on *P. falciparum* infection of mosquitoes. Unlike WM4, WM382 is found to inhibit both PMIX and X and potentially blocked transmission as gametocytes treated with WM382 failed to produce oocysts in the midgut of infected mosquitoes. Taken together, these results suggest that, in *P. falciparum*, PMIX function is important for oocyst development in mosquitoes. However, we cannot rule out the possibility that WM382 is active against other plasmepsins such as PMVI, which has been shown to be essential for *P. berghei* transmission to mosquitoes (145). Previously, PMIX is shown to not be expressed in *P. berghei* gametocytes (151) but its translation may be initiated at gamete formation and its function important for processing and function of proteins such as MTRAP, a protein essential for gamete egress from erythrocytes and transmission to mosquitoes (195, 196). This would be consistent with our results showing that WM382 and not WM4 was able to block transmission of *P. falciparum* to mosquitoes suggesting PMIX plays a key role in processing essential proteins for this step. However, according to our *in vivo* study with mice infected with *P. berghei*, WM4 is also able to block transmission as well (Fig 5.5) suggesting that there could be other factors, such as differences between species, involved. Gametocyte development in *P. berghei* progresses faster than *P. falciparum*, as it usually takes almost 17 days in order to obtain mature gametocyte in *P. falciparum* while, in *P. berghei*, 48 hours is all it takes. This significant difference might be the reason why WM4 acted differently between 2 parasites species. In our *P. falciparum* experiment, we were unable to include our antimalarials too early as it will block asexual parasite egress and impact on the ability of the gametocytes to develop independently of any PMIX/PMX-specific function. Without stress caused by increased parasitemia, blood stage parasites are unable to commit into gametocytogenesis as egress is blocked. This gap between early and late stage gametocyte is much shorter in *P. berghei* and, if PMX turned out to be early stage gametocyte specific protein, it will be easier for WM4 to target and block PMX compared to *P. falciparum*. Taken together, PMIX might not be the only vital plasmepsin that responsible for the transmission. Due the differences between species, more investigations are needed

in order to determine the mechanism of action of PMIX and X and their roles during mosquito stage development.

In summary, a novel class of compounds has been discovered that can cure both *P. berghei* and *P. falciparum* infection in mouse models. These compounds are active against both PMIX and X in blood and mosquito stages of *Plasmodium*. Due to the availability of PMIX/X dual and PMX selective inhibitors, we are able to determine the role of these proteases and it has been demonstrated that PMIX and PMX are master regulators responsible for processing and activation of key proteases, ligands and other proteins that are required for essential steps in *Plasmodium* invasion, egress, and development. Also, it is very important to mention that effect in early gametocytes in *P. falciparum* has not been tested yet. This is one of the limitation due to our current protocol for gametocyte production. Once we are able to produce synchronized gametocytes and test our compounds on early stage gametocytes, it can possibly explain the differential transmission blocking effect between *P. falciparum* and *P. berghei*.

Chapter 6 – General Discussion

Aspartyl proteases in *Plasmodium* parasite

Over the last decade, due to the global distribution of insecticide-impregnated bed nets and the introduction of artemisinin-based combination therapy (ACT), as recommended antimalarial treatment, malaria incidence has been significantly decreased from 1 to 2 million deaths in 2000 to an approximately half million in 2017 (197). Unfortunately, these strategies are not enough to eradicate malaria as mosquitoes are becoming increasingly resistant to insecticides (198) and artemisinin resistance is emerging (199). Given that many antimalarial drug development programs currently in clinical trials rely on artemisinin analogues and ACTs (200), it is crucial to develop drugs with novel mechanisms of action in order to stay ahead in our fight against drug resistance.

Aspartic proteases, also known as plasmepsin in *Plasmodium* species, are enzymes proven to be well suited for target-based drug development due to the knowledge of their enzyme mechanisms and active site structures. Furthermore, several aspartic proteases in *Plasmodium* species are found to be involved in a variety of pathways that are essential for parasite survival in both sexual and asexual stages. Over the last few years, there has been significant progress in the development of efficient genetic methods to modify the parasite, including several conditional approaches. This progress has allowed some of the different plasmepsins to be validated as drug targets.

In this study, we characterized the biological function of plasmepsin VI (PMVI) in *P. falciparum*. The equivalent PMVI in *P. berghei* has previously been shown to be essential for sexual development and transmission from infected mosquitoes to the rodent host (145). Moreover, we also evaluated the importance of PMIX and X during mosquito stages using the novel antimalarials WM4 and WM382. These aspartic proteases were found to be essential for asexual stage development of *P. falciparum* and sexual development of *P. berghei* as previously demonstrated in previous studies with competitive PMIX and X inhibitor, 49C (151, 183).

