IDENTIFICATION OF POTENTIAL TARGETS FOR RETROVIRAL PROTEASE INHIBITORS IN *Plasmodium berghei*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my dear parents John and Loyce Onchieku; my brothers Edgar, and Aggrey as well as my sisters Fanish, Isabellah and Linet. Your continued support, prayers and encouragement gave me strength to reach this far and successfully complete this thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS……..**.....**Acquired Immune Deficiency Syndrome

CADD…….**.....**Computer-Aided Drug Discovery

CDC……...Centers for Disease Control

CO_2……..……...Carbon (IV) Oxide

CBRD……..Centre for Biotechnology Research and Development

CTMDR…….**.....**Centre for Traditional Medicine and Drug Research

CVS……..…….…….Concurrent Versioning System

DDI1……..…….DNA Damage Inducible Protein-1

dNTPs…….……Deoxyribonucleotide Triphosphate

DPI…….**.....**Days Post Infection

DV**.....**Digestive Vacuole

ED…….……Effective Dose

EDTA……..**.**Ethylenediaminetetraacetic acid

gDNA…….…..Genomic DNA

HAP ……..**.**Histo-aspartic Protease

DHFR…….……Human dihydrofolate Reductase

HIV……..……...…Human Immunodeficiency Virus

KEMRI……...…. Kenya Medical Research Institute

KO……Knock out

LP...……Lopinavir

$MgCl_2$…….…..Magnesium Chloride

NaHC03……..Sodium Hydrogen Carbonate

NCBI……..National Center for Biotechnology Information

Pb……..…..*Plasmodium berghei*

PBS…….….Phosphate Buffered Saline

PCR……Polymerase Chain Reaction

Pf..……...……*Plasmodium falciparum*

PIs ….……..……..Protease Inhibitors

PM…….…….….Plasmepsin

QSAR……...…….Quantitative Structure-Activity Relationship

RT……...……...…….Ritonavir

RMSD…….……...……. Root Mean Square Divisions

SQ……..…….……..Saquinavir

TB……Terrific Broth

TE……..……..Tris/EDTA

WHO…….……World Health Organization

WT…….……Wild Type

ABSTRACT

Retroviral protease inhibitors (RPIs) such as lopinavir (LP) and saquinavir (SQ) are active against *Plasmodium* parasites. However, the exact target(s) for these RPIs in the *Plasmodium* parasites is unknown. We hypothesized that LP and SQ suppress parasite growth through inhibition of aspartyl proteases. Using reverse genetics approach, the study embarked on separately generating transgenic parasite lines lacking Plasmepsin 4 (PM4), PM7, PM8, or DNA damage-inducible protein 1 (Ddi1) in the rodent malaria parasite *Plasmodium berghei ANKA*. The suppressive profiles of the LP/Ritonavir (LP/RT) and SQ/RT as well as antimalarials; Amodiaquine (AQ) and Piperaquine (PQ) were then tested against the transgenic parasites in the standard 4-day suppressive test. The Ddi1 gene proved refractory to the deletion, thus essential for the asexual blood stage parasites. Study results revealed that deletion of PM4 significantly reduces normal parasite growth rate phenotype (P = 0.0032). Unlike PM4_KO parasites which were less sensitive to LP and SQ (P = 0.0364, P = 0.0303), the suppressive profiles for PM7_KO and PM8_KO parasites were comparable to those for the WT parasites (P = 0.938 - 0.559, P = 0.6634 - 0.2013). This finding suggests potential role of PM4 in the LP and SQ action. Further analysis using modelling and molecular docking studies revealed that both LP and SQ had high binding affinities (-6.3 kcal/mol to -10.3 kcal/mol) towards the *Plasmodium* aspartyl proteases. It was concluded that PM4 plays an important role in assuring asexual stage parasite fitness and might be mediating LP and SQ action**.** The indispensable nature of the newly identified Ddi1 gene warrants further studies to evaluate its role in parasite asexual stage survival as well as its candidature as a target for RPIs.

CHAPTER ONE: INTRODUCTION

1.1 Background to the study

Malaria and HIV/AIDS are two most important challenges for public health stability and are responsible for more than two million deaths yearly (WHO, 2015); sub-Saharan Africa being the most affected. The two diseases share common vulnerability determinants, referred to as diseases of poverty, and their geographical overlap has raised opportunities and concerns for potential immunological, social, therapeutic and clinical interactions (WHO, 2015). At the clinical level, findings from Rutto *et al* (2015) & Whitworth *et al* (2000) showed that deteriorating immunity as a result of HIV-1 infection increases malaria clinical episodes and parasitaemia. Moreover, people living with HIV/AIDS, co-infected with malaria show increased recrudescence of malaria(Whitworth *et al*., 2000)

On the other hand, *in vitro, in vivo* and clinical studies have demonstrated that antiretroviral therapy, especially retroviral protease inhibitors (RPIs), exert a potent effect against both antimalarial drug-sensitive and drug-resistant *P. falciparum* as well as reduction in the incidence of malaria (Achan *et al.*, 2012; Andrews *et al*., 2006; Hobbs *et al*., 2009; Nsanzabana & Rosenthal, 2011; Parikh *et al*., 2005; Skinner-adams, Mccarthy, Gardiner, Hilton, & Andrews, 2004). A lopinavir–ritonavir–based regimen reduced the incidence of malaria by 41% as well as reduction in the risks of malaria recurrence in HIV-infected children under five years in Uganda. The findings were attributed to either a synergistic association between lopinavir and lumefantrine (an

antimalarial drug used in combination with artemether), reduced lumefantrine metabolism as well as direct antimalarial activity of the antiretroviral therapy (Achan *et al*., 2012). The latter hypothesis has been extensively supported by a number of *in vitro* and *in vivo* studies which have indicated that retroviral PIs exert significant activity against growth of *P. falciparum* parasites.

In their studies, Nsanzabana and Rosenthal (2011) tested seven RPIs and all of them inhibited the development of cultured *P. falciparum* parasites at pharmacologically relevant concentrations, lopinavir being the most potent compound as well as demonstrating moderate synergy with lumefantrine. Furthermore, using rodent-malaria parasites, *P. yoelii* liver burden was attenuated by 93% after oral administration of 100mg/kg lopinavir with 50m/kg ritonavir (Hobbs *et al*., 2009). There was also a significant reduction in median peak parasitaemia in mice infected with *P. chabaudi* AS after treatment with saquinavir, ritonavir and lopinavir, administered singly or in combinations (Andrews *et al*., 2006). These interactions between the malaria parasites and RPIs have provided further impetus for investigations into the anti-parasitic mechanism of action of these RPIs.

RPIs are used as second-line therapeutic agents in the treatment of HIV-1 patients and are inhibitors of retroviral aspartyl proteases (Abbenante, Fairlie, Abbenante, & Fairlie, 2005; Patick & Potts, 1998). Aspartyl proteases play key roles in the biology of humans, retroviruses such as HIV as well as parasites such as *Leishmania* sp., and *Plasmodium* sp (Ghosh, Mannhold, Kubinyi, & Folkers, 2011). In *P. falciparum*, there are 11 aspartyl proteases, ten of which are pepsin-like proteases

(plasmepsins; PMs) while the remaining is a retropepsin-like protease. Seven of the ten genes that encode pepsin-like proteases (PMs I–IV, PM V, IX and X) are expressed in the intraerythrocytic stages while PMs VI, VII and VIII are either expressed in the sexual stages or in the exoerythrocytic stages (Banerjee *et al*., 2002). PMs I–IV are located on chromosome 14 of *P. falciparum* and are localized in the digestive vacuole (DV), thus play a role in host hemoglobin digestion (Banerjee *et al.*, 2002; Mastan, Kumari, Gupta, Mishra, & Kumar, 2014; Moura, Dame, & Fidock, 2009; Nair, Singh, Angira, & Thiruvenkatam, 2016; Skinner-adams *et al.*, 2004). Whereas majority of studies on aspartyl proteases have focused on the pepsin-like proteases, especially PMs I–IV, the findings indicate that no single DV plasmepsin is likely to be the primary target of the RPIs due to functional redundancy (Omara-Opyene *et al*., 2004), necessitating studies involving inhibition of multiple PMs (Bonilla, Bonilla, Yowell, Fujioka, & Dame, 2007).

In *P. berghei,* there is only PMIV ortholog of the DV PMs that is situated on chromosome 10 (Liu, Robbins, Marzahn, & Mcclung, 2015). Therefore, PMIV is also functionally orthologous and is presumed to be independent of all other food vacuole PMs. PMIV seems to be a more attractive drug target since it is functionally superior, it is the only one found in all species of *Plasmodium* and that the paralogs in *P. falciparum* are just derivatives (Dame *et al.*, 2003; Moura *et al.*, 2009). The sensitivity of PMIV-mutant *P. berghei* to RPIs is yet to be determined

The eleventh protease is a retropepsin-like aspartyl protease called Ddi1 that bears a retroviral domain in its sequence. Ddi1 is a relatively poorly characterized

protease in *Plasmodium* parasites but has been shown that a Ddi1-like protein in *L. major* is an active aspartyl protease and is able to hydrolyze specific aspartyl protease peptide substrates (Perteguer *et al*., 2013). Besides, results from White, Powell, & Berry (2016) indicated that the Ddi1-like protein in *L. major* is a potential target for RPIs. This proposition is augmented by the fact that this protein is the only aspartic proteinase encoded by the *L. major* genome (White *et al.*, 2016).

Like in *P. falciparum, P. berghei* presents a putative Ddi1 ortholog at chromosomes 14 and 10 respectively and is shown to be highly expressed in the erythrocytic stages of their growth (PlasmoDB, 2016a). Blood stages of *P. falciparum* is associated with immense significance in clinical manifestations of human malaria. The protein is found in all *Plasmodium* species and is thought to be a funtionally important protein in ubiquitin mediated proteolysis pathways (PlasmoDB, 2016a, 2016b). There is currently no data available interrogating this protein in *Plasmodium* parasites as a potential target for RPIs.

1.2 Statement of the problem

Incidences of parasite tolerance and resistance to the ACTs were first reported in western Cambodia and has since spread to other parts in Asia. This region is historically recorded as a site of emerging resistances to the previous first-line antimalarial therapies which later rapidly spread across the African countries. Thus the resistance trend poses an eminent danger to malaria elimination efforts in Africa where malaria transmission is consistently high. Since the options of drugs for which the human malaria parasite *Plasmodium falciparum* has not evolved resistance is rapidly diminishing, new and

rational approaches to the prevention and treatment of malaria infections are urgently needed.

In vitro, in vivo and clinical studies have demonstrated that RPIs have significant activity against growth of *Plasmodium* parasite. Attempts have been made towards understanding the mode of action of these RPIs in suppressing growth of *Plasmodium* parasites. However, to date, there is no single study that has conclusively determined the mechanisms of action of these drugs. This study interrogated selected *Plasmodium* aspartly proteases as potential targets for the RPIs.

1.3 Justification of the study

In the wake of parasite resistance to the current anti-malarial drugs, development of novel agents that act on enzymes essential for parasite metabolism, such as proteases, are attractive targets for anti-malarial drug development. However, development of such agents require initial validation of the enzymes as potential targets. This study used *P. berghei* and PlasmoGEM genetic modification vectors to evaluate the essentiality and suitability of PM4, PM7, PM8 and Ddi1as potential targets for RPIs. The study hoped to provide basis for the development of such inhibitors as antimalarial therapy.

1.4 Hypothesis

RPIs do not suppress the growth of malaria parasite through inhibition of PM4, PM7, PM8 or Ddi1.

1.5 Main Objective

To determine the potential targets for RPIs in *Plasmodium berghei ANKA.*

1.5.1 Specific Objectives

1. To separately generate PM4, PM7, PM8 or Ddi1-knockout *Plasmodium berghei ANKA* mutant parasites
2. To determine the growth rate phenotype of wild type and the knockout *Plasmodium berghei ANKA* mutant parasites
3. To determine the sensitivity of wild type and the knockout *Plasmodium berghei ANKA* mutant parasites to retroviral protease inhibitors
4. To correlate the sensitivity of wild type and the knockout *Plamodium berghei ANKA* mutant parasites with *in silico* RPIs' predicted binding affinities to PM4, PM7, PM8 and Ddi1.

CHAPTER TWO: LITERATURE REVIEW

2.1 Global burden of malaria

Notwithstanding the immense investments in malaria programs to date, it remains to be major global health problem in most regions of the world including Africa, Asia and parts of the Eastern Mediterranean Region (Breman *et al*., 2006; WHO, 2017b). The global distribution of malaria is a function of the species of the *plasmodium* parasites, exposure, the climatic conditions and the mosquito vectors available in those regions as well as the social, economic and political development status of these regions (CDC, 2016b; Parham *et al.*, 2015; WHO, 2017b). Anopheles mosquitoes are the principle vectors of malaria transmissions, and the intensity of transmission increases with mosquito preference to bite humans as well as increase in mosquito lifespan; for complete parasite development. Besides, climatic conditions that favor multiplication and survival of mosquitoes enhance the transmission of malaria. This is normally observed in periods just after a rainy season. In fact, the selective human-biting habit and the long lifespan of the vectors in Africa is solely associated with the high burden of the disease in the region (WHO, 2017b).

Latest reports indicate that there was an average of 216 million cases of new malaria infections throughout the world in 2016, 90% of which occurred in Africa, especially sub-Saharan Africa. 7% of these new cases occurred in South-East Asia Region while the remaining 3% were recorded in Eastern Mediterranean Region. Sub-Saharan Africa continues to bear the highest burden of the disease and, out of the estimated 445 000 malaria mortalities in 2016, over 90% of the cases occurred in this

region, especially in children under 5 years of age. 292 000 children in Africa succumbed to malaria out of the estimated 306 000 mortalities worldwide in 2016 alone. Country-level trends showed that, in 2016, the concentration of malaria burden was most felt in 15 countries, which are mainly found in Africa and made up to 80% of global malaria incidences and 78% of all mortalities (WHO, 2017b).

Out of the five *Plasmodium* parasite species that cause malaria in human beings, *P. falciparum* and *P. vivax* pose the highest threats, with *P. falciparum* accounting for the highest shares of the disease burden while *P. vivax* accounting for the highest geographical distributions of the disease (Bousema & Drakeley, 2011). In Africa, *P. falciparum* is the common malaria parasite and is responsible for majority of infections and almost all deaths (WHO, 2017b). It is estimated that around 2.4 billion people risk malaria transmission as a result of *P. falciparum* with an average of 400 million clinical cases and 1 million deaths every year (Guerra *et al*., 2008; Hay *et al*., 2010). On the other hand, despite the low malaria mortality cases associated with *P. vivax,* it is geographically wide spread. It is estimated that 2.9 billion people risk malaria infections from *P. vivax* with an approximate annual maximum of 300 million clinical episodes (Guerra *et al*., 2010; Mueller *et al*., 2009).

