INVESTIGATION OF PYRONARIDINE RESISTANCE IN *PLASMODIUM BERGHEI* ANKA ISOLATES.

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2016

Investigation of pyronaridine resistance in *Plasmodium berghei* anka isolates.

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology.

2016

DECLARATION

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

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

This thesis is devoted to my beloved wife Lydiah, daughter Abigael and entire family of Ann and late John Kimani Gathuo.

ACKNOWLEDGEMENT

Working on the Master of Science has been a wonderful and often overwhelming experience for me. To achieve this, I am indebted to many people.

First of all, I am deeply grateful to my supervisor **Dr. Joseph Ng'ang'a;** for introducing me to the world of academic research. Working on this research project under your intellectual guidance has been a delightful learning experience.

Prof. Daniel Kariuki; your insights on my work formed a pillar of my thinking towards science. Thank you for the encouraging discussions while visiting you.

Dr. Daniel Kiboi; to work with you has been a real pleasure to me, with heaps of fun and excitement. You have been a steady influence on my career; you have oriented and supported me with promptness and care, and have always been patient and encouraging in times of new ideas and difficulties. Above all, you made me feel a friend, which I appreciate from bottom of my heart.

I express my sincere appreciation to **Mr. Francis Kimani** for his kind assistance. You also gave me an opportunity to work in your laboratory. I thank you!

In **KEMRI**, I am indebted to all staffs and students I had the pleasure to work with. In particular, special thanks to Carolyne, Thaddeus, Justus and Catherine, for your moral support.

Am grateful to Karatina University fraternity for granting me a chance to write this thesis while in service. Without your support, this would have been unachievable.

Special thanks to **Mr. Stephen Gathuo** and his family for giving a chance to be part of their family; your **UNWAVERING** moral and financial support cannot go unnoticed. You made me realize my educational dream. God bless you.

I thank the **National Council of Science and Technology** for providing financial support for completion of this work. Without the research fund assistance, this would have been unachievable.

Lastly, all thanks to **ALMIGHT JEHOVAH** for the work would not have been possible without **HIS** endless mercies, steadfast love and boundless blessings.

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ABBREVIATIONS AND ACRONYMS

2% RT	2% Relapse Technique
4-DT	4- Suppressive Day Test
ABC	ATP Binding Cassette
ACTs	Artemesinin based Combinational Therapies
ACUC	Animal Care and Use Committee
AQ	Amodiaquine
ARMD	Accelerated Resistance to Multi-drug
ASN/MQ	Artesunate/Mefloquine
ATM/LM	Artemether-Lumefantrine
ATPase	Adenosine Triphosphatase
CM	Cerebral Malaria
CQ	Chloroquine
D	Density
DEAQ	N-desethylamodiaquine
DHA/PQ	Dihydroartemisinin/Piperaquine,
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
Dtmp	deoxy-Thymidine Monophosphate
dUMP	deoxy-Uridine Monophosphate
DV	Digestive Vacuole
ED	Effective Dose
FACS	Fluorescence-Activated Cell Sorting
GFP	Green Fluorescent Protein
HLF	Halofantrine
HRP-II	Histidine-Rich Protein-2
I	Index of resistance
IL	Interleukin
INF	Interferon
IRS	Indoor-Residual Spraying

ITNs	Insecticide Treated Nets
KDa	KiloDalton
KEMRI	Kenya Medical Research Institute
LLINs	Long-Lasting Insecticidal Nets
LM	Lumefantrine
MFQ	Mefloquine
MMV	Medicines for Malaria Venture
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
PBS	Phosphate Saline Buffer
PCR	Polymerase Chain Reaction
PfCRT	*P. falciparum* CQ Resistance Transporter
PfDHFR	*P. falciparum* Dihydrofolate Reductase
PfDHPS	*P. falciparum* Dihydropteroate Synthase
PfEMP-1	*P. falciparum* Erythrocyte Membrane Protein-1
PfMDR-1	*P. falciparum* Multidrug Resistance -1
PfNHE	Sodium Hydrogen ion Exchanger-1
PQ	Piperaquine
PRBCs	Parasitized Red Blood Cells
PRD	Pyronaridine
PRD/ASN	Pyronaridine/Artesunate
PSG	Phosphate Saline Glucose
PVP2	V-type H+ ATPase
p.i	Post-infection
QBC	Quantitative Buffy Coat
QN	Quinine
RDT	Rapid Diagnostic Tests
SCC	Scientific Coordinating Committee
SERCA	Sarcoplasmic Endoplasmic Reticulum Atpase
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-Pyrimethamine

THF	Tetrahydrofolate
TNF	Tumor Necrosis Factor
tRNA	Transfer Ribonucleic Acid
TS	Thymidylate Synthase
WHO	World Health Organization