Role of plasmepsins in asexual stages

Degradation of erythrocyte contents

In order to grow within erythrocytes, parasites import proteins from the host cytosol into the food vacuole where they are degraded by a panel of proteases into single amino acid. This pathway, also known as the hemoglobin degradation pathway, supplies amino acid for protein synthesis and provides space within the erythrocyte for the parasite to grow. Due to the high level of proteolytic redundancy built into this pathway, it indicates that this process is important for parasite development, however, it also implies that individual proteases are not likely to be essential.

Four aspartic proteases that includes PMI, II, IV and histo-aspartic protease (HAP), previously known as PMIII, are expressed in blood stage parasites and responsible for the degradation of hemoglobin into oligopeptides (201). Although PMI and II initiate this hemoglobin degradation pathway (202, 203), they are proteolytically activated by falcipains (papain-like proteases) (204). Falcipain inhibition leads to accumulation of undigested hemoglobin in the food vacuole resulting in an enlargement of this organelle (205). While each individual falcipain could be genetically disrupted without significant effects on parasite growth, no double or triple falcipain knockout (KO) has been reported, suggesting that, as a family, these proteases could be essential. However, individual KO of the genes encoding each of the four food vacuole plasmepsins does not affect parasite growth and parasites lacking all four plasmepsin genes (quadruple KO) are viable but grow significantly slower *in vitro* and are more sensitive to cysteine protease inhibitors (133, 206). This data suggests that these food vacuole plasmepsins are not optimal drug targets (207). However, the importance of a gene might be underestimated when evaluated in laboratory-adapted strains growing under optimal conditions as opposed to an *in vitro* setting.

Modifying the infected erythrocyte

Modification of host erythrocyte is a crucial step for blood stage parasites. In order to survive and develop within infected erythrocyte, the *Plasmodium* parasite remodels the erythrocyte cytosol and membrane extensively to obtain metabolites, adhere to epithelial cells and evade host immune system. During this process, several of parasite proteins are exported into infected erythrocyte through translocation of parasitophorous vacuole (PV) proteins across the parasitophorous vacuole membrane (PVM) via the PTEX complex (*Plasmodium* translocon for exported proteins) (208). Most proteins exported via this pathway contain a PEXEL (protein export element) motif downstream of the secretory signal peptide. This motif is critical for protein export mechanism as it is required to be processed by PMV, an aspartic protease located within endoplasmic reticulum (ER) (134, 135). Once cleaved by PMV, the N-terminal sequence on exported protein is exposed and can likely be recognized by PTEX. As a majority of proteins exported into erythrocyte cytosol or membrane are cleaved by PMV, this protease was likely to be a promising target as its inhibition would block a variety of biological processes such as protein trafficking to the infected host cell surface, metabolite import, hemoglobin internalization or Maurer's cleft formation.

Another mechanism by which the parasite releases proteins into the host cell is through secretion of rhoptry proteins at the time of invasion. These proteins have been shown to be implicated in PV formation, erythrocyte membrane modification and nutrient uptake (209). Many rhoptry proteins contain a pro-peptide downstream of the signal peptide that is proteolytically removed during merozoite maturation (210, 211). PMX is believed to be vital during this process as it is found to be expressed in blood stages and needed for the activation of subtilitin-like protease 1 (SUB1) (151). Although, SUB1 has been shown to cleave some of rhoptry proteins *in vitro* (212), it is not known whether there are other proteases that also involve in this processing and more studies are required in order to fully understand the mechanism of action between these proteases.

Getting in and out of host erythrocyte

Once the *Plasmodium* merozoite has egressed from infected erythrocytes, they are vulnerable to the host immune system and are viable for a short time during which they need to find and invade a new erythrocyte. Due to this short time, egress and invasion are tightly coordinated regulated processes with proteolysis playing a major role. Proteases are believed to be vital in these pathways due to: 1. disruption of the PV and erythrocyte membranes are required for parasite egress, 2. general cysteine and serine protease inhibitors block egress, and 3. the protein coat that covers the merozoite is proteolytically shed during invasion. With data from recent proteomic studies, more than 180 *Plasmodium* and host proteins are identified to be cleaved during the last 6 hours prior to parasite egress. These include not only erythrocyte membrane and cytoskeletal proteins expected to be degraded during schizont rupture, but also proteins that are directly implicated in egress and invasion, and factors involved in protein trafficking and early parasite development within erythrocytes. Despite the fact that some of the key proteases responsible for egress and invasion are already identified, the development of conditional KO and knockdown (KD) systems are allowing us to understand the precise biological and molecular functions of these essential enzymes.