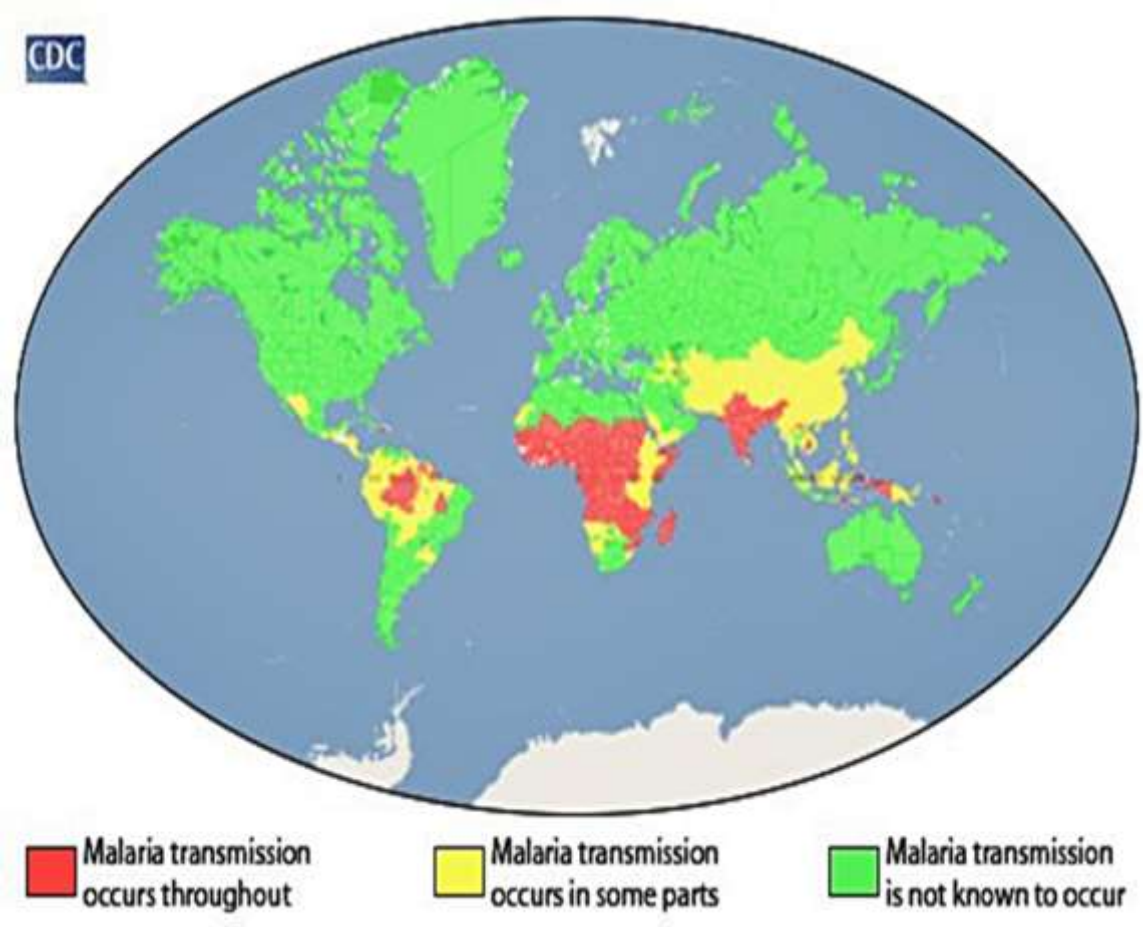


Figure 1: World geographical distribution of malaria, Source; (CDC, 2016b)

2.2 Antimalarial drugs and emerging resistance

Perhaps the greatest obstacle facing malaria management worldwide, and contributing significantly to its burden, is the emergence, spread and intensification of human parasite resistance to antimalarials (Blasco, Leroy, & Fidock, 2017). For decades, chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) have been used as antimalarial drugs in the world. However, due to parasite resistance to these drugs, the spectrum of antimalarial drugs was widened by development of drugs such as mefloquine, atovaquone, proguanil and pyrimethamine; drugs that have since lost ability to inhibit

parasite growth (Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick, 2002). Incidences of parasite resistance to all the drugs have always been first reported in Southeast Asia in Western Cambodia and the phenomenon has always spread to other regions, including Africa which bears the highest burden of the disease (Noedl *et al.*, 2008; WHO, 2017b).

After the discovery of artemisinin derivatives such as artesunate, artemether, and dihydroartemisinin in the 1990s, artemisinin-based combination therapies have been recommended as first-line treatments for human malaria parasite in most malaria-endemic countries. Together with other malaria management strategies, artemisinin-based combination therapies have greatly reduced malaria-related mortalities (Abbas *et al*., 2007). Unfortunately, this milestone in malaria control and elimination is now threatened by the emergence of artemisinin resistance. Incidences of parasite resistance to artemisinin-based drugs were first reported in western Cambodia and has since spread to other parts in Asia. This region is historically recorded as a site of emerging resistances to the previous first-line antimalarial therapies (Ashley *et al*., 2014; Noedl *et al*., 2008; Phyo *et al*., 2012). Resistance to artemisinin-based drugs has been associated with a point mutation in the "propeller" region of the kelch protein gene (k13) (Talundzic, Okoth, & Congpuong, 2015). Molecular surveillance studies have traced the K13-propeller mutations in Thailand, Myanmar, Vietnam, Laos, India, China and Bangladesh (Ashley *et al.*, 2014; Blasco et al., 2017; Fairhurst & Dondorp, 2016; Talundzic *et al.*, 2015). This trend poses an eminent danger to Africa, which bears the

highest cases of malaria infections (Talisuna *et al*., 2012). Therefore, new approaches to the prevention and treatment of malaria infections are timely.

2.3 Malaria and HIV interactions

Malaria and HIV/AIDS are the two most important challenges for public health stability and are responsible for more than four million deaths yearly, sub-Saharan Africa being the most affected (WHO, 2005). The two diseases share common vulnerability determinants, referred to as diseases of poverty, and their geographical overlap has raised opportunities and concerns for potential immunological, social, therapeutic and clinical interactions (WHO, 2005). At the clinical level, it has been shown that deteriorating immunity as a result of HIV-1 infection increases malaria clinical episodes and parasitaemia. This association tends to increase with advancing immunosuppression. Moreover, people with HIV/AIDS, co-infected with malaria show increased malaria recrudescence (Whitworth *et al*., 2000).

On the other hand, both clinical and *in vitro* studies have demonstrated that RPIs exert a potent effect against both antimalarial drug-sensitive and drug-resistant *Plasmodium* parasites. The RPIs have been shown to have a positive effect on the incidence of malaria. A lopinavir–ritonavir–based regimen reduced the incidence of malaria by 41% as well as risks of malaria recurrence in HIV-infected children under five years in Uganda. The findings were attributed to either the synergistic association between lopinavir and lumefantrine, reduced lumefantrine metabolism as well as direct RPIs activity on the parasites (Achan *et al*., 2012). The latter hypothesis has been extensively supported by a number of *in vitro* studies which have documented that RPIs

exert significant activity against growth of *P. falciparum* parasites. Besides, the previously published effects of RPIs on cytoadherence and phagocytosis, Skinner-adams *et al*. (2004) reported the first study showing that RPIs (saquinavir, ritonavir, and indinavir) directly inhibit the *in vitro* growth of both drug-sensitive and drug-resistant *P. falciparum* parasites at clinically relevant concentrations.

Subsequently, a wider range of RPIs were shown to be active and inhibited the development of cultured *P. falciparum* parasites at pharmacologically relevant concentrations, lopinavir being the most potent compound. Importantly, lopinavir was found to be active against *P. falciparum* at concentrations lower than those achieved with standard dosing of lopinavir-ritonavir. Besides, lopinavir exhibited moderate synergy with the lumefantrine (Nsanzabana & Rosenthal, 2011).

2.3 Human malaria species

Traditionally, only four species of *Plasmodium* are recognized as causes of human malaria. These species include *P. falciparum*, *P. vivax, P. malariae* and *P. ovale*. However, in the recent past, there has been an upsurge of malaria infections as a result of *P. knowlesi*, a simian species in South-East Asia (Antinori, Galimberti, Milazzo, & Corbellino, 2012; CDC, 2016c)

Tropical and subtropical regions are highly infested with *P. falciparum.* Africa is a typical example of such regions harboring the highest densities of this species (Guerra *et al*., 2008). *P. falciparum* is the principle cause of severe malaria due to its multiplication rate in blood and can also cause cerebral malaria when infected parasites clog in the brain (CDC, 2016c; Gething et al., 2011). In 2002, the sequence of the whole

P. falciparum genome was published, giving way for major molecular struggles against this lethal species (Antinori *et al*., 2012).

P. vivax has the widest geographical spread, present throughout the tropics with highest densities in Asia and Latin America. In most cases, the parasite causes benign tertian fever and is relatively not life-threatening. It preferentially invades reticulocytes, a fact that is associated with the limited reproductive capacity with a maximum of 2% parasitaemia in relation to circulating red blood cells. The sequence of *P. vivax* was published in 2008 and bears a higher nuclear genome compared to that of *P. falciparum* (Antinori *et al*., 2012).

P. malariae is well scattered in all malaria endemic regions and causes "quartan malaria". It has a preference for invasion of older erythrocytes (Antinori *et al*., 2012; Chiang, Bujnicki, Su, & Lanar, 2017). Compared to other regions, *P. malariae* is milder and causes more infections in southwest Pacific and in sub-Saharan Africa, with low parasite densities per microliter. This phenomenon is as a result of slow parasite development and the preference for invasion of older erythrocytes (Antinori *et al*., 2012; Mueller, Zimmerman, & Reeder, 2007).

P. ovale is not a common species and is confined to areas in tropical Africa, the Philippines, eastern Indonesia and New Guinea. West and Central Africa has a *P. ovale* malaria prevalence of more than 10% (Mueller *et al*., 2007).

P. knowlesi, a simian *Plasmodium*, was first reported in 2004 and is mainly found in Southeast Asia. The parasite is associated with zoonotic malaria and exploits

Anopheles leucosphyrus (forest-dwelling mosquitoes) as vectors. It is yet to be detected in Africa (Singh & Daneshvar, 2013).

2.4 Rodent malaria parasites

Rodent malaria parasites have been extensively relied upon as practical model organisms for experimental studies of human malaria. These parasites show high similarity to human malaria parasites in terms of the genetic composition, structure and physiology as well as their lifecycles (Craig *et al*., 2012; Otto *et al*., 2014). The current research surrounding human malaria has led to adoption of four African rodent malaria species for use in the laboratories. These parasites include, *P. berghei, P. chabaudi, P. yoelii* and *P. vinckei* (Otto *et al*., 2014). *P. chabaudi* is extensively used to study mechanisms of drug resistance and immune evasion (Langhorne, Ndungu, Sponaas, & Marsh, 2008; Schneider *et al*., 2012). *P. yoelii* is used to study the biology of liver stages of parasite growth and immunity associated with the liver stages (Miguel, Maria, & António, 2011). In contrast, *P. berghei* has a preference for infection of reticulocyte and is often associated with severe malaria in mice. It has been used to study drug resistance as well as experimental cerebral malaria (Craig *et al*., 2012; Langat, Kiboi, Irungu, & Kimoloi, 2012).

P. berghei was first isolated in 1948 from blood of a thicket rat and shows an infection preference to rats, hamsters and mice. In the laboratory, the parasites can be transmitted by *Anopheles stephensi* mosquitoes and it infects reticulocytes (Culleton, 2005). So far, there are five isolates of *P. berghei;* ANKA, K173, KSP11, NK65, and LUKA which have been confirmed to be genetically identical (Saul, Prescott, Smith,

Cheng, & Walliker, 1997). The ability to study *P. berghei* parasites throughout the whole life cycle compounded with the availability and use of reverse genetics technologies have made it easier to perform functional screens. This parasite provides the most robust platform for manipulations of the genome with a high degree of transfection efficiency (Gomes *et al*., 2015; Otto *et al*., 2014). In *P. falciparum*, there is no efficient reverse genetic techniques that can be used to carry out functional studies (Balu & Adams, 2006). Genetic manipulations such as gene knockouts have been successfully undertaken in *P. berghei.* For instance, PMIV-deficient mutants have been created and have shown to have be associated with reduced virulence (Spaccapelo *et al*., 2010). The availability of these robust reverse genetics techniques has yet to be widely used to test sensitivity of the mutants to different drugs.

2.5 Life cycle of the malaria parasite

The life cycle of human malaria parasite species is fairly complex and involves an exogenous sexual phase which is commonly referred to as sporogony and an endogenous asexual phase, referred to as schizogony. The sporogony cycle accounts for the development and multiplication of the parasites in female *anopheles* mosquitos while the schizogony cycle develops in humans (Antinori *et al.*, 2012; CDC, 2016a). The latter phase initiates the human malaria infection after inoculation of sporozoites by a female anopheles mosquito, where it undergoes both liver stages and blood stages which account for the clinical manifestations of malaria. The sporozoites enter parenchymal cells of hepatocytes where they develop and multiply in a process referred to as preerythrocytic schizogony. The exoerythrocytic schizogony is characterized by

multiplication and differentiation of the sporozoites in the liver giving rise to tissue schizonts which harbor thousands of hepatic merozoites. In *P. falciparum,* the exoerythrocytic schizogony takes a minimum of six days and the hepatic merozoites may count up to 30 000. The schizonts, containing hepatic merozoites, rupture and release merozoites into the bloodstream (CDC, 2016a). This marks the end of the tissue phase of the schizogony cycle. However, for *P. ovale* and *P. vivax,* the mature schizonts form hypnozoites, a phenomenon that leads to relapse (Antinori et al., 2012; CDC, 2016a).

The erythrocytic schizogony is initiated after infection of the red blood cells by the merozoites via an actomyosin motor system (Antinori et al., 2012) . After invasion, the ring stage develops into trophozoites. All *Plasmodium* species have the same and constant period from infection by mosquito bite through to the development of trophozoites. This period is referred to as the "prepatent period" and takes 9 days in *P. falciparum* (Antinori *et al*., 2012). Maturation of trophozoites leads to the production of blood schizonts with each schizont containing up to 36 merozoites which are released to the blood stream after rapture of the red blood cells. These merozoites re-initiate a new erythrocytic schizogony cycle by infecting other erythrocytes (CDC, 2016a). Alternatively, some merozoites differentiate into macrogametocytes (female) and microgametocytes (male) which accounts for the sporogonic cycle when ingested by a female anopheles mosquito (CDC, 2016a).

After ingestion of an infected blood meal, there is fusion of the male and female gametes forming a zygote which evolves into a slowly motile and elongated ookinete.

Ookinetes actively invade and penetrate the mosquito midgut epithelium and develops into oocysts. The mature oocysts rapture and release sporozoites which migrate to the acinal cells of salivary glands in readiness for a new infection cycle (Bousema, Okell, Felger, & Drakeley, 2014; CDC, 2016a).

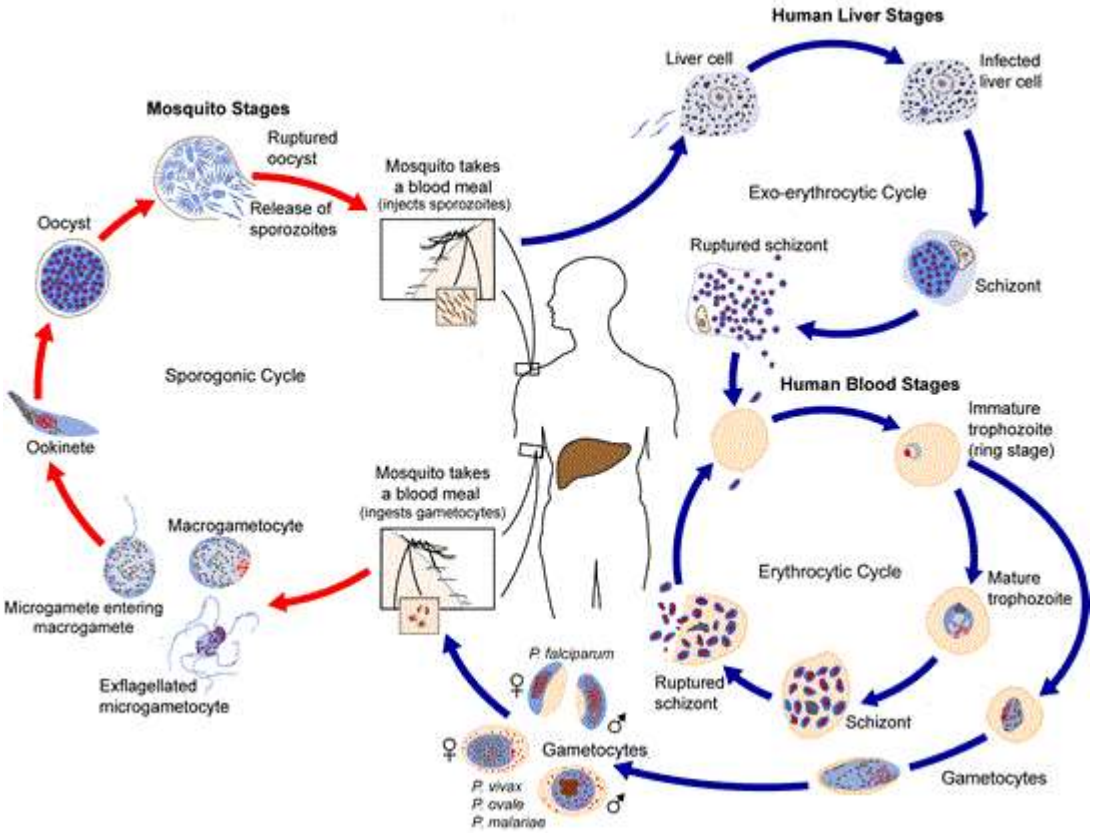


Figure 2: Lifecycle of malaria parasite, Source; (CDC, 2016a)

Despite the similarities observed in all *Plasmodium* species, there are very specific differences that are evident in some parasites. *P. berghei* is one of the species that presents such difference as it shows a strong preference for invading of reticulocytes (Chris, 2016). This leads to production of more merozoites in reticulocytes compared to

those that grow in mature erythrocytes. As a response to unavailability of reticulocytes, during the course of an infection, *P. berghei* lines invades normocytes (Chris, 2016) In addition, unlike *P. falciparum* where commitment to sexual differentiation happens before maturation of the schizonts, sexual commitment in *P. berghei* takes place in the trophozoite stages (Chris, 2016). Besides, in *P. berghei*, there is asynchrony in the different blood developmental stages as well as a short period of development of mature gametocytes from merozoites which is only 26-30 hours (Chris, 2016).

2.6 Hemoglobin digestion

The erythrocytic schizogony is an important phase for parasite growth, maturation and asexual replication. The erythrocytic cycle comprises of three morphologically well-defined stages which include the ring, trophozoite, and schizont stages. All these stages develop in blood, a cell that contains a single major protein called hemoglobin. Out of these three stages, the trophozoite stage which lasts for 10–12 hours is characterized by highest metabolic activities that include ingestion and degradation of host erythrocyte hemoglobin in acidic digestive vacuoles via a cytosome (Klemba, Beatty, Gluzman, & Goldberg, 2004). In *P. falciparum*, degradation of hemoglobin takes place primarily in trophozoites and early schizonts (Rosenthal, 1998). Essentially, the limited ability of the parasites for exogenous uptake from host plasma or *de novo* synthesis of amino acids for their protein synthesis, growth and reproduction, prompts the parasites' resort to host hemoglobin digestion. It is estimated that the parasites degrade up to 75% of the hemoglobin in infected erythrocytes (Goldberg, Slater, Cerami, & Henderson, 1990).

Malaria parasite hemoglobin degradation is a massive and complex catabolic process that is vital during intra-erythrocytic development. This process takes place in an acidic DV and is mediated by the concerted action of nearly a dozen proteases. An ordered pathway exists that efficiently catabolizes hemoglobin to yield amino acids that the parasite uses as a nutrient source (Goldberg, 2005). With regard to the enzymology of hemoglobin degradation, two families of proteases play prominent roles. Four aspartic proteases called plasmepsins, aminopeptidases, metalloprotease falcilysin, and three cysteine proteases called falcipains are found in the food vacuole (Daniel E. Goldberg, 2005). All of them are capable of degrading hemoglobin or globin. The presence of multiple plasmepsins with overlapping specificity and function and multiple falcipains that overlap each other is strategic to increase parasite fitness (Liu, Istvan, Gluzman, Gross, & Goldberg, 2006; Omara-Opyene *et al*., 2004). Therefore, given the importance of food vacuole proteases in hemoglobin digestion, these enzymes are attractive drug targets (Qidwai, 2015; Rosenthal, 1998).

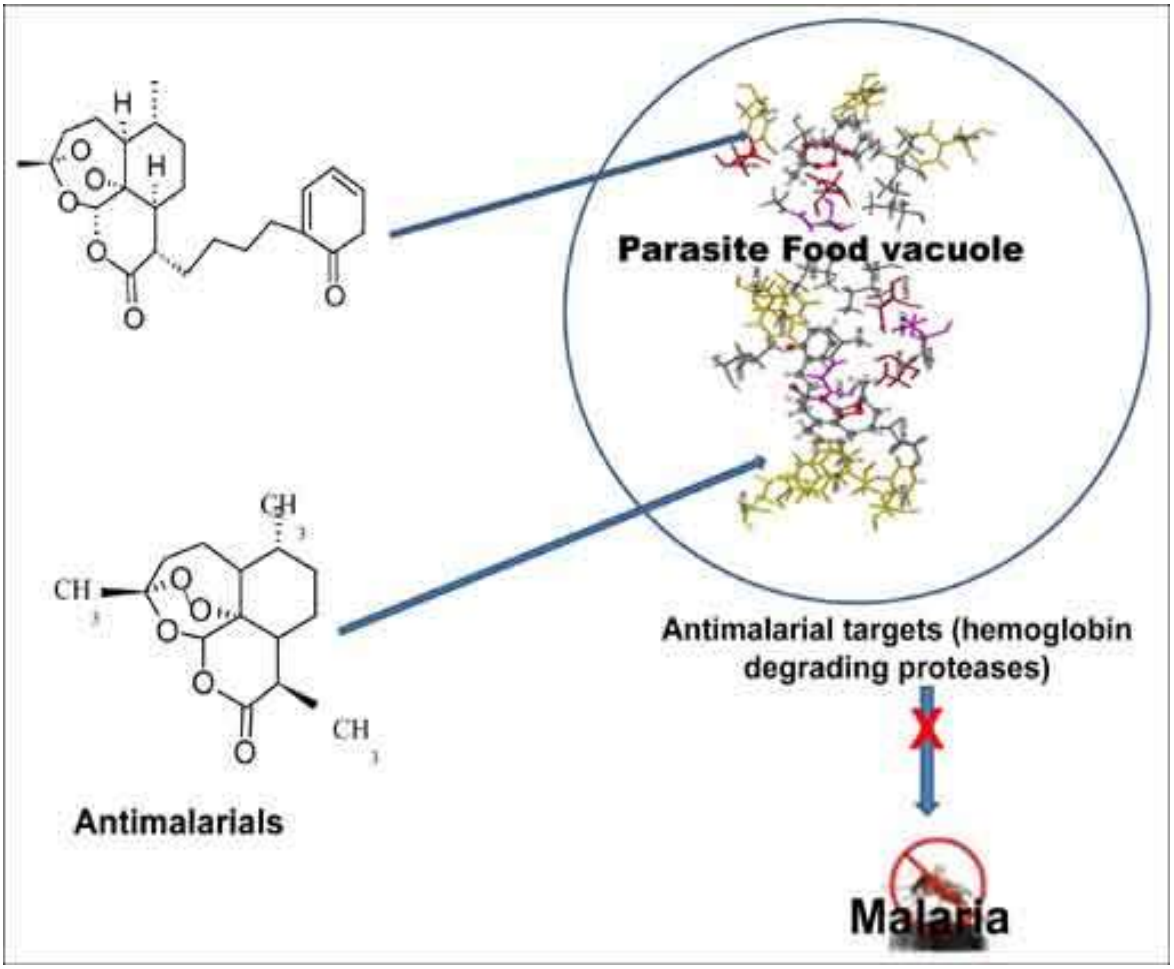


Figure 3: Graphical representation of potential drugs targets in hemoglobin degradation pathway, Source; (Qidwai, 2015)

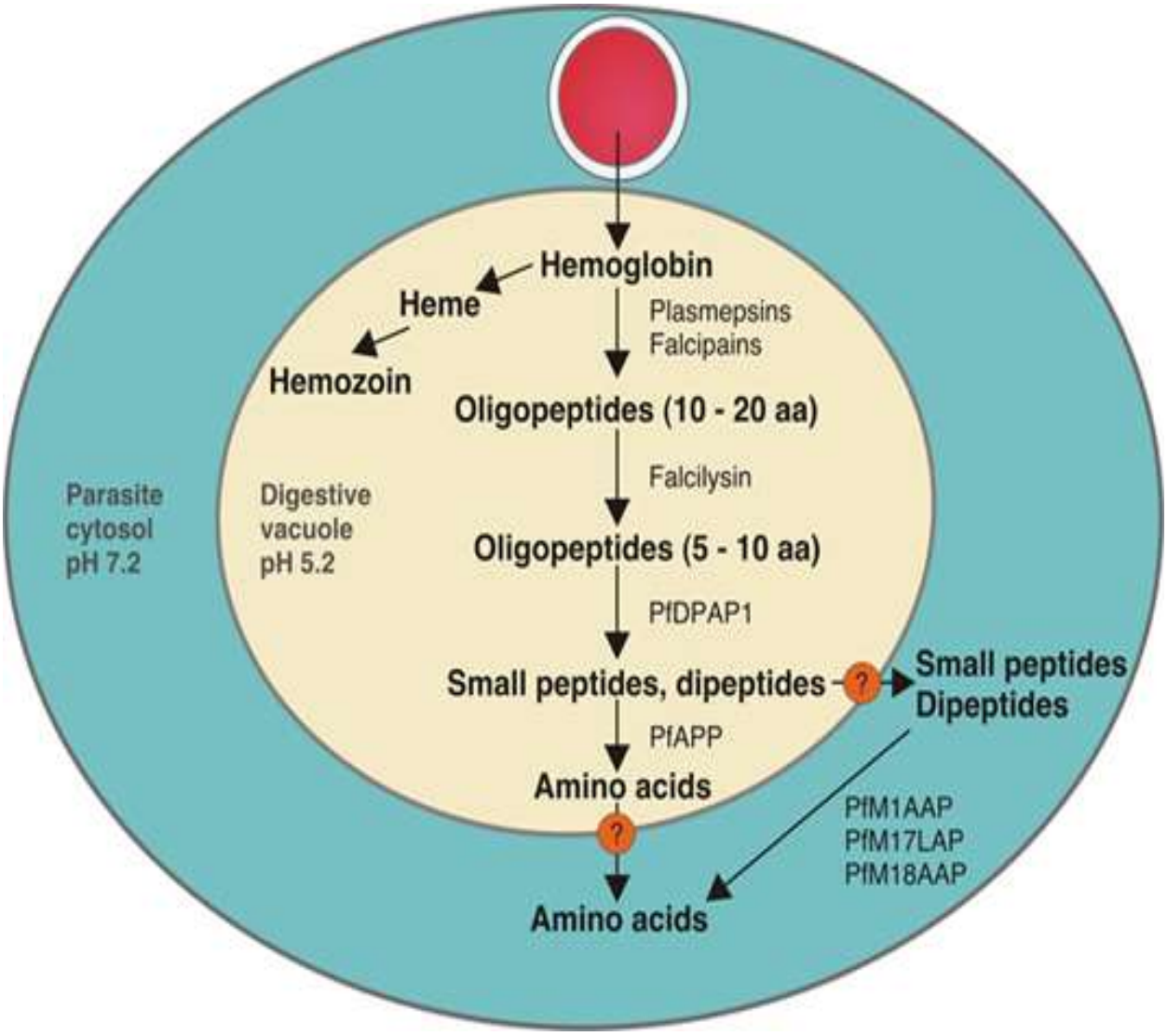


Figure 4: Hemoglobin degradation pathway, Source; Wunderlich, Rohrbach, & Dalton, 2012

During the hemoglobin degradation process, free heme is released. Free heme is potentially toxic to the parasites due to its ability to destabilize and lyse membranes, as well as inhibiting the activity of several enzymes (Teixeira, Gomes, & Gomes, 2011; Wunderlich *et al.*, 2012). To counter this, the parasite has evolved a detoxification system resulting in the formation of hemozoin, a malarial pigment. So far, some

antimalarials such as chloroquine and other quinolines inhibit this pigment formation, as well as the heme degradative processes and thereby prevent the detoxification of heme (Teixeira *et al.*, 2011; Wunderlich *et al.*, 2012).

2.7 Aspartyl proteases

Aspartyl proteases are involved in a number of important metabolic processes in living organisms. Parasite aspartyl proteases are responsible for semi-ordered pathway for hemoglobin digestion in order to secure nutrition for intraerythrocytic malaria parasites and subsequent rapturing of the host erythrocytes (Rosenthal, 1998). In addition, recent literature has indicated that some aspartyl proteases are implicated in ubiquitin binding pathways (PlasmoDB, 2016a, 2016b). In *P. falciparum*, there are eleven aspartyl proteases, ten of which belong to the A1 family of proteases while the remaining putatively belongs to the A2 family of proteases. The A1 family of proteases are pepsin-like, commonly referred to as plasmepsins (White *et al*., 2016), while the remaining protease in A2 family is a retropepsin-like protease.

The ten plasmepsins that have been identified in *P. falciparum* are; PfPM I - X. PfPM III, which is also referred to as histo-aspartic protease (HAP), has an unusual active site with a histidine in place of one of the two standard catalytic aspartates present in the other plasmepsins in the same class (Banerjee *et al*., 2002; Cai *et al*., 2011). These plasmepsins were formerly known as “acid proteases” or “carboxyl proteases” because of their preferred activity in an acidic pH. Essentially, they are divided into two distinct classes: PM I-IV are located next to each on chromosome 14, have a single exon and are

intronless. They are thought to exist as a result of tandem gene duplications for parasite fitness. PM V-X are representatives of ancestral type of plasmepsins and functions of some of them are yet to be authoritatively elucidated (Cai *et al*., 2011).

Virus-encoded proteases represent essential enzymes for all retroviruses. Retroviral aspartyl proteases, referred to as retropepsins, undoubtedly represent the most thoroughly studied proteolytic enzymes in the history of science. Retroviral aspartyl proteases of HIV have been studied extensively as they are essential for conversion of immature virions into mature, infectious virus. This has led to development of specific inhibitors of these proteases and have proved to be highly effective antiviral agents (Konvalinka, Kräusslich, & Müller, 2015). Besides, these proteases are also virulence factors implicated in the mechanisms of host colonization by the yeast *Candida albicans* in different types of candidiasis, as such they are targeted in the development of treatment for and immunization against this common fungal infection (Vilanova *et al*., 2004). Research showing the importance of aspartyl proteases in many biological functions of *Leishmania* sp., *Plasmodium* sp. and HIV, and being targets of some clinically useful drugs in the market today (Alfonso & Monzote, 2011); suggests that these enzymes are appealing to those seeking novel anti-parasitic drugs.