Proteases involved in parasite egress

Cyclic GMP (cGMP) level is found to be significantly increased prior to egress. The increase in cGMP level activates cGMP-dependent protein kinase G, which is required for the secretion of proteins from apical organelles (exonemes and micronemes) into the PV and onto the merozoite surface (213). Upon secretion, several substrates that are important for egress and invasion are processed by SUB1 (214). The members of serine repeat antigen (SERA) family are also cleaved and processed by SUB1. In *P. falciparum*, there are 9 SERA proteins, each containing a papain-fold catalytic domain. However, only SERA 6, 7, and 8 are predicted to be active while the other 6 (SERA 1 to 5 and 9) are having serine instead of catalytic cysteine. SERA5 and SERA6 are the most abundant SERAs in blood stages and the only 2 that refractory to genetic KO,

suggesting that they are essential (215). However, data from recent studies with DiCre conditional KO found that whilst SERA5 is not essential, parasites lacking its function egress prematurely leading to a significant decrease in invasion. Therefore, SERA5 is likely a pseudoprotease that regulates the timing of egress to coincide with the formation of fully mature and invasive merozoites (216).

Breakdown of the parasitophorous vacuole and erythrocyte membranes allows parasites to egress from infected erythrocytes. Previous studies have shown that SUB1 and SERA6 are essential and required during these processes (217). SUB1 is required for PVM breakdown while SERA6 is responsible for the disruption of erythrocyte membrane but not the PVM. The interaction between SUB1 and SERA6 is confirmed by the complementation of the SERA6 KO parasites with different mutants. The data obtained from the complementation experiment strongly indicates that proteolytic activation of SERA6 by SUB1 is needed for erythrocyte membrane breakdown (212, 218, 219). However, it is not yet clear whether SUB1 or SERA6 are directly involved in the degradation of the erythrocyte cytoskeleton. It is also important to mention that SUB1 is also expressed in liver stages where it has also been shown to play a role during egress using a conditional KO approach in *P. berghei* (220). However, conditional KO of *PbSUB1* was also shown to arrest schizont development and merozoite maturation within hepatocyte (221). Taken together, these data indicate that SUB1 might play additional functions in liver stages compared to those in the erythrocytic cycle.

MSP1 is also considered as another essential protein during the parasite egress as it can trigger a conformational change that allows it to bind to spectrin (222). Interestingly, parasites that express MSP1 mutants with SUB1 cleavage sites that are processed less efficiently egress significantly more slowly, suggesting that its cleavage might be a rate-limiting step in parasite egress. MSP1 is also the most abundant merozoite surface protein and provides a scaffold for the formation of MSP1/6/7 complex. This complex was previously believed to be essential for erythrocyte binding by released merozoites and invasion. However, an attempt to convert MSP1 to a soluble PV protein that is no longer GPI-anchored to the merozoite

surface was performed using conditional truncation of MSP1. Surprisingly, these parasites have only a two-fold decrease in parasite replication rate, suggesting merozoites lacking MSP1 can still invade host erythrocytes.

Despite the fact that there are many proteins that are essential for egress, PMX appears to be a master regulator. As previously mentioned, it has been previously demonstrated that PMX processes SUB1, which is required for maturation of MSP1, SERA5, and SERA6 and essential for parasite egress (151). Moreover, in our study, we are able to demonstrate the importance of PMX during egress of *P. falciparum* by using our novel antimalarials WM4 and WM382 (Fig 5.1). Our data confirmed that PMX is essential for blood stage parasites because WM4 (PMX specific inhibitor) and WM382 (PMIX and X dual inhibitor) blocks egress and further development.

Proteases involve in merozoite invasion

Besides their roles during invasion, proteases are also needed for merozoite maturation within infected erythrocyte. This step is crucial as it ensures that, once egressed, these merozoites can effectively invade host erythrocytes before they can be detected by the host immune system. The current model for erythrocyte invasion involves initial recognition and attachment to the erythrocyte surface, reorientation of the apical end towards the erythrocyte membrane, active invagination and invasion of the erythrocyte membrane using an actin/myosin motor, formation of the parasitophorous vacuole, and sealing of the parasitophorous vacuole membrane and erythrocyte membrane (223, 224). Many of the proteins involved in these events, including rhoptry, microneme, and surface proteins, are processed during merozoite maturation. However, with the exception of a few proteins (mainly SUB1 substrates), the significance of these cleavage events, or the proteases mediating them, are still unknown. Although MSP1 does not appear to be not strictly required for invasion, SUB1 processing of MSP1 and MSP7 likely makes merozoites more invasive. SUB1 is also found to cleave several rhoptry proteins such as RAP1, RhopH3, and RAMA. These proteins are important for invasion as well as PV proteins and erythrocyte membrane proteins (212).