2.7.1 Plasmepsins

Hemoglobin degradation takes place in an acidic compartment, referred to as the digestive vacuole (DV) and the digestion plays an important role in the intraerythrocytic stages of malaria parasite development (Wunderlich et al., 2012). This semi-ordered digestion process acts as a source of amino acids and is thought to help maintain

intracellular osmolarity during rapid parasite growth. This process is thought to involve the action of four aspartyl proteases, referred to as plasmepsins (PMs). These enzymes have received considerable attention as potential antimalarial drug targets. The catabolism of hemoglobin in the DV is thought to occur in a semi-ordered sequence of proteolytic events involving *P. falciparum* plasmepsins PfPM I, II, HAP and IV. Previous researchers have indicated that expression of Pf PM I, II, HAP, IV, V, IX and X takes place in the erythrocytic stage, whereas the expression of PfPM VI, VII, and VIII happens either in the sexual stages or in the exoerythrocytic stages of parasite growth (Banerjee *et al*., 2002; Omara-Opyene *et al*., 2004). PfPM I is transcribed in the early ring stage followed by PfPM II, which is optimally expressed in the trophozoite stage. HAP and PfPM IV are detectable as from the trophozoite stage and all the four persist to schizogony (Ersmark, Samuelsson, & Hallberg, 2006).

In the hemoglobin degradation pathway, two homologous aspartyl proteases; PfPM I and II have been implicated in the initial hemoglobin degradation process by cleaving the native hemoglobin molecule in a highly conserved Phe^{33}–Leu^{34} bond in the hinge region. Subsequently, this initial hydrolysis renders the protein a partially degraded molecule, which after unwinding, becomes susceptible to further downstream proteolysis by the other digestive vacuole proteases (Banerjee, Francis, & Goldberg, 2003). Besides being acted upon by cysteine proteinases, aminopeptidases and metalloprotease falcilysins, the partially degraded hemoglobin also gets denatured by HAP and PfPM IV (Banerjee *et al*., 2003). These two digestive vacuole plasmepsins share approximately 60% sequence identity with PfPM1 and PfPM2 and have similar

targeting pro-regions (Banerjee *et al.*, 2003). HAP is an active enzyme despite having an unusual active site with a histidine in place of one of the two standard catalytic aspartates present in the other PMs in the same class (Banerjee *et al*., 2003). The other 6 genes which encode PfPMs V–X, are not clustered together and have different expression patterns. PfPM V is expressed over the course of asexual intraerythrocytic development. Expression of PfPM V is very low in the ring stages of parasite development and increases regularly through schizogony. It is an essential protease as it is involved in processing of effector proteins for export to colonized erythrocytes (Hodder et al., 2015). These actions help the parasites to remodel their niche in order to induce severe virulence and evade host immune defense (Hodder *et al*., 2015; Klemba & Goldberg, 2005; Sleebs *et al*., 2014). PMVII and PMVIII are expressed in gametocyte and sporozoite stages. Whereas the role of PM VII is dispensable (Mastan *et al*., 2014), PM VIII is critical for sporogonic stages of malaria parasite life cycle (Mastan, Kumar, Dey, Arun, & Mishra, 2017). Both PMs IX and X are critical for parasite survival. PM IX is plays an essential role during invasion of the erythrocytes while PM X is critical in the mosquito-infective stages (Li, Bounkeua, Pettersen, & Vinetz, 2016; Nasamu *et al*., 2017).

The DV PMs have long been studied as potential candidates for drug targets and subjected to both biochemical and functional studies with the wish that blocking them would hamper hemoglobin degradation and lead to parasite growth attenuation (Spaccapelo *et al.*, 2010). Surprisingly, the systematic inhibition of either single or multiple combinations of the PM genes has not yielded any striking growth defect.

Presumably, this is because of the functional redundancy in the DV PMs, the redundant enzyme system involved in hemoglobin digestion and the presence of multiple pathways in the uptake of extracellular amino acids. This enzyme system involve falcipains, metalloproteases, and aminopeptidases (Omara-Opyene *et al*., 2004; Spaccapelo *et al*., 2010). However, of all the single-PM knockouts, PfPM IV or quadruple-PM disruptions shows the highest and statistically significant reduction in hemozoin formation, indicating that hemoglobin degradation was impaired (Bonilla *et al.*, 2007). Therefore, although the DV PMs are not essential, they contribute significantly to the development and fitness of the parasite. If the DV PMs are valid targets for the development of antimalarial compounds, more than one may need to be targeted by a drug. Recently, PfPM II amplification has been shown to be a surrogate molecular marker associated with piperaquine resistance *(Witkowski et al*., 2016).

While the four DV PM genes cluster on the chromosome 14 of *P. falciparum*, expressing PfPM I, II, fHAP and IV, there is only one identified DV PM (PM IV) in each of the other human malaria *Plasmodium* parasites. In *P. berghei*, PM IV (PbPM IV) ortholog on chromosome 10 shares the highest sequence similarity with the DV PMs of human malaria parasites. Previous work on the PbPM IV shows that it plays an important role in rodent malaria pathogenesis. Besides having a modest effect on the intraerythrocytic parasite development, PM IV-knockout mutants of *P. berghei* manifests attenuated virulence and confers protective immunity in the host against wild-type plasmodium parasites (Liu *et al*., 2015; Spaccapelo *et al*., 2010). A growing evidence has indicated that PM IV is the only DV-specific PM found in all *Plasmodium*

sp. Therefore, it is the original digestive vacuole PM ortholog that gave birth to the paralogs found in *P. falciparum* via gene duplication processes (Moura *et al.*, 2009).

2.7.2 DNA damage-inducible protein 1

Ddi1 proteins contain a conserved domain homologous to one in retroviral aspartyl protease, and it has been suggested that the corresponding protease activity could be involved in a novel, ubiquitin-dependent proteolytic pathways (PlasmoDB, 2016b; White *et al*., 2016). Recently, studies have demonstrated that a *L. major* ortholog of the yeast Ddi1 protein can complement protein secretion phenotype of *Saccharomyces cerevisiae* Ddi1 knockout, and that a number of RPIs avert the resultant complementation phenotype (White *et al.*, 2016). This phenomenon suggests that inhibition of the *Leishmania* Ddi1 ortholog might be the mechanism by which RPIs mediate their anti-parasitic actions (White *et al*., 2016). Besides, it has been shown that Ddi1is an active aspartyl proteinase and in *L. major* and showed optimum hydrolytic activity between pH 2.5 and 5.0 (Perteguer *et al*., 2013). In yeast *S. cerevisiae,* the Ddi1 protein is involved in cell cycle control, protein targeting to the proteasome, and suppression of protein secretion from the cell (White *et al*., 2016).

Plasmodium parasites also have the retroviral-like protease which is shown to be highly expressed in the blood stages of *P. falciparum* growth. Blood stages of *P. falciparum* is associated with immense significance in clinical manifestations of malaria. To date, no sinlgle study has chracterized the functions of Ddi1 protein in *Plasmodium* parasites and its importance to parsite survival is yet to be determined. Thefereore,

identification of the functions of this gene in malaria parasites and evaluating its candidature as a potential drug target is timely.

2.8 Gene knock out

2.8.1 PlasmoGEM gene targeting vectors

*Plasmo*GEM vectors are genetically modified clones from a *P. berghei* ANKA genomic DNA library that are constructed in the pJAZZ ®-OK NotI vector. The *P. berghei* ANKA genomic DNA library clones have been converted into gene targeting vectors (tagging and knock-out vectors) using a combination of Recombineering and Gateway technologies that give rise to *P. berghei* selective marker hdhfr/yfcu. These vectors have long homology arms, which enhance integration and recombination frequency (Pfander *et al*., 2012; Schwach *et al*., 2015). The hdhfr gene confers resistance to both pyrimethamine and WR99210

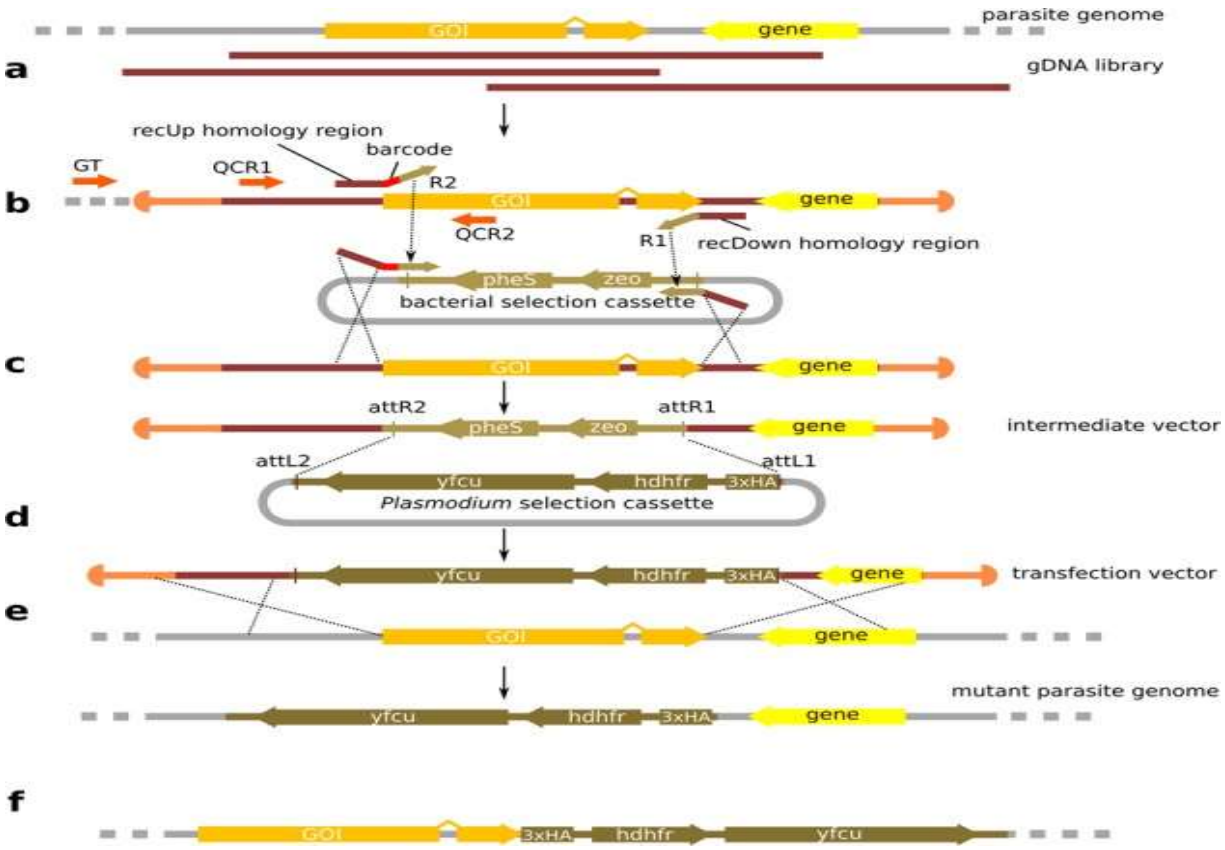


Figure 5: Graphical overview of vector design (Pfander *et al.*, 2012)

The vectors have corresponding primer pairs used in genotyping the DNA of transgenic parasites. The primers have unique designations for confirmation of the transfection vector backbone (selectable marker cassette), vector integration as well as the status of the wild type (WT) locus.

Table 1: ***Primers for Quality Control***

Purpose	**Primer pair**
Proper vector integration	GT GW1/2
Detection of the transfection vector	QCR2 GW1/2
Detection of WT gene locus	QCR1 QCR2

2.9 *In silico* methods in drug discovery

Drug discovery and development is a tedious and costly process. The integration of computer-based approaches to experimental methods has come in handy to accelerate the process of drug development. Therefore, computer-aided analysis is a critical aspect in modern drug design and development. Computer-aided drug discovery/design (CADD) filters large libraries of compounds into smaller sets that can be easily tested experimentally, directs lead compound optimization studies as well as designing of novel compounds (Ferreira, Dos Santos, Oliva, & Andricopulo, 2015; Sliwoski, Kothiwale, Meiler, & Lowe, 2013). CADD methods are broadly divided into ligand-based drug design and structure-based drug design.

2.9.2 Ligand-based computer-aided drug design

Ligand-based computer-aided drug design is an approach that is dependent on knowledge of known molecules that interact with biological targets of interest. The design is mainly used when there is lack of information about the receptor 3D structures (Sliwoski *et al.*, 2013). This ligand-based approach exploits tools such as pharmacophore modeling and quantitative structure-activity relationship (QSAR) in provision of predictive models for drug design (Sliwoski *et al*., 2013). The molecules are represented in a way that only the most important physicochemical features that enable interactions are retained while those properties that are not relevant to interactions are discarded.

2.9.2 Structure-based computer-aided drug design

Structure-based computer-aided drug design depends on structural knowledge of the target protein that is used in calculation of interaction energies for all compounds or molecules tested (Ferreira *et al.*, 2015). It is based on the hypothesis that effective interaction between a molecule and a specific protein to give a desired effects depends on the ability of the molecule to favorably interact with a particular binding site on that protein. Therefore, the structural data enables formulation of ligands with specific stereochemical and electrostatic characteristics in order to achieve high receptor binding affinities as well as reduction of off-target effects (Ferreira *et al*., 2015). The availability of 3D structures of the target protein enables thorough examination of the binding site topology, such as presence of sub-pockets, clefts and cavities as well as electrostatic properties (Ferreira *et al.*, 2015).

Molecular docking is a major tool used in structure-based computer-aided drug design. It has ability to forecast, with a significant degree of accuracy, the orientation of small-molecule ligands within appropriate receptor binding cavity. Molecular docking determines the predicted binding modes of lead compounds and measures the degree of binding affinity for the receptors (Ferreira *et al.*, 2015; Sliwoski *et al.*, 2013).

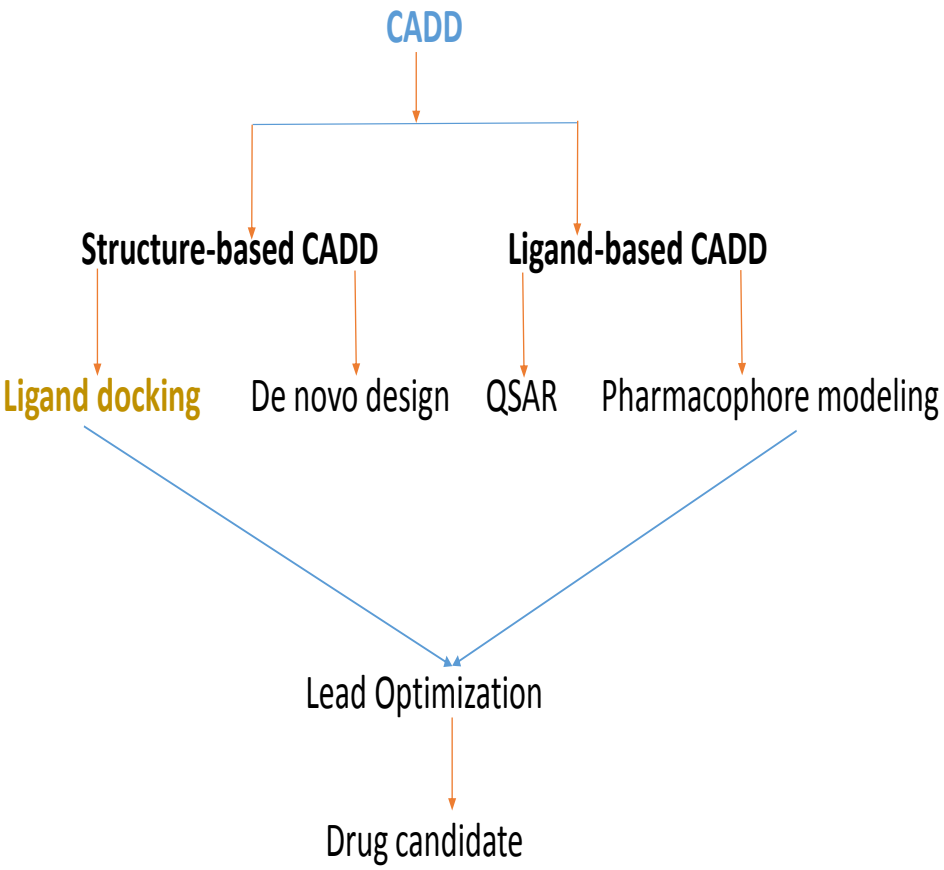


Figure 6: Overview of *in silico* methods in drug discovery

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

The study was carried out at the Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI) and at the Centre for Traditional Medicine and Drug Research (CTMDR), in Kenya Medical Research Institute (KEMRI) Headquarters, Nairobi, Kenya.

3.2 Parasites, hosts and drugs

A transgenic *P. berghei* ANKA strain 676m1cl1{PbGFP-LUC(con)} which expresses a fusion protein GFP-Luciferase (Franke-fayard *et al.*, 2004; Kiboi *et al.*, 2009), maintained in cryopreserved stocks in KEMRI, was used in this study. The knock out vectors were kindly provided under a material transfer agreement with the PlasmoGEM project at the Wellcome Trust Sanger Institute (PG-MTA-0093).

Male Swiss Albino mice (6-7 weeks old), weighing 18-20g were acquired from the KEMRI animal house facilities and used as models in this study. The animals were kept in the animal house in standard polypropylene cages and fed on commercial rodent feed and water *ad libitum*. The cages measured 290 × 220 × 140 mm and held a maximum of 8 mice.

The most potent anti-parasitic retroviral protease inhibitors (Lopinavir{LP} and Saquinavir {SQ}) and Ritonavir (RT) were purchased from *Sigma-Aldrich (USA)*. Ritonavir was used as a booster as it inhibits cytochrome P450-3A4 (CYP3A4), a liver enzyme that metabolizes protease inhibitors. All protocols were in accordance with prior

approvals obtained from the KEMRI's Scientific Ethical Review Unit (SERU; 3572) and Animal Care and Use Committee (IACUC).

Table 2. ***Retroviral protease inhibitors***

Inhibitor Name	CAS Number
Lopinavir	192725-17-0
Saquinavir	149845-06-7
Ritonavir	155213-67-5

3.3 Collection of *P. berghei* schizonts for transfection

The collection of *P. berghei* schizonts for transfection was done using standard protocols as described by Janse, Ramesar, & Waters, (2006). For each of the vectors, at least three mice were used for schizont culture. *P. berghei* parasites were propagated by intraperitoneal (IP) injection into the mice and were harvested for *in vitro* asexual stage parasite culture at 3% parasitaemia using cardiac puncture. The asexual stage parasites (rings) were cultured *in vitro* at 37°C in tightly sealed and gassed flasks containing 100ml of schizont culture medium (72 ml RPMI1640, 25 ml freshly thawed FBS, 1ml of antibiotic Pen/Strep (1:100 Penicillin/streptomycin) and 2ml 0.5M $NaHC0_3$). Each flask contained 2ml of blood and were incubated overnight, in a shaker incubator.

After 22 hours, 500µl of the mature schizonts were harvested by spinning for 5s at 16,000g, smears made and analyzed to confirm presence and quality of schizonts under a microscope; ×100. The rest of the mature schizonts were purified by nycodenz

density-gradient centrifugation using a Nycodenz-PBS solution. Centrifugation was done for 20 min at 450*g* in a swing-out rotor at room temperature. The schizonts (collected from the brown interlayer) were diluted in complete culture medium in 50 ml falcon tubes and re-distributed in Eppendorf tubes (one per transfection). The parasite pellets were prepared by centrifugation at 300g for 8 min and left in the medium supernatant, awaiting transfection.

3.4 Preparation of transfection PbGEM vectors

The gene knock out vectors (PbGEM-039254, PbGEM-086320, PbGEM-057101 and PbGEM-097527), that contain a human dihydrofolate reductase (hdhfr) selection cassette were provided by the PlasmoGEM, Wellcome Trust Sanger Institute (http://plasmogem.sanger.ac.uk/). The vectors were maintained in glycerol stocks of *Escherichia coli (E. coli)*.

Table 3: Target genes and corresponding PbGEM knockout vectors

Gene Name	Gene identifier(s)	Knockout Vector (ko)
DNA-damage inducible protein	PBANKA_103320	PbGEM-097527
Plasmepsin 4	PBANKA_103440	PbGEM-039254
Plasmepsin 7	PBANKA_051760	PbGEM-086320
Plasmepsin 8	PBANKA_132910	PbGEM-057101

3.4.1 Starting *E. coli* culture from glycerol stocks

Three (3) ml of each plasmid construct in *E. coli* starter culture from cryopreserved glycerol stocks were cultured in 3 ml of Terrific Broth (TB) solution with kanamycin (30 µg/ml) for 5 hours at 37 °C. The 5-hour cultures were transferred to 100 ml of TB solution with kanamycin (antibiotic; 30 µg/ml) and cultured overnight at 37^0C at a speed of 220g. Each of the overnight bacteria culture was aliquoted into two 50ml

falcon tubes and centrifuged for 30 min at 4000 rpm at 4^0C. The pellet was stored for plasmid extraction.

3.4.2 Plasmid isolation

Plasmid DNA was isolated using QIAfilter Plasmid Midi Kit. Briefly, the *E. coli* bacterial pellet was re-suspended in 6 ml Buffer P1 (re-suspension buffer containing 50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Buffer P2 {lysis buffer containing 200 mM NaOH and 1% SDS (w/v)} was added, mixed thoroughly, and incubated at room temperature (15–25°C) for 5 min. Six (6) ml of pre-chilled Buffer P3 (neutralization buffer containing 3.0 M potassium acetate, pH 5.5) was added, mixed thoroughly and the lysate incubated at room temperature for 10 min. After washing, the plasmid DNA was eluted with 5 ml of Buffer QF, precipitated by adding 3.5 ml isopropanol, mixed, and centrifuged at 15,000g for 30 minutes at 4°C. The DNA pellet was washed with 2 ml 70% ethanol and centrifuged at 15,000g for 10 minutes. The DNA pellet was air-dried for 10 minutes and re-dissolved in 150 µl of PCR water. Diagnostic PCR, using QCR2/GW2 primer pairs, was carried out in order to confirm presence of the resistance marker cassette. The PCR amplicons were resolved in a 1% gel electrophoresis as described in section 3.8.

3.4.3 Restriction digestion of plasmid DNA

Approximately 10 µg of each vector DNA underwent a NotI restriction digestion at 37°C in a 100 µl digest volume. This digestion was meant to release constant arms of the vector backbone. Briefly, 11 µl of 3M sodium acetate and 250 µl of cold 96% ethanol solution were added to each of the 100 µl of restriction digest solution and the

mixture kept at -20^{0}C overnight. The vector DNA pellets were prepared by spinning at 13000 rpm for 30 min at 4^{0}C, washed with 250 µl of 70% ethanol solution and span again at 13000 rpm for 2min at 4^{0}C. The pellets were dried at 65^{0}C for 5 min, dissolved in 10 µl of water and the concentration measured using a Nano-Drop. The DNA pellets were stored at -20^{0}C, ready for insertion into the purified schizonts.

3.5 Transfection and selection of genetically transformed parasites

The schizonts in the Eppendorf tube were harvested by centrifugation for 5s at 16,000*g* in a microcentrifuge and supernatant discarded. For every transfection, 20µl of schizont pellet was used. Each pellet was re-suspended in 100µl AMAXA supplemented nucleofector solution 88A6 containing 10 µl of the purified vector DNA solution. Exactly 100µl of each solution (vector DNA /nucleofector solution and schizonts) were pipetted into a Lonza electroporation cuvette and transfection carried out using program U33 for 5s. The transfection solution was collected in thin plastic pipettes, mixed with 150uL RPMI prepared in an eppendorf tubes and loaded into insulin syringes (30Gx3/16"). Two mice in the induction chamber were immediately injected with 150 µl of each transfection solution mixture intravenously (IV). The transfection and injection procedures were repeated for all the knock out vectors.

The knock out parasites as well as wild type (WT) parasites were subjected to *in vivo* selective pressure by pyrimethamine (7 mg/ml) treatment of mice, a day after infection. Mice treatment was continued for a period of 9 days. It should be noted that the knock out vectors contain a selectable marker, (human dihydrofolate reductase; *hDHFR*), for pyrimethamine that enables positive selection *in vivo.*

3.6 Recovery of transgenic parasites

Thin blood smears were made from one droplet of tail blood of the infected mice and parasitaemia was measured. After mice anesthesia with intraperitoneal (IP) administration of pentobarbital at 70mg/kg (Gargiulo *et al*., 2012), blood was collected by cardiac puncture using a 1-ml syringe containing 0.1 ml of heparin stock solution. A 0.3 ml of each blood sample was transferred to separate eppendorf tubes for genotype analysis while the rest of the blood was stored for drug profile studies.

3.7 Extraction of parasite DNA (pDNA) from blood

Each of the 0.3ml of blood was span for 1 minute at 500g and the pellet transferred to 15 ml centrifuge tubes containing 10 ml of cold (4 °C) erythrocyte lysis buffer. The mixture was incubated on ice for 25 minutes. The parasites were harvested by centrifuging at 4°C for 10 minutes at a speed of 500g. The pellet was washed in 10 ml PBS and re-suspended in the remaining 2ml of PBS. The pDNA was purified according to the QIAamp DNA Blood Mini Kit protocols.

3.8 Genotype analysis of pDNA

Diagnostic PCR was used to genotype the transgenic lines alongside WT pDNA using primer pairs; QCR2/GW2, GT/GW1 or GW2 and QCR1/QCR2. The primer pairs are specific for the vector resistance marker cassette, for integration of the insert into the correct locus, and the status of wild type (WT) locus. QCR1 and QCR2 anneals to the genomic sequence of the targeted locus, GT anneals to the genomic sequence outside of but preceding the region covered by the gDNA clone, while GW1/2 anneals to the sequence of the selection cassette. PCR was done using 2xGoTag Green master mix for

thirty cycles with an annealing temperature of 50°C and an extension temperature of 62°C. The PCR amplicons were resolved in a 1% agarose gel electrophoresis.

Table 4: Primer pairs used in PCR amplification of gDNA isolated from PM4_KO parasites

Purpose	**Primer pair**	**Primer Sequence**
Vector integration	GT GW1	AGACAAACTTTGCCCACAACA CATACTAGCCATTTTATGTG
Vector resistance marker cassette	QCR2 GW2	AAGGGCCGCAACAACATTCA CTTTGGTGACAGATACTAC
WT locus	QCR1 QCR2	TGAACTTGACCCAGTCGGCGG AAGGGCCGCAACAACATTCA

Table 5: Primer pairs used in PCR amplification of gDNA isolated from PM7_KO parasites

Purpose	**Primer pair**	**Primer Sequence**
Vector integration	GT GW2	AGCTGCCTATGAAGGAGGGA CTTTGGTGACAGATACTAC
Vector resistance marker cassette	QCR2 GW2	AAGGGCCGCAACAACATTCA CTTTGGTGACAGATACTAC
WT locus	QCR1 QCR2	TGGATGGACGCTAGGACAAGT ATGTGCATGGAAACAGGGTT

Table 6: Primer pairs used in PCR amplification of gDNA isolated from PM8_KO parasites

Purpose	**Primer pair**	**Primer Sequence**
Vector integration	GT GW1	TCCAATGGCCCAACAACCTTTCGA CATACTAGCCATTTTATGTG

Vector resistance marker cassette	QCR2 GW2	AGGTGCACTTATACCGATTCTCA CTTTGGTGACAGATACTAC
WT locus	QCR1 QCR2	TCGAGTATTGCAGGACCCCA AGGTGCACTTATACCGATTCTCA

Table 7: PCR master mix preparation (per reaction)

Reagent	**Quantity**
NF_H_2O	9.5µl
2xGoTag Green (*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations)	12.5µl
Primers (forward and reverse)	1µl
DNA template	1µl

PCR was done using 2xGoTag Green master mix for thirty cycles with an annealing temperature of 50°C and an extension temperature of 62°C. The reaction was held at 4°C after completion of the reaction.

3.9 Agarose gel electrophoresis of PCR products

One percent (1%) agarose gel was prepared by mixing one gram of agarose powder and 100ml of 1× TBE buffer in an Erlenmeyer flask. The mixture was quickly dissolved by heating in a microwave oven for one and half minutes. The mixture was cooled to 50°C; 3µl of SYBR® Safe DNA Gel Stain was added and mixed well before gelling. A 5 µl of each pDNA sample was mixed approximately with 1 µl of 5× loading dye and loaded into individual wells in the gel. The gels were run at a constant voltage (100 volts) for 45 minutes, viewed by a digital gel imager and analyzed.

3.10 Preparation of test drugs

On the day of administration, all the test drugs (LP, SQ, RT, Amodiaquine; AQ and Piperaquine; PQ) were prepared by solubilizing them in a solution consisting 70% Tween-80 (density=1.08gml-1) and 30% ethanol (density=0.81 gml-1) and diluted 10 fold with double distilled water.

3.11 Inoculation, grouping and dosing of mice

Each of the mutant parasite lines (PM4_KO, PM7_KO and PM8_KO) as well as WT parasites were separately IP inoculated into three mice and parasitaemia allowed to build up for four days. Blood smears were taken on the fourth day to determine parasitaemia. Thereafter, blood was obtained from each set of mice and diluted with PBS to 1%. The diluted solution was then used to infect test mice. For LP/RT and SQ/RT, groups of eight mice were used (four mice for each mutant parasite line and the other four for the wild type parasite). For AQ or PQ, groups of sixteen mice were used (eight mice for mutant parasite line and the other eight for the wild type parasites for two different AQ and PQ dosages). Each control group had also four mice. Each study mice was IP inoculated with 200µl of 1×10^5 of the 1% parasitized erythrocytes (iRBCs) from infected donor mice, on the first day (day 0; D0). Two hours after parasite inoculation, four mice from the groups received 0.2ml of LP/RT; 40mg/kg.10mg/kg, SQ/RT; 50mg/kg.5mg/kg, AQ; 2.5 mg/kg, AQ; 1.25mg/kg, PQ; 2.5mg/kg and PQ; 1.25mg/kg, once daily oral gavage. Treatment was continued for three consecutive days (making a total of four consecutive treatment days; from D_0 to D_3).

3.12 Monitoring of parasitaemia (Four-day {4-D} suppressive test)

Drug activity was determined using the 4-D suppressive test (Fidock *et al*., 2004). On the fifth day (day 4: D_4), Giemsa stained blood smears, prepared from blood collected from the saphenous vein, were prepared and parasitaemia determined by microscopic examination (x100).

Percentage (%) chemosuppression (parasite reduction) of each drug dose tested was determined using the following equation:

% chemosuppression = {(Parasitaemia in negative control - Parasitaemia in study group)/Parasitaemia in negative control} x 100.

3.13 Molecular docking

3.13.1 Homology modelling

To predict the 3D structures of the *P. berghei* proteins (Ddi1; PBANKA_103320/0, PM4; PBANKA_103440/0, PM7; PBANKA_051760/0 and PM8; PBANKA_132910/0, best structures with the highest similarity to their sequences available in *Plasmodium* database http://plasmodb.org/ and NCBI protein database http://ncbi.nlm.nih.gov were selected as templates. The homology models were predicted using SWISS-MODEL (Biasini *et al*., 2014) available at: https://swissmodel.expasy.org/. The SWISS-MODEL was a preferred modelling server because it annotates essential cofactors and ligands as well as quaternary structures, allowing for modelling of complete structures with less complex software packages (Biasini *et al.*, 2014). The structures were downloaded and saved in PDB format.