During invasion, the protein coat of the merozoite is shed by *Plasmodium* rhomboids (ROM) and SUB2. In *P. falciparum*, ROM1 localizes to the apical end of merozoites where it can process apical membrane antigen 1 (AMA1) *in vitro* (225, 226). AMA1 is a transmembrane micronemal protein that is secreted onto the merozoite surface right before egress. It is one of the core components of the tight junction, a protein complex that links the parasite myosin/actin motor to the erythrocyte surface. AMA1 bridges interactions between components of the motor and rhoptry-derived proteins that are inserted into erythrocyte membrane after reorientation (227, 228). The forward motion is generated by the movement of tight junction (from the apical to the posterior end of the merozoite) and the proteolytic cleavage of parasite-erythrocyte interactions along the moving junction.

ROM1 is not the only rhomboid protein that is involved in invasion of *P. falciparum*. ROM4 is localized to the merozoite surface and cleaves the transmembrane domain of adhesin proteins, that are important for parasite attachment to the erythrocyte surface (225). It has been shown that ROM4 cleaves EBA175, an adhesin required for the sialic acid dependent invasion pathway (229). This ROM4 mediated cleavage of EBA175 is essential as it has not been possible to mutate its rhomboid cleavage site.

The other important sheddase for erythrocyte invasion is SUB2, a transmembrane Ca^{2+} dependent protease that is secreted from micronemes into the merozoite surface (230). It is responsible for shedding the MSP1/6/7 complex, AMA1, and PTRAMP (231). Like other essential proteins, SUB2 is also refractory to genetic deletion suggesting an essential role.

Whilst ROM1, ROM4, and SUB2 are essential PMX appears to be a master regulator of erythrocyte invasion as it processes AMA1 (151). We also found that PMX can also cleave and activate SUB2 (unpublished data), whose inhibition then blocks shedding of AMA1, MSP1, and PTRAMP during invasion (232). Moreover, PMX also plays a key role in processing members of the EBL and Rh protein families, which are also

required erythrocyte invasion. Unfortunately, how these PMX-mediated processing events affect the function of these proteins still remains largely unknown. However, these processing events by PMX may play an important role in maturation of the proteins so they can be displayed on the merozoite surface to interact with their receptors. It is also important to mention that Rh5, a protein in Rh family that is also cleaved by PMX, is a leading malaria vaccine candidate and plays an essential role, when complexed with CyRPA and Ripr, in binding to the basigin, an erythrocyte surface protein (189-191). During invasion, PMX can target and process Rh5 at the N-terminal region. This N-terminal region has been reported to bind to Pf113, a GPI-anchored protein that tethers the Rh5/CyRPA/Ripr complex to the plasma membrane (192). This particular step is critical for the invasion as it has been reported that cleavage of Rh5 by PMX at N-terminal domain would release Rh5/CyRPA/Ripr complex from Pf113 at the merozoite surface. Without this PMX function, Rh5/CyRPA/Ripr complex would not be able to insert into the erythrocyte membrane resulting inability to invade (189).

PMIX function during invasion is not well understood but it also plays an important role in ring stage development after successful invasion. RAP1 and RON3 are identified as substrates of PMIX and they are essential for ring stage development (193, 194). RAP1 is released into the PV during invasion and, together with its partner RAP2 or RAP3, play a role in establishing this compartment. RON3, on the other hand, is deposited in the PV and essential for development beyond ring stage and functions with the PTEX translocon in the export of parasite proteins to the cytosol of host erythrocyte, and is required for transport of glucose from the host cell into the PV (194). Taken together, PMIX and X have a broad function in the activation of many proteins involved in egress, invasion, and parasite development, suggesting their importance as drug targets (151, 183).