3.13.2 Structure validation

To determine the quality of the modelled structures, the models were analyzed using PROCHECK (Laskowski, Macarthur, Moss, & Thornton, 1993) and PRoSA-web (Wiederstein & Sippl, 2007)). PROCHECK evaluates the general stereochemistry of the protein while PRoSA-web checks for potentials errors in the 3D structures using Z-score as a means of scoring. To determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D), Verify_3D (Bowie, Lüthy, & Eisenberg, 1991) was used.

3.13.3 Ligand selection

To perform docking experiments, the most potent anti-parasitic retroviral protease inhibitors (LP and SQ) were selected as test ligands. The molecular structures of the two selected ligands were downloaded from the ChemSpider (http://www.chemspider.com/); an online database that provides access to unique chemical compounds (Williams & Pence, 2010).

The CADD Group's Chemoinformatics Tools and User Services, available at: https://cactus.nci.nih.gov/translate/, was used to convert the chemical structures from Mol2 format into PDB format for compatibility with the docking software.

3.13.4 Binding site analysis and ligand docking

The modelled structures and binding sites were visualized in PyMOL version 1.6.x (Delano, 2002). Optimization of the grid box parameters of the receptors and the ligands was executed using scripts within AutoDock Tools (ADT). The files were then saved in PDBQT format and their corresponding coordinates rewritten into a

configuration file used for docking. . The configuration file specified the pdbqt files for both the ligand and the proteins as well as the docking parameters (dimensions and spacing angstrom). Optimization of the inputs involved removal of water molecules from the receptor and addition of missing hydrogen ions. Docking was carried out using Autodock Vina (Forli et al., 2016; Trott & Olson, 2010), for docking of a single ligand with a single receptor. Autodock Vina employs the Lamarckian genetic algorithm (LGA). HIV-1 aspartyl protease whose 3D structure was available in the protein data bank (PDB; http://www.rcsb.org/pdb/explore.do?structureId=2hvp) was also included. The binding geometries (binding energies and the positional root-mean-square deviation; RMSD) of the ligands and the proteins were displayed on an output file. A ligand orientation with low binding energy indicates better affinity towards a receptor.

3.14 Data Management Plan

3.14.1 Data presentation and statistical analysis

The parasitaemia data from microscopy was recorded in Microsoft Excel spreadsheets and analyzed using R software. Box Plots and Violin Plots were generated to portray the KO growth trends and parasite densities in presence and absence of the test drugs. Using the Student's t-test in the Stata version 15.1 software, we computed means of percentage drug activity/percentage parasitaemia from each mouse in the treated group and compared them to that from mice in the control group. The significance for all tests was set at 5% (p-value <0.05). For molecular docking, the ligand-receptor binding affinities in kcal/mol were analyzed and used to indicate the most favored reactions.

3.15 Ethical consideration

All protocols were conducted in accordance with prior approvals obtained from the Kenya Medical Research Institute (KEMRI)'s Scientific Ethical Review Unit (SERU; 3572) and the KEMRI Animal Care and Use Committee (ACUC).

3.16 Sources of funding

This project has been funded by the African Union Commission under the Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI) and the AFRICA-ai-JAPAN innovation research funds.

CHAPTER FOUR: RESULTS

4.1 Generation of PM4, PM7, PM8 and Ddi1 knock out parasites

To understand the essentiality of the genes of interest in parasite metabolism, the effect of gene deletion on parasite survival was evaluated. Genes encoding PM4, PM7 and PM8 proteases were successfully knocked out and the respective mutant parasites generated. Three successive attempts to delete the Ddi1 failed to recover parasite twenty days post infection suggesting that the Ddi1 gene was refractory to deletion and thus might be essential for the asexual blood stage parasite growth. The independent transfection vectors harbor significantly longer homology arms and integrate more efficiently providing a more stringent test for essentiality. We then proceeded to genotype the PM4, PM7 and PM8 KO parasite lines using three sets of primers; QCR2/GW2, GT/GW1(2) and QCR1/QCR2. As expected, using the standard GW2 and the vector specific QCR2 primers, we obtained a fragment of 1kb, 0.8kb, and 1kb in PM4, PM7 and PM8 (KO) parasite lines (Fig 7) indicating the presence of the vector within the parasite. The PCR amplification using vector specific quality control primers, the QCR1 and QCR2 only amplified in the WT parasite line but failed to amplify in the KO lines confirming successful deletion of the respective genes. To further confirm correct integration of the specific vector into the correct chromosome and position, we utilized the standard primers GW1 or GW2 and the vector-specific primers GT. As expected, we obtained a PCR product of 2.9kb, 2.1kb and 2.6kb for PM4, PM7 and PM8 KO lines respectively (Fig 7).

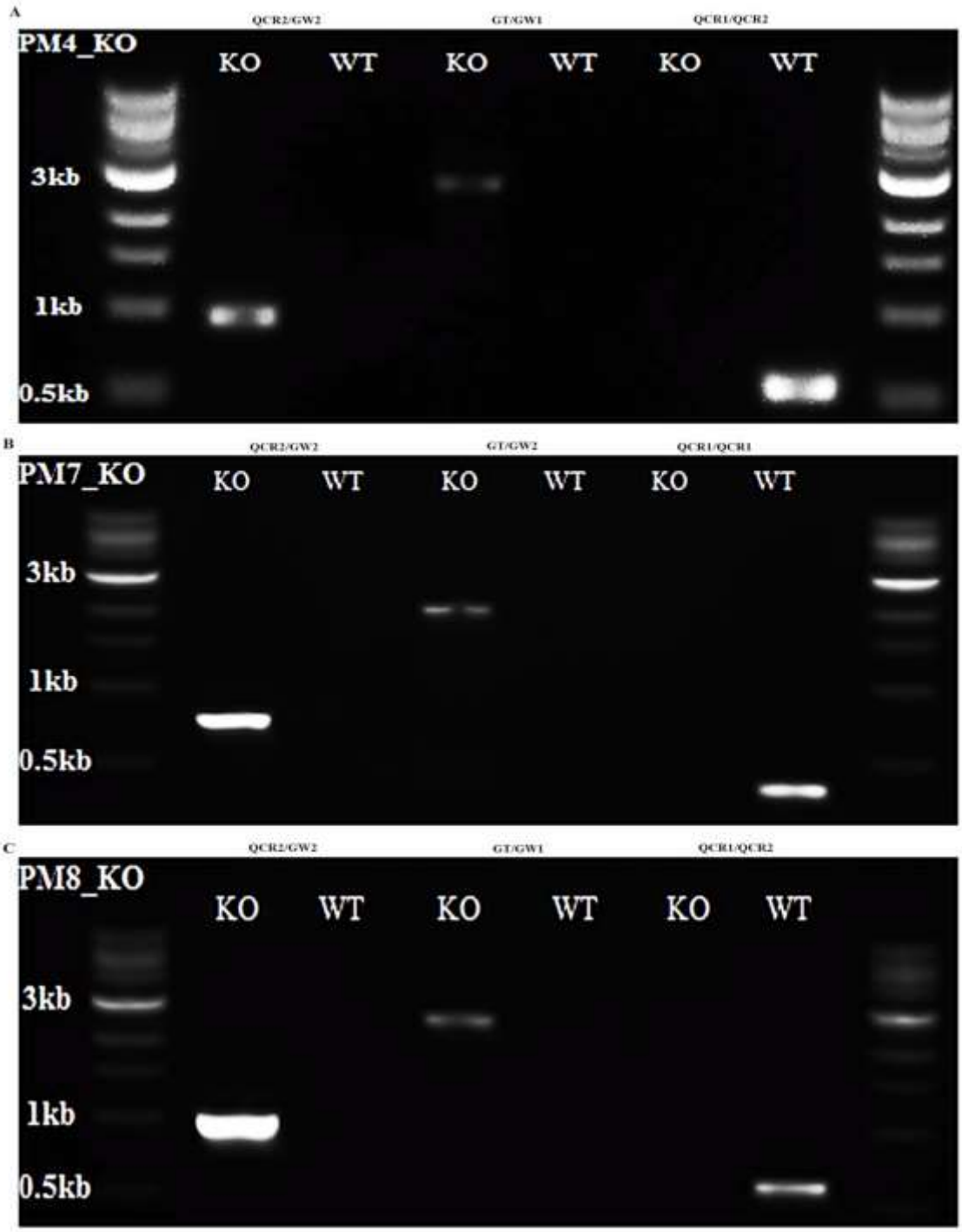


Fig 7: PCR amplification of gDNA from knock out lines; (A) PM4_KO, (B) PM7_KO and (C) PM8_KO, and WT parasite lines.

The PCR amplification used three sets of primer pairs; QCR2/GW2, GT/GW1(2) and QCR1/QCR2. The PCR products were resolved in a 1% agarose gel electrophoresis. KO indicates the sizes of DNA fragments amplified from KO parasite lines while WT shows DNA fragment sizes from the control WT parasites. The amplification confirmed the presence of the vector selection marker cassette, correct vector integration in the parasite genome and excision of the WT gene locus.

4.3 Evaluation of growth rate phenotypes of the KO parasites

To evaluate the effect of the gene knock out on the growth rate phenotypes, the *in vivo* asexual stage parasite density for all the transgenic and WT parasites were assessed, five days after inoculation in mice (Day 4 post-infection; D4 PI). Our growth rate phenotype results revealed that deletion of PM4 significantly reduces normal parasite growth rate phenotype by 58% (P = 0. 0.003), suggesting its substantial contribution to the fitness of the asexual stage parasites. On the contrary, PM7_KO and PM8_KO parasite lines exhibited significantly increased growth rate phenotypes compared to the WT parasites (P = 0.007 and P = 0.0007 respectively) at day four post-infection (D4 PI) (Fig 8).

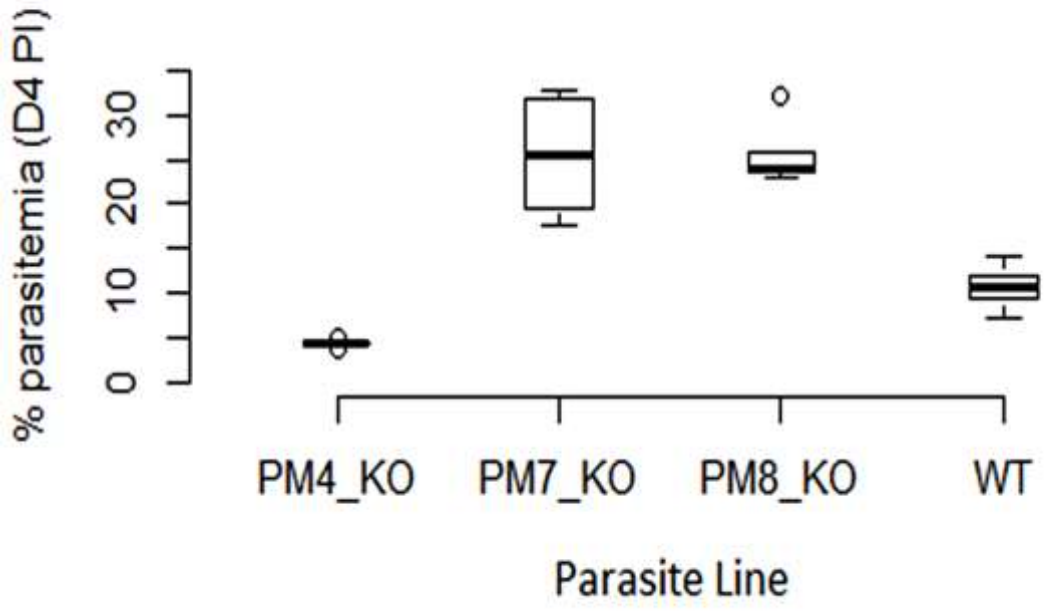


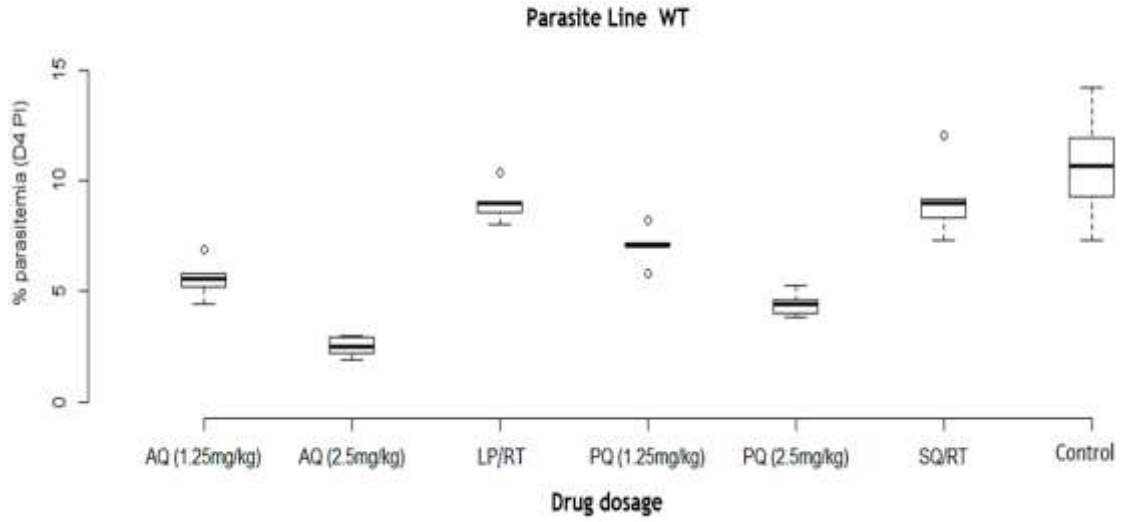
Fig 8: Growth rate phenotypes of the transgenic lines and WT parasites D4 PI.

A box plot showing the median percentage parasitaemia in mice infected with the PM4_KO, PM7_KO or PM8_KO parasite line relative to the wild-type (WT) parasite line as measured on day 4 post parasite inoculation. Compared to the WT parasites, PM4_KO parasites acquired a reduced growth rate phenotype while PM7_KO and PM8_KO parasites attained an increased growth rate phenotype.

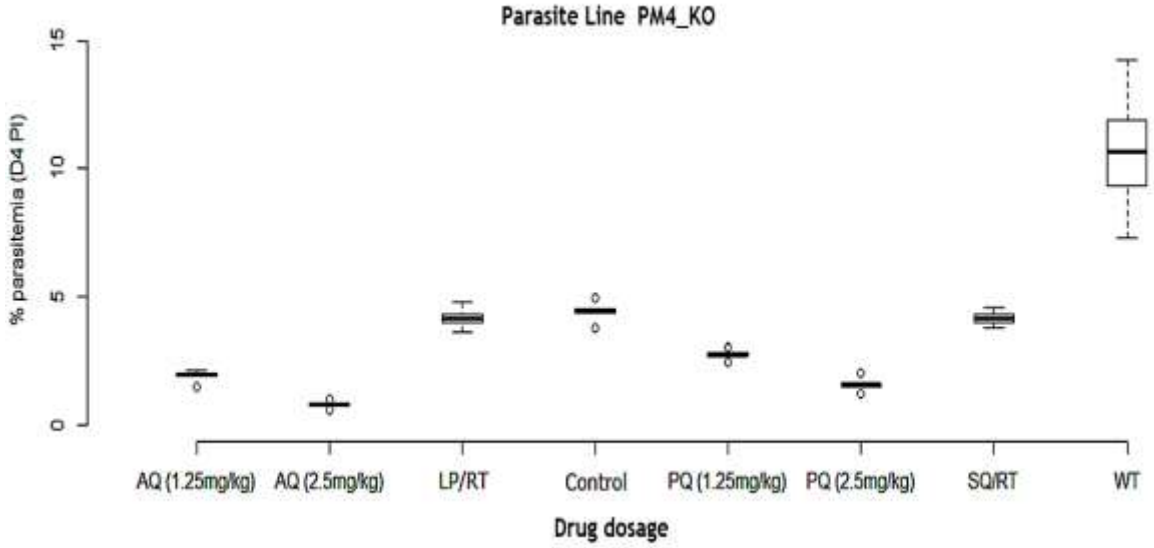
4.4 Drug sensitivity profiles (4-D suppressive test)

We assayed whether the deletion of the PM4, PM7 or PM8 affected sensitivity to LP, SQ, PQ and AQ. To estimate the sensitivity profiles of the LP and SQ against the transgenic parasites, parasite densities for both the mutant and WT parasites were assessed five days after inoculation of parasites and administration of the drugs to the mice (D4 PI). The LP/RT reduced the PM4_KO mutants' parasitemia by 5.01% compared to a 15.71% reduction in the WT parasites (P = 0.036). Similarly, the SQ/RT reduced PM4_KO mutants' parasitemia by 5.29% compared to a 14.12% reduction in the WT parasites (P = 0.030). However, the percentage activity for both the LP/RT and SQ/RT against the PM7_KO and PM8_KO parasites were comparable to the activity of the WT parasites. LP/RT or SQ/RT reduced PM7_KO and PM8_KO parasites' parasitaemia by an average of 16% (P = 0.938 - 0.559) and 20% (P = 0.6634 - 0.2013) respectively, compared to a 15% reduction in the WT parasites. As expected, the antimalarial drugs (AQ and PQ) were active against both mutant parasites and WT parasites and the results showed that the drugs exerted almost the same pressure in suppressing the growth of both the mutant and WT parasites, D4 PI (Fig 9).

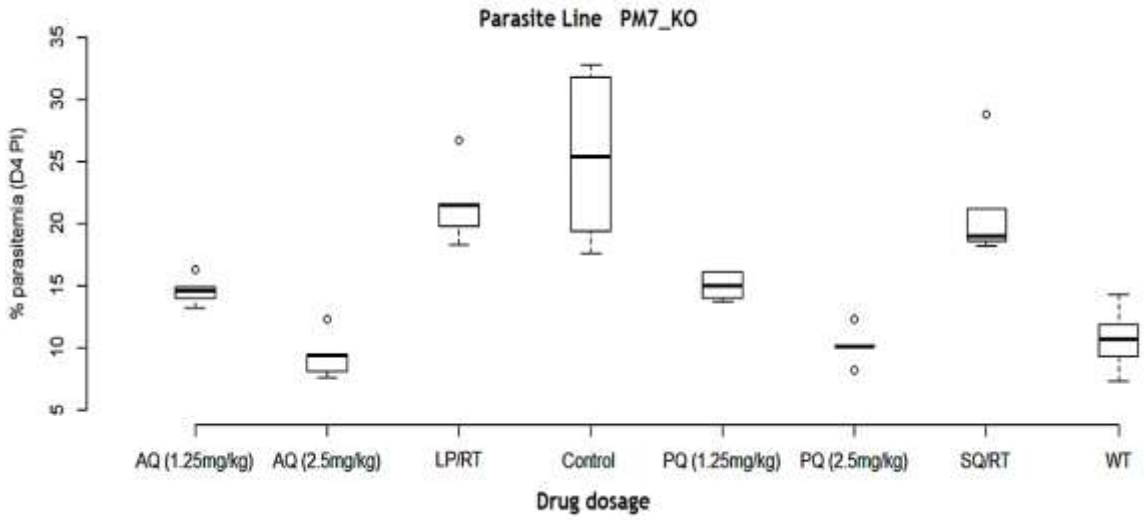
A.


Parasite Line WT
% parasitemia (D4 PI)
0
5
10
15
AQ (1.25mg/kg)
AQ (2.5mg/kg)
LP/RT
PQ (1.25mg/kg)
PQ (2.5mg/kg)
SQ/RT
Control
Drug dosage

B.


Parasite Line PM4_KO
% parasitemia (D4 PI)
0
5
10
15
AQ (1.25mg/kg)
AQ (2.5mg/kg)
LP/RT
Control
PQ (1.25mg/kg)
PQ (2.5mg/kg)
SQ/RT
WT
Drug dosage

C.



D.

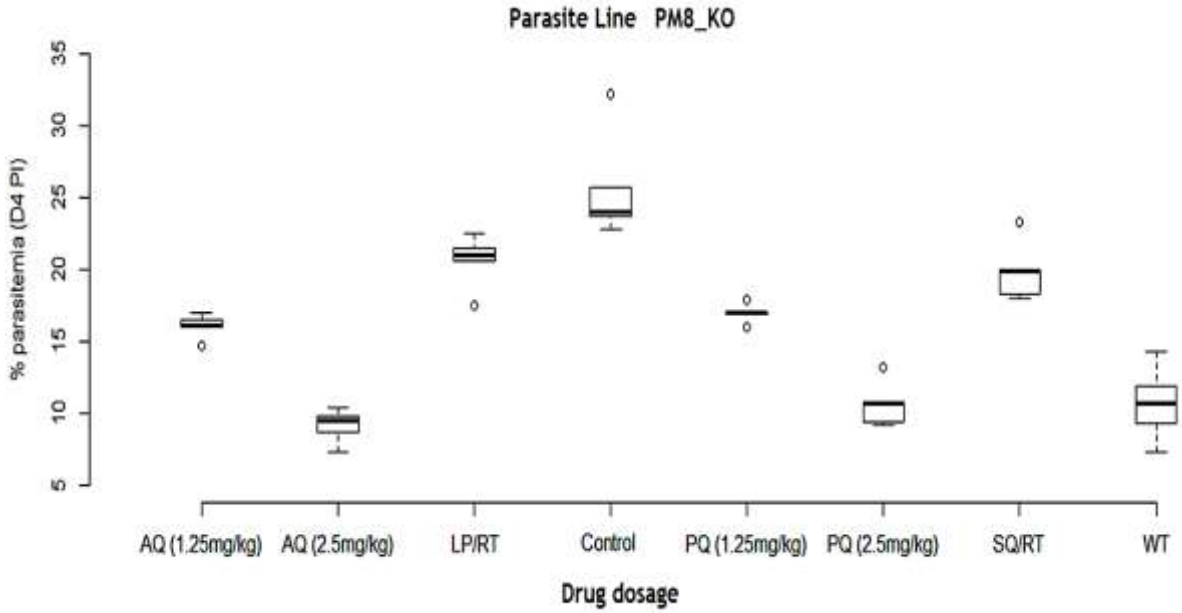


Fig 9: Box Plots showing median suppressive LP, SQ, AQ and PQ profiles against (A) WT parasites (B) PM4_KO (C) PM7_KO, and (D) PM8_KO lines.

(A) The wild type (WT) parasite line displayed normal growth pattern and the expected susceptibility to amodiaquine (AQ), piperaquine (PQ), LP/RT and SQ/RT. (B) The PM4_KO parasite line exhibited a significant reduction in the growth outline and a significant loss of susceptibility to LP/RT and SQ/RT, but not to AQ or PQ drugs. (C) The PM7_KO and (D) PM8_KO parasite lines displayed rapid growth pattern as compared to the WT parasite line but retained susceptibility to AQ and PQ as well as to the RPIs; LP/RT and SQ/RT.

4.5 Validation of the modelled 3-D structures

The degree of correctness for all the modelled structures was successfully validated. The structures of the modelled proteins were within the range of scores typically found with experimentally defined proteins. The averaged 3D-ID values were obtained from VERIFY 3D while PROCHECK confirmed residue positioning in the 3D structures (Table 8).

Table 8: Validation of the modelled 3D structures of the *P. berghei* aspartyl proteases

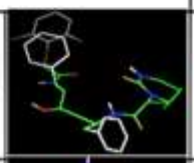
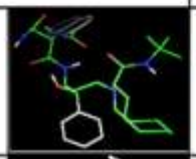
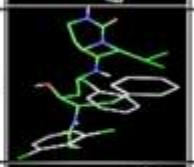
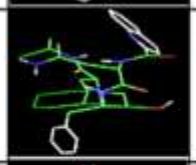
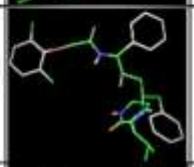
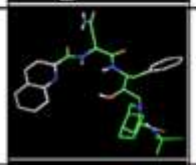
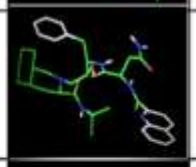
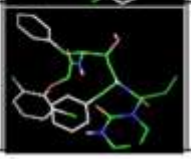
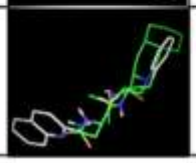
Protein	Gene ID	Z-score	Averaged 3D-1D score >= 0.2 (%)	Residues in most favored regions (%)	Residues in additional allowed regions (%)	Residues in disallowed regions (%)
Ddi1	PBANKA_103320	-5.09	84.17	90.5	8.7	0.0
PMIV	PBANKA_103440	-9.11	97.59	90.9	8.8	0.0
PMVII	PBANKA_051760	-7.94	82.24	85.8	11.7	0.9
PMVIII	PBANKA_132910	-7.52	87.40	86.3	10.9	0.6

The 3D structures of the proteins were predicted using SWISS-MODEL and their quality validated using PROCHECK, for stereochemical evaluation, PRoSA-web, for detection of potential errors in the 3D structures and verify_3D, for compatibility of the 3D structures with their respective amino acid sequences.

4.6 Molecular docking of a single ligand with a single receptor

To evaluate the protein binding affinity, ligand conformations were determined by the ADT and the best modes (mode 1) were used to determine the affinities. All the protein binding complexes showed high binding affinities with the highest affinity of -10.6 kcal/mol and the lowest at -6.3 kcal/mol. Unlike Ddi1 which had similar binding energy as HIV-1 asp protease for LP (-6.3 kcal/mol), the binding affinities of LP and SQ to PM4, PM7 and PM8 were higher than the affinity of the two drugs to the HIV-1 aspartyl protease. PM4 had the lowest binding energy; -9.3 and -10.6 kcal/mol for LP and SQ respectively, indicating better binding affinity towards the drugs (Table 9).

Table 9: Ligand best docking modes with respective binding energies towards PM4, PM7, PM8, Ddi1 and HIV_Asp

PROTEIN	LIGAND			
	Lopinavir		Saquinavir	
	Ligand Mode	Binding Affinity (kcal/mol)	Ligand Mode	Binding Affinity (kcal/mol)
PM4	1 	-9.3	1 	-10.6
PM7	1 	-8.4	1 	-8.5
PM8	1 	-9.1	1 	-9.4
DDI1	1 	-6.3	1 	-7.0
HIV_Asp	1 	-6.3	1 	-7.9

Shown are binding geometries for docking of a single ligand with a single receptor. LP or SQ were docked into the modelled 3-D structures of PM4, PM7, PM8, Ddi1 or HIV_Asp using Autodock Vina. A ligand-receptor complex with low binding energy indicates better affinity. LP and SQ recorded the lowest binding energy with the PM4 suggesting better affinity to the protein. The LP and SQ yielded higher binding affinity to both PM7 and PM8 as compared to the known RPIs target; the HIV Aspartic protease (HIV Asp).

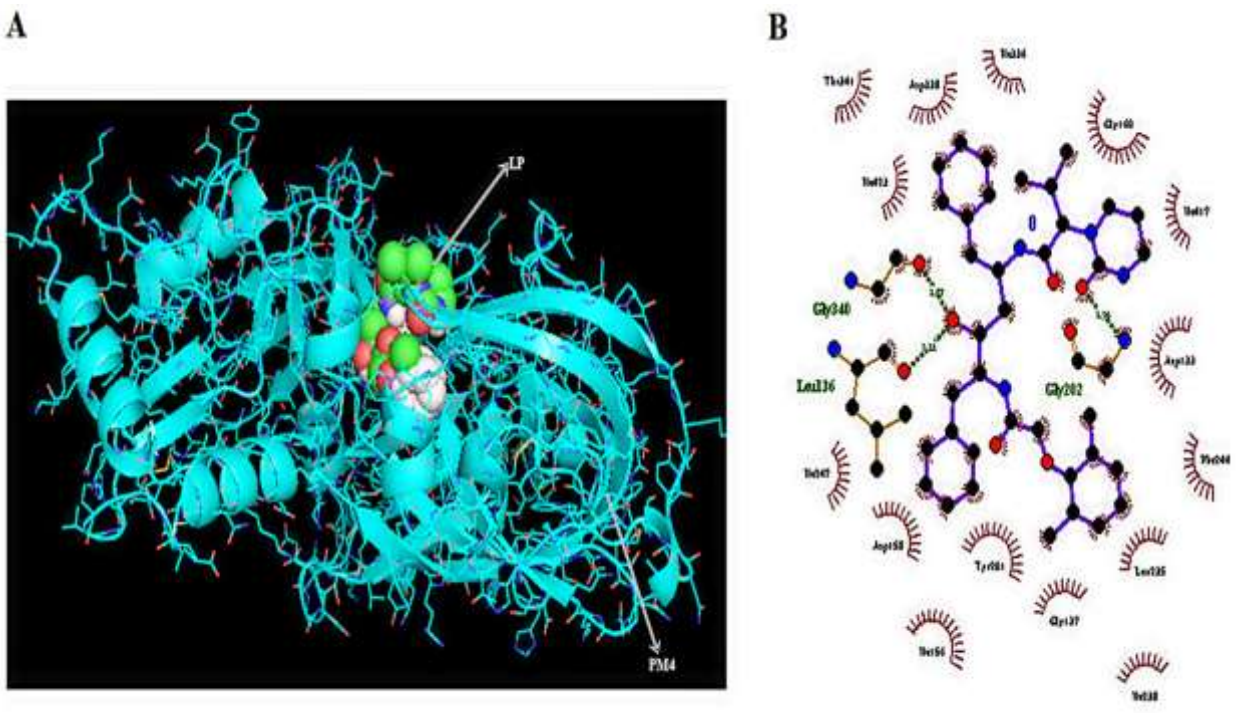


Fig 10: **Interaction of LP with the PM4**

(A) Crystallographic structure of PM4 in complex with LP as visualized using PyMOL. (B) 2D LP-PM4 interaction diagram with the oxygen atoms shown in red, nitrogen atoms shown in blue while hydrogen bonds are shown as olive green dotted line. The interaction plots were generated using LigPlot+.

CHAPTER FIVE: DISCUSSION

Widespread resistance of the malaria parasite to most first-line drugs and the emergence of resistance against artemisinin-based combination drugs have made the identification and validation of novel antimalarial targets extremely urgent. Proteases are proven drug targets as evidenced by the use of protease inhibitors such as LP and SQ for the treatment of HIV/AIDS. This study considered both *Plasmodium* pepsin-like proteases; PM4, PM7 and PM8 as well as a retropepsin-like protease (Ddi1) as potential targets for RPIs. We investigated the essentiality of PM4, PM7, PM8 and Ddi1 by creating transgenic lines and used the lines to understand the possible mechanisms of action of LP and SQ.