Parasite sexual stages development

The mosquito midgut marks the interface between the human host and the insect vector for *Plasmodium* spp. According to this, they represent important aspects to

study a broad range of biological processes, including stage conversion and parasite/host co-adaptation. These midgut stages also represent a major bottleneck during the life cycle of *Plasmodium* as they have to survive for up to 24 hours outside host cell, expose themselves to various risk factors such as components of human immune system included within bloodmeal, natural midgut microbial flora in mosquito midgut, and the mosquito innate immune system. These aspects can lead to a 300-fold decrease in parasite survivability during transmission of *Plasmodium* to the mosquito. Due to this unique feature, midgut stages are prime targets for transmission blocking intervention strategies aimed to inhibit spread of the parasite by the mosquito. Several proteins that are involved in critical processes such as gametogenesis and conversion of the zygote to ookinete are identified as targets for transmission blocking vaccines or drugs. However, more work is needed to piece together the puzzle in order to reveal the big picture with respect to their role in malaria parasite development in the mosquito midgut.

Gamete formation

Conversion of gametocytes to gametes is activated within the mosquito midgut. During this period, activated gametocytes round up, egress from the enveloping erythrocytes and transform into fertile female macrogametes and male microgametes. Gametocytes then egress from the erythrocyte by an inside-out mechanism, during which the PVM ruptures prior to the opening of erythrocyte membrane. This PVM rupture is a Ca^{2+} -independent process and occurs at multiple locations at once (233). The osmiophilic bodies, ie. electron dense vesicles, can be detected and accumulate beneath the rupture sites and disappear simultaneously with the disintegration of the PVM while the activated gametocytes are rounding up (reviewed in (234).

Osmiophilic bodies can be found in both male and female gametocytes. However, in males, they appear to be smaller and less abundant than female osmiophilic bodies (235). Female osmiophilic bodies contain gametocyte-specific proteins including Pg377 (235-237) and GEST (gamete egress and sporozoite traversal) (238), which are discharged into the PV after gametocyte activation. Gametocytes lacking GEST

are found to have defects in egress and are stuck within the PV (238, 239) while Pg377 is found to play an important role in egress of *P. berghei* but not of *P. falciparum* gametocytes (235, 240).

Once ready to egress, an egress vesicle is released into the cytoplasm of host erythrocyte. These vesicles contain the plasmodial pore-forming perforin PPLP2, a protein that is able to perforate the erythrocyte membrane to release contents within the erythrocyte cytoplasm. The gamete is then enveloped by the erythrocyte membrane only and, approximately 15 minutes after uptake by the blood-feeding mosquito, the erythrocyte membrane opens and releases the fertile gamete (reviewed in (234)).

Exflagellation is the process when activated male gametocytes replicate their genome three times, turning from haploid to octaploid (241, 242) to mitotically produce eight flagellar microgametes. Flagellum formation requires axonemal assembly from basal bodies, which involves the centriole/basal body protein SAS-6 (243). Axoneme motility, on the other hand, is regulated by the conserved Armadillo-repeat protein PF16, and *P. berghei* parasites lacking this protein show abnormal flagellar movements (244). During exflagellation, microgametes detach from the residual body and is freely motile in search of a macrogamete. Several proteins have been shown to be involved in the attachment between micro- and macrogametes including the LCCL-domain proteins (termed CCp proteins in *P. falciparum*) and the 6-cys motif proteins Ps47, Ps48/45 and Ps230 (245-249). The latter two proteins are considered as promising candidates for transmission blocking vaccines (reviewed in (250)). It has recently been shown that in *P. falciparum* Ps230, Ps48/45 and the 6 CCp proteins assemble to multi-protein complexes, which are linked via P25 to the macrogamete surface; reviewed in (251).

The fertilization process begins with the fusion of the two gamete plasma membranes, and the axoneme and attached male nucleus enter the female cytoplasm. The gamete fusion is mediated by microgamete protein GCS1 (generative cell specific 1) and disruption of this gene in *P. berghei* resulted in male sterility and blocked fertilization

(252, 253). After gamete fusion, nuclear fusion ensues, and over the next three hours, meiosis occurs, and the zygote becomes tetraploid (242), a process involving the NIMA-related kinases Nek-2 and Nek-4 (254, 255). Parasite tetraploidy persists throughout the ookinete stage until sporozoite budding in the oocyst restores the haploid state (242).

Zygote to ookinete conversion

Development of zygotes and their transformations into ookinetes is accompanied by strict regulations of transcript expression. A *de novo* synthesis study in zygotes identified 91 proteins synthesized only after fertilization, most of which are involved in motility and invasion (256). Data from cross-fertilization study demonstrated that zygote/ookinete stage exhibits a maternal phenotype either from maternal mRNA inheritance or transcription of the maternal alleles, while the respective paternal alleles are silenced in these stages (257). Gene expression is further regulated by the apicomplexan transcription factor AP-O, which associates with more than 500 genes important for ookinete development, motility, midgut penetration and protection against mosquito immunity (258, 259). Among the genes identified are those encoding the IMC components like GAP40, 45, and 50, perforins PPLP3-5, the putative ookinete-secreted proteins PSOP1, 2, 6, 7, and 12, the secreted ookinete adhesive protein SOAP, the von Willebrand factor A domain-related protein WARP and for ookinete surface-associated proteins like POS1-10 or P25 and P28 (258). P25 and P28 are targets of highly effective transmission-blocking antibodies, which interfere with oocyst development. The proteins exhibit multiple and partially redundant functions, for example, they play a role in ookinete survival in the mosquito midgut, traversal of the epithelium and ookinete-oocyst transformation; reviewed in (250).

At the beginning of transformation from zygote to invasive ookinete, the retorts develop and form the new apical complex and IMC. Previous studies also found that, during midgut stages, downregulation of GAP45 leads to defect that zygotes are unable to transform into ookinetes (256). Lipid modification is another factor that important during conversion from zygote to ookinete as palmitoyl-S-acyl-transferase DHHC2 is

also found to be essential for this process as well (260). Moreover, the sexual stage-specific actin isoform actin 2, which was originally reported to play a role in male gametogenesis of *P. berghei*, is additionally important for ookinete formation (261, 262).

Besides lipidations, post-transcriptional modifications via phosphate group transfer are important for ookinete formation. *P. berghei* parasites lacking the protein kinase PK7 or the cyclin G-associated kinase GAK show severely reduced ookinete numbers (263). Furthermore, deletion of the Shewanella-like protein phosphatase (SHLP1), PPM2 or the kelch-like domain-containing phosphatase PPKL results in impaired *P. berghei* ookinete development, and phenotypes include impaired IMC and microneme formation (264-267).

The maturation of ookinetes is completed between 19 and 36 hours post-bloodmeal, and the ookinetes then quickly exit the midgut lumen (268-270). Ookinete motility appears to be regulated by cGMP levels, because disruption of the gene encoding GC β in *P. berghei* impaired ookinete gliding (271, 272). A similar phenotype was observed, when an inhibitor of the cGMP-dependent protein kinase PKG was added to the midgut stages; hence, an essential role of this kinase in ookinete motility is suggested (272). Also, the activity of CDPK3 is required for *P. berghei* ookinete motility and engagement with the mosquito midgut epithelium (273, 274), suggesting that besides cGMP, Ca²⁺ is important for ookinete motility. In accord with these findings, quantitative phosphoproteomics comparing *P. berghei* ookinetes sensitive and resistant to PKG inhibitors demonstrated that the kinase is involved among others in phosphorylation of IMC components like GAP45 and IMC1b as well as of enzymes involved in the inositol phospholipid metabolism, like phosphoinositol kinases, which in consequence results in the maintenance of high cytosolic Ca²⁺ levels (275).

An impressive number of micronemal proteins important for midgut traversal were identified in the past. CTRP, once secreted, inserts into the ookinete surface to form a molecular link between the epithelium and the actin/myosin motor and hence to mediate motility (276-280). Furthermore, 3 of the 5 plasmodial perforins were assigned

to mediating midgut traversal via breaching of the epithelial membranes, that is, PPLP3-5. Other microneme proteins assigned to midgut traversal of *P. berghei* ookinetes include SOAP, WARP and CelTos (cell-traversal protein for ookinetes and sporozoites) (145, 258, 277, 281-285).

One last challenge that the ookinete has to overcome before exiting the midgut lumen is the peritrophic membrane. This structure, secreted by the midgut epithelium, forms within 1-2 days post-blood meal. It consists of chitin and cross-linked proteins and functions in protecting the midgut from food particles as well as microbial infections; reviewed in (286). In order to breach through the peritrophic membrane before it has fully matured, the ookinetes secrete a chitinase able to hydrolyze the polysaccharide (287-289). Lack of this enzyme leads to reduced ookinete midgut infection (290, 291).