We successfully generated PM4, PM7 and PM8-deficient parasites but the gene encoding Ddi1 proved refractory to the deletion. The Ddi1 protein belongs to the A2 family of proteases, a retropepsin-like protease and is an active and functional aspartyl protease in *Leishmania major* parasites (Perteguer *et al.*, 2013). It has been suggested that it is involved in a novel, ubiquitin-dependent proteolytic, cell cycle control and reduction of extracellular protein secretion in *Saccharomyces cerevisiae* (Gabriely, Kama, Gelin-licht, & Gerst, 2008). Recently, studies have shown that an ortholog of yeast Ddi1 in *L. major* and human can complement protein secretion phenotype in Ddi1-deficient *S. cerevisiae* cells and that RPIs forestall the resultant complementation phenotype (White *et al.*, 2016). The retropepsin-like family of proteases includes retroviral aspartyl proteases, retrotransposons and DNA-damage-inducible proteins in eukaryotic cells. From this study, the high binding affinities of the Ddi1 to the LP

coupled with its potential essential role in the growth of the asexual blood parasite suggest that chemical compounds that inhibit the Ddi1 might form next generation of novel drugs.

We further used the generated transgenic lines (PM4_KO, PM7_KO and PM8_KO) to investigate the effect of gene knock out on the parasite growth rate phenotypes. We have demonstrated that, whereas PM7 and PM8 genes are dispensable during the asexual stages of parasite development, they exhibit an indirect interaction with asexual stage expressions. The loss of PM7 and PM8 genes increased the growth rate of asexual blood stage parasites suggesting that the deletion confer a growth advantage to the KO parasite. At D4 PI, the parasitemia for the PM7_KO and PM8_KO parasites was 2.5-fold higher than that of the WT parasites. Further, we observed that, despite the PM4 being a dispensable gene, its deletion significantly reduces parasite asexual stage growth rates. The parasitemia for PM4_KO parasites was 2-fold lower than that of the WT parasites, suggesting a substantial contribution to the fitness of the parasite within the red blood cells. Whereas *P. falciparum* expresses four digestive vacuole (DV) PMs; (PM 1, 2, histo-aspartic protease; HAP and PM4), there is only one identified DV PM in *P. berghei*, PM4. Therefore, successful deletion of PM4 in *P. berghei* suggests that the parasite could be relying on other enzyme pathways for hemoglobin degradation, a source of amino acids for the intraerythrocytic parasite growth. These PM4 findings are consistent with the findings from (Bonilla *et al.*, 2007), who documented that a quadruple knock out of all the DV PMs (PM1, PM2, PM4 and HAP) in *P. falciparum* does not completely impair parasite growth. However, the effect

of the quadruple gene KO on drug susceptibility was not determined. With regard to the enzymology of hemoglobin degradation, plasmepsins, aminopeptidases, metalloprotease, falcilysin, and falcipains are found in the DV and are capable of degrading hemoglobin. The presence of multiple enzyme pathways with overlapping specificity and function is strategic to increase parasite fitness (Omara-Opyene *et al*., 2004; Spaccapelo *et al*., 2010).

Our drug profile results show that the activity of all the tested drugs against PM7_KO and PM8_KO parasites was nearly equivalent to the WT parasites. The activity of LP/RT and SQ/RT ranged between 15% and 21% against both the mutant parasites and WT parasites. These findings augment the fact that the two genes are not expressed in the erythrocytic stages of parasite growth (Banerjee *et al*., 2002; Omara-Opyene *et al.*, 2004). Our study focused on the asexual blood stage parasites; both the LP and SQ are predicted to act on the parasite within the erythrocytes, thus although the LP and SQ may bind PM7 and PM8, the inhibition effect may not be observed in the asexual parasites within the erythrocytes. Studies involving PM7 and PM8 have shown that they play an essential role in the sporogonic stages of malaria parasite development. PM7 proteins are expressed in *P. falciparum* ookinetes and that antibodies directed against these proteins reduced parasite transmission to *Anopheles* mosquitoes when used at high concentration in mosquito infection experiments (Mastan *et al.*, 2017).

On the contrary, compared to the WT parasites, the LP/RT or SQ/RT activity against PM4_KO lines was 3-fold lower, demonstrating a potential role in the LP and SQ action. The PM4 is the only DV specific PM found in all *Plasmodium* species

suggesting an essential role in the growth of the asexual blood stage parasite. PM4 is the original DV PM that gave birth to the paralogs found in *P. falciparum* (Moura *et al.*, 2009).

Further analysis using modelling and molecular docking studies revealed that LP or SQ have high binding energies towards the *Plasmodium* aspartyl proteases. In fact, the drugs' binding affinity towards PM4, PM7, PM8 or Ddi1 were higher than the affinity towards the HIV-1 aspartyl protease. However, because parasites contain many proteases with functional redundancy (Wunderlich *et al*., 2012), unlike retroviruses, the *in vivo* activity of LP and SQ against the parasites is not proportional to the *in silico* binding affinities. The high binding affinity of LP or SQ towards PM4 correlates with the reduced sensitivity of PM4_KO parasites to the drugs. However, because of the redundant enzyme system involved in hemoglobin digestion for parasite asexual stage development, inhibition of PM4 does not completely deprive of parasite survival. The favorably low binding energy (high affinity) shown by LP or SQ towards PM7 or PM8 (-8.4 kcal/mol to -9.4 kcal/mol) does not correlate with the *in vivo* drug suppression of asexual stage parasites because they are not essential and are not expressed during the erythrocytic asexual stages of parasite growth.

CHAPTER SIX: CONCLUSION AND RECOMMENDATION

The deletion of PM4 confers significant growth disadvantage. The PM4_KO parasite is less sensitive to LP and SQ. We thus argue that since the PM4 participates in the essential haemoglobin degradation pathway, then the protein might be mediating the action of LP or SQ in suppressing parasite growth. The growth rate attenuation might help in the development of host adaptive immunity against subsequent challenge with wild type parasites. The high binding affinity of the LP or SQ towards the PM4 protein affirms the "targetability" fact. However, the presence of alternative enzymes for haemoglobin degradation provides the asexual blood stage parasites with an advantage albeit with cost in the growth rate.

The deletion of the Ddi1 gene seemed toxic to the growth of the asexual blood stage parasites. The Ddi1 gene has a pepsin_retropepsin-like domain, with a catalytic motif, within its sequence. Interestingly, this motif is conserved in all *Plasmodium* species. Therefore, the gene is essential for parasite survival and thus can be a focus for rational drug development in the fight against the malaria parasite. Future work tailored towards understanding its functions in *Plasmodium* parasites and its candidature as a target for the RPIs are needed.

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APPENDICES

Appendix 1: Interaction of the aspartyl proteases with LP or SQ

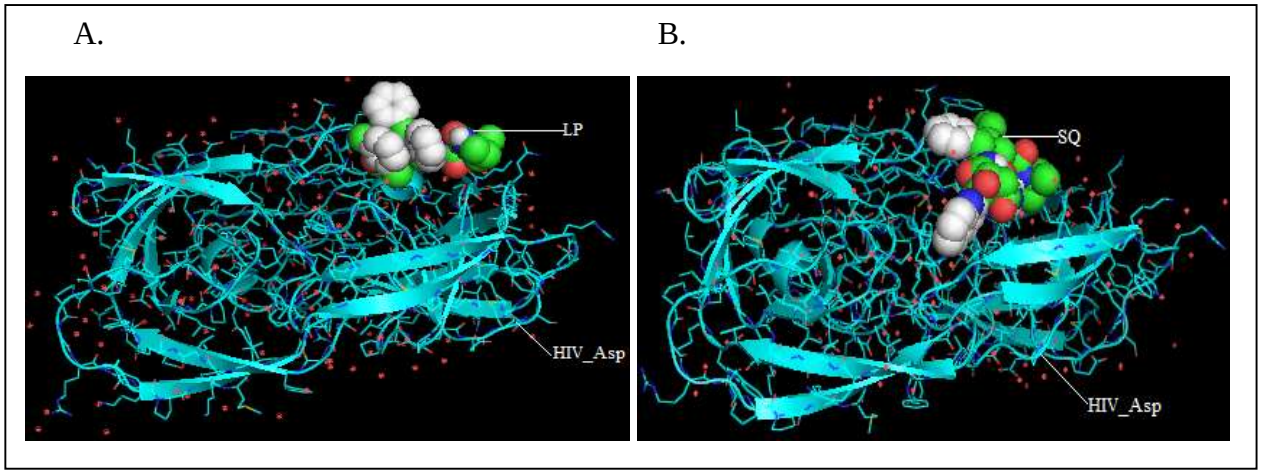

Fig 11: Structure of HIV-1 asp protease in complex with LP (A) and SQ (B) docked in their binding pockets

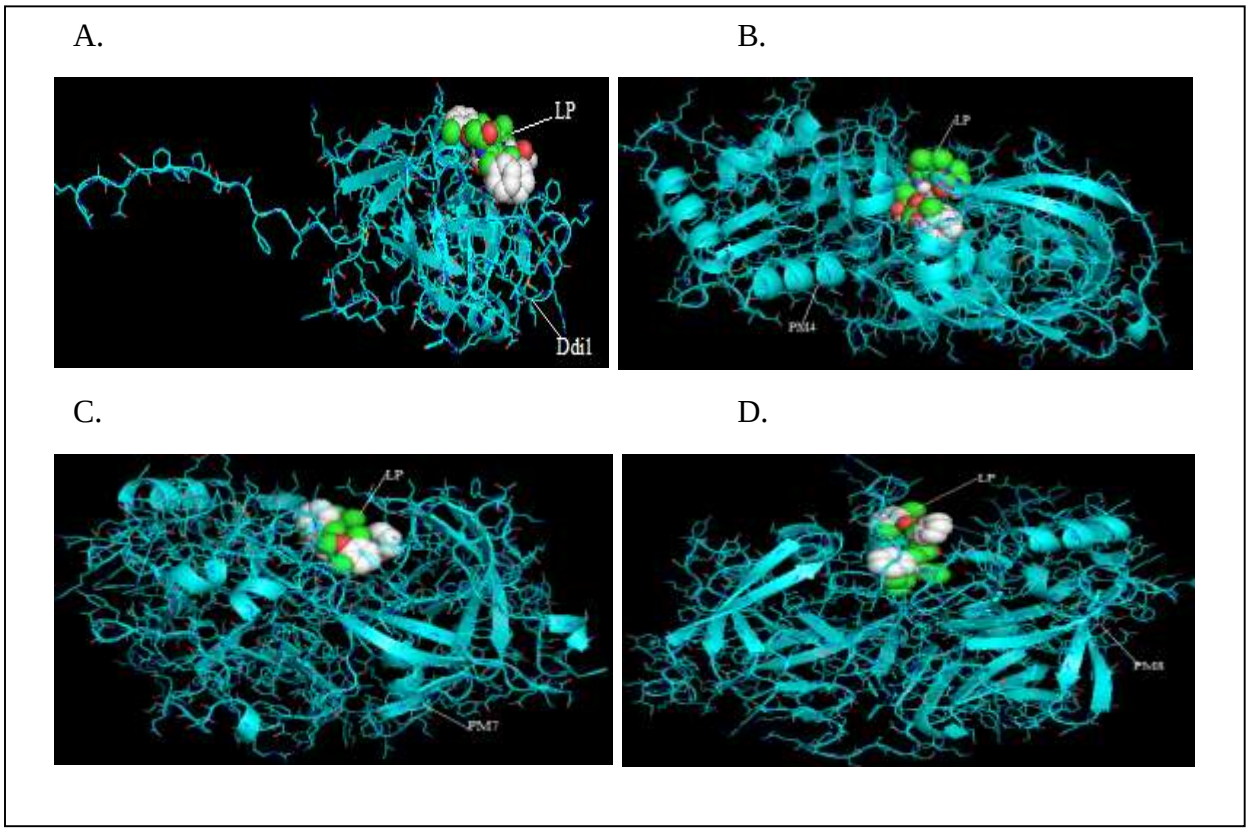

Fig 13: Structure of Ddi1 (A) PM4 (B) PM7 (C) and PM8 (D) in complex with LP, docked in their binding pockets

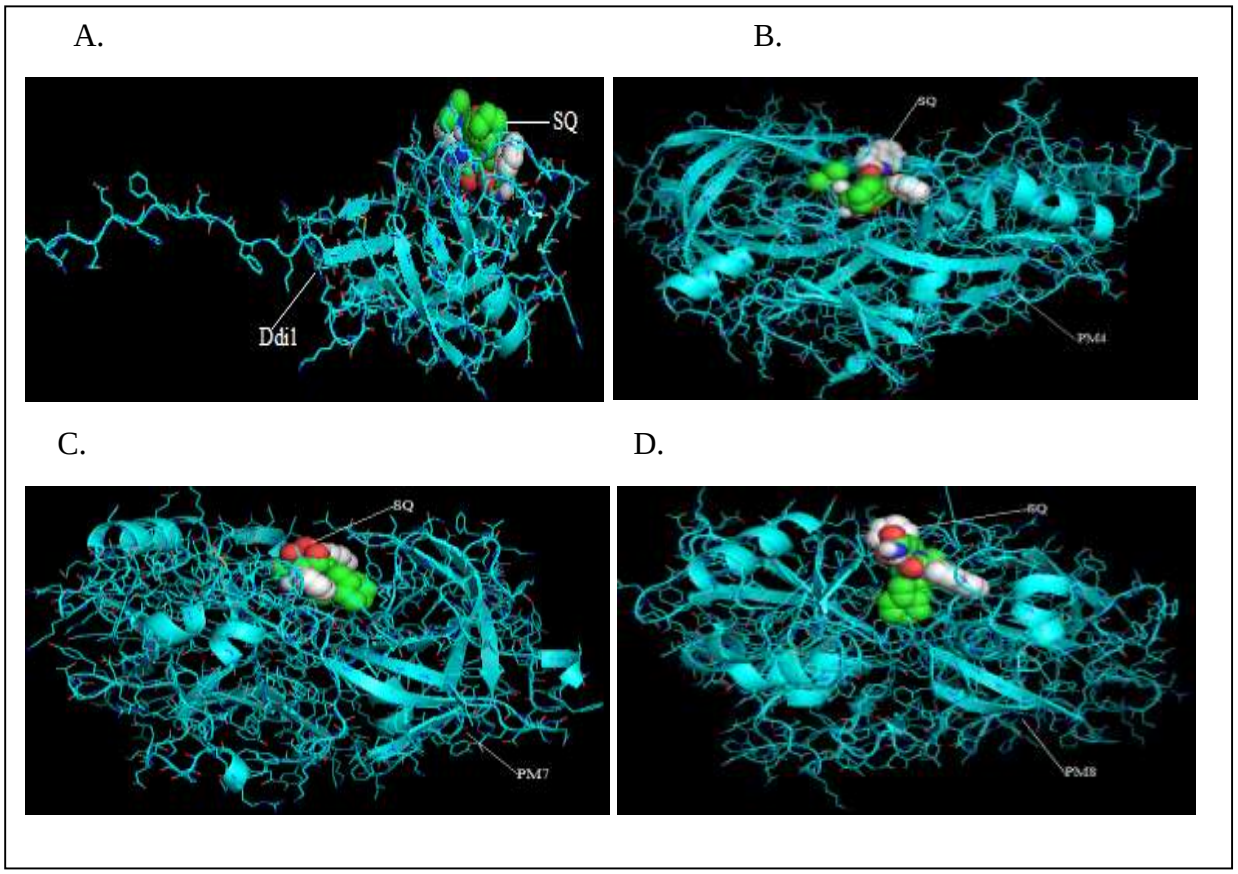

Fig 14: Structure of Ddi1 (A) PM4 (B) PM7 (C) and PM8 (D) in complex with SQ, docked in their binding pockets

Appendix 2: Crystallographic structure of PM4, PM7, PM8 or Ddi1 in complex with LP