The role of plasmepsins during sexual stages

There are five plasmepsins that express within sexual stage life cycle including PMVI, VII, VIII, IX, and X. Among these, PMVII is the only plasmepsin that shown to be non-essential for parasite survivability as disruption of PMVII gene in *P. berghei* has no effect in blood or mosquito stages (148). PMVI is also dispensable during the blood stage but lack of PMVI significantly decreases the number of sporozoite in the salivary gland of *P. berghei* infected mosquitoes suggesting that it should plays an important role in sexual stages (145). PMVI is originally believed to be involved in sporozoite development as data from *PMVI* gene disruption in *P. berghei* showed that parasites are able to form oocysts normally at the outer layer of mosquito midgut. However, within these oocysts, sporulation, an important process that occurs within oocyst and gives birth to sporozoites, is does not occur resulting in significantly reduced sporozoites in salivary glands. In our study, we showed that PMVI was required for transmission of *P. falciparum* as the number of sporozoite in salivary gland was significantly decreased when mosquitoes were mosquitoes infected with parasites lack of PMVI (Fig 3.4b). We also found that a small number of sporozoites can egress from the oocyst into the mosquito hemolymph (Fig 3.5). These phenotypes were confirmed by complementation of PMVI knock-in parasites indicating that in *P.*

falciparum PMVI likely plays other roles. Based on our results, PMVI in *P. falciparum* could be involved in egress of sporozoites rather than specifically for sporulation. If PMVI is involved in egress of sporozoites then additional work focusing on egress-related proteins such as SERA8 (292) need to be investigated in order to fully understand the biology and molecular function of PMVI in sexual stage of *P. falciparum*.

We also studied the function of PMIX and X which are essential for both asexual and sexual stages (151, 183). In this study, our data showed that the antimalarial compounds WM4 (PMX specific inhibitor) and WM382 (PMIX and X dual inhibitor) are valuable compounds that block transmission of *P. falciparum* and *P. berghei* to mosquitoes demonstrating the importance of PMIX and X. In *P. falciparum*, we found that treatment of late-stage gametocytes with WM382 blocks oocyst formation in the mosquito midgut which results in failure of transmission into mosquito (Fig 5.4). In contrast, when treated with WM4, *P. falciparum* parasites show a slightly decreased number of midgut oocyst compared to wild-type (Fig 5.3). Taken together, these data indicate that PMIX is important for oocyst formation in *P. falciparum*. However, we cannot rule out the possibility that PMX could also be important since it has been previously demonstrated to be involved in gametocyte egress (151). In order to answer this question, we evaluated the transmission blocking activity of WM4 and WM382 in *P. berghei*. Interestingly, we found that, instead of WM382 alone, both WM4 and WM382 are highly potent at blocking oocyst formation and, therefore, blocking transmission (Fig 5.5). This could be due to the difference between species as *P. berghei* gametocytes develop more rapidly than *P. falciparum*. It was also previously shown to block gametocyte egress in *P. berghei* (151). Moreover, as we mentioned earlier, PMX is required for SUB2 activation. Once activated, SUB2, a protein located in the secretory osmiophilic bodies of gametocytes, is secreted from ookinetes during invasion of midgut epithelial cells (240). Taken together, these data indicate that the differences observed for WM4 and WM382 in *P. berghei* and *P. falciparum* may be a result of species-specific factors. More information is needed and we should focus on proteins that are functionally different between *P. berghei* and *P. falciparum*. For example, Pg377 seems to be an interesting candidate as it previously reported to be

female gametocyte-specific protein like PMX (151, 235-237) and demonstrated to play an important role in egress of *P. berghei* but not of *P. falciparum* gametocytes (235, 240).

In summary, our work has described the activity of two antimalarial compounds with a novel mechanism of action with one being PMX specific and whilst the other is dual acting against both PMX and X. Both compounds show an impressive transmission blocking effect against both *P. berghei* and *P. falciparum* suggesting they are great candidates for development as potential antimalarials. We also demonstrated the significance of 3 plasmepsins that are required for transmission in *P. falciparum*. Taken together, there are 4 plasmepsins that are essential during the transmission of *Plasmodium* parasites. Plasmepsin VIII is found to be responsible for sporozoite motility which make it essential for the transmission of parasite from mosquito to human host. Plasmepsin VI is also important for transmission of parasite from mosquito to human as it is required for sporozoite to develop and egress from oocyst. Plasmepsin IX and X, on the other hands, are involved in the transmission from human to mosquito as, once inhibited or genetically knocked out, parasite is unable to form an oocyst in the outer layer of mosquito midgut and preserve its infection. We still don't know why Plasmepsin X seems to be much more important in *P. berghei* compared to *P. falciparum* but this could be due to many factors which needed to be study later on in order to fully understand the mechanism of actions of both plasmepsins and how can we benefit from them.

Sexual stage

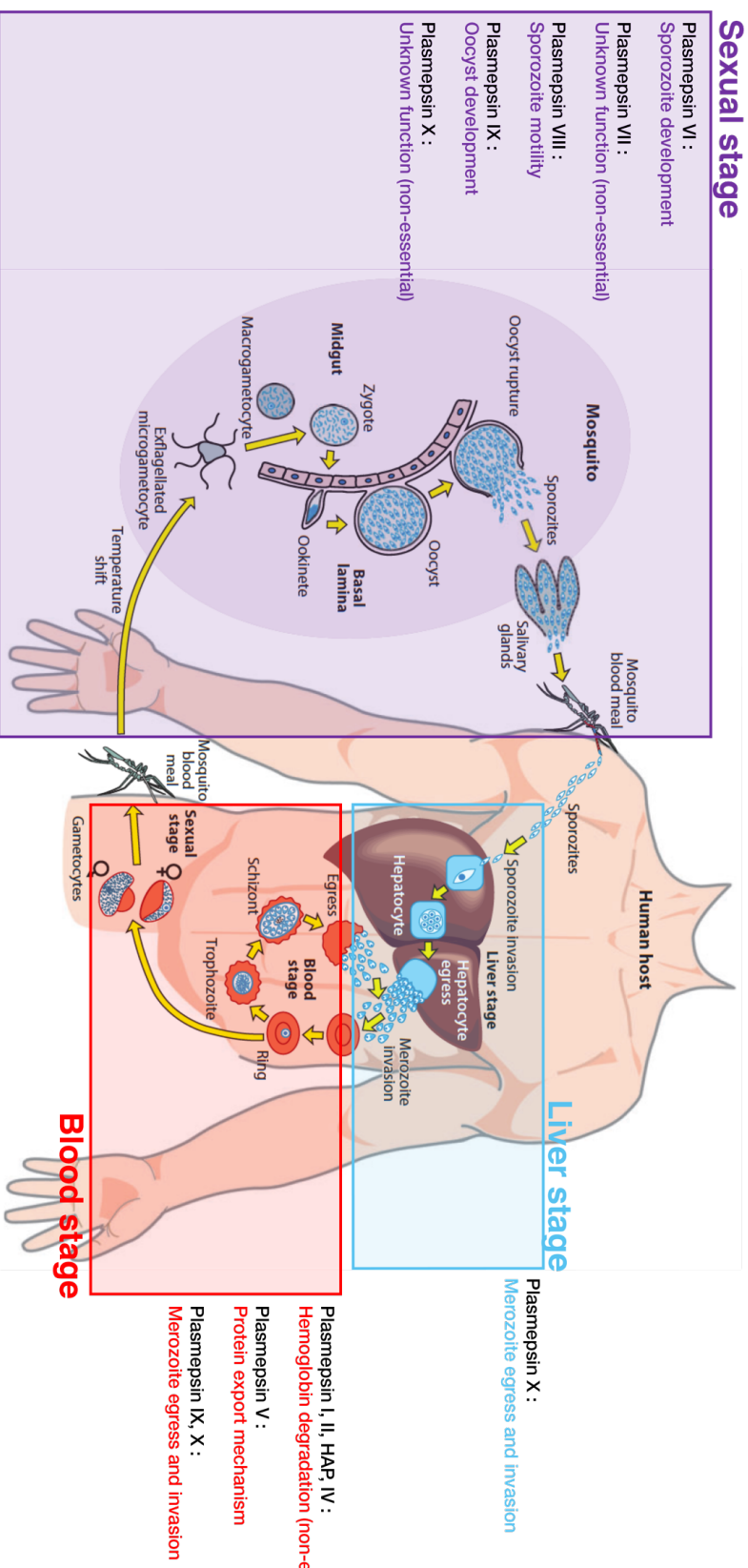


Figure 6.1 The roles of plasmepsins in *P. falciparum*

Plasmepsins are expressed throughout the whole parasite life cycle. 5 of them are considered essential and are required for parasite to maintain their infectivity and survive within its host. In human, plasmepsin IX and X has been demonstrated to be essential as they involve with the merozoite egress and invasion while plasmepsin V is important for protein export mechanism with infected erythrocyte. Here in this study, we have demonstrated 2 more plasmepsins that are significantly involved with the transmission process of *P. falciparum* to mosquito. Plasmepsin VI is required for sporozoite development and egress while plasmepsin IX is essential for oocyst formation in the outer layer of midgut. According to our data, these plasmepsins represent a good potential target for drug development and transmission-blocking strategies (adapted from (10)).

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