ABSTRACT

Malaria is a major worldwide health problem and the control of the disease is compromised by acquisition and spread of *Plasmodium falciparum* resistance to multiple antimalarial drugs, which has caused an increase in malaria related morbidity and mortality. In response, Artemisinin based Combination Therapies (ACTs) have been implemented in almost all malaria endemic areas in an attempt to reduce emergence of resistance. For instance, a combination of Artesunate (ASN)-Pyronaridine (PRD) was recently prequalified by WHO drug as an alternative for treatment of malaria in African setting. However pyronaridine, a benzonaphthyridine derivative has a long half-life, consequently predisposed to high selection pressure, hence high chances of emergence of resistance. To counter this problem, the mechanisms of resistance of existing drugs need to be fully elucidated. As a first step towards understanding resistance, the study first selected pyronaridine resistance by submitting *Plasmodium berghei* ANKA line *in vivo* to increasing Pyronaridine concentration for 20 successive passages over a period of six months. The effective doses that reduced parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) determined in the standard four-day suppressive test for the parent line were 1.83 and 4.79 $mgkg^{-1}$, respectively. After 20 drug pressure passages, the ED_{50} and ED_{90} increased by 66 and 40 fold, respectively. To determine the stability of resistance, the parasites were first cloned by limiting dilution, then grown in absence of drug for five passages and cryo-preserved at -80^{0}C for one month. The effective doses determined later found that the resistance phenotypes remained stable. Using PCR amplification and sequencing, the coding sequence of *Plasmodium berghei* multidrug resistance gene 1 (*PbMDR-1*) was analyzed, which is orthologous to the gene associated with quinoline resistance in *Plasmodium falciparum.* The results did not show any nucleotide polymorphism within the coding region. Further broader sequencing is therefore recommended in order to associate *PbMDR-1* gene to PRD resistance in *Plasmodium berghei.* Nonetheless, additional possible candidate genes involved in pyronaridine drug resistance should be explored.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is one of world's most widespread life threatening hematoprotozoan parasitic infection transmitted to people through the bites by infected species of *anopheline* mosquitoes. In humans, malaria is caused by four main species of *Plasmodium* parasites; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. Recent research recognized that *Plasmodium knowlesi*, the macaque monkey malaria parasite, also infects humans (Cox-Singh *et al*., 2008). The effects of *P. falciparum* malaria are mostly experienced in Sub-Saharan Africa, where young children under 5 years and pregnant women bear the biggest disease burden. *Plasmodium falciparum* is responsible for most malaria related deaths and morbidity (Murray *et al*., 2010; WHO, 2011). In the early stages, malaria symptoms are sometimes similar to those of many other infections caused by bacteria or viruses. Most symptomatic malaria infections are uncomplicated and manifest as fever, chills, malaise, often abdominal discomfort and mild headache**.** In Kenya more than four million cases of malaria are reported annually. A 5.1% mortality rate has been reported among patients admitted with severe malaria (Robi *et al*., 2010). Although *Plasmodium falciparum* is the species most frequently associated with severe malaria and accounts for 80-90% of cases in Kenya, *P. malariae*, *P. ovale* and *P. vivax* also exist in the country (WHO, 2006). Vector species in Kenya are members of the *Anopheles gambiae* complex and *Anopheles funestus*. Transmission patterns of the disease in Kenya are influenced by rainfall, vector species, intensity of biting, and altitude. Stable malaria occurs in most parts of Coast, Nyanza, and Western Provinces (Robi *et al*., 2010).

Despite efforts to control the disease, malaria is among the top three infectious diseases and the most deadly tropical disease (Sachs and Malaney, 2002). Existing malaria control strategies include integrated approaches such as vector control using insecticides and chemoprophylaxis. However, effective vaccine development against human malaria parasites is in advance stage awaiting license (Breman and Plowe, 2009; Vaughan and

Kappe, 2012). Insecticide Treated Nets (ITNs) are low cost and highly effective method for reducing the incidence of malaria but development of resistance to insecticides is a challenge (Balter, 2000; Curtis and Mnzava, 2000). Despite advances in vector control and effective vaccines development, chemotherapy remains the principal mode of malaria control (WHO, 2014).

Early diagnosis and prompt treatment is one of the principal technical components of the global strategy to control malaria (WHO, 2000). Unfortunately, resistance to the most commonly used antimalarial drugs has emerged globally in *Plasmodium falciparum*, and resistance has rendered these drugs useless in many endemic areas (Laxminarayan, 2004). Drug resistance causes treatment failure and can lead to death and further rapid spread of resistance (Alonso *et al*., 2004). For instance, over a half century, chloroquine (CQ) was mainstay of malaria chemotherapy and control, providing effective, safe, affordable and widely available treatment across Africa (Wellems and Plowe, 2001). The progressive failure of CQ led to promotion of Sulfadoxine/Pyrimethamine (SP) as the first line drug combination (Trape, 2001; Hyde, 2005). However, its resistance emerged and spread rapidly reducing its therapeutic life (Gregson and Plowe, 2005). In response to increasing resistance, effective, safe, affordable first line treatment and strategies against resistance are of essence if the battle against malaria infection is to succeed (WHO, 2006). ACTs have been evaluated for malaria treatment and control (WHO, 2003; Yeung *et al*., 2004), currently, the ACTs form WHO recommended treatment gold standard against *P. falciparum* malaria. In most ACTs, the artemisinin derivative is a short half-life drug while the partner drug is a long half-life drug (WHO, 2006). In low malaria transmission settings, this constitutes a minor problem but in high transmission areas the success of ACTs remains a big challenge (Sisowath *et al*., 2005) due to pharmacokinetics incompatibility.

Several forms of artemisinin combinational therapies (ACTs) have been evaluated, in which the regimen uses a double combination therapy geared towards delay or circumventing resistance altogether (Olliaro and Wells, 2009). These include; Artemether/Lumefantrine, (ATM/LM), Dihydroartemisinin/Piperaquine, (DHA/PQ) (Bassat *et al*., 2009), Artesunate/Mefloquine (ASN/MQ) (Ashley *et al*., 2008) and

recently WHO prequalified combination therapy, Pyronaridine/ Artesunate (PRD/ASN) (Ramharten *et al*., 2008; MMV, 2010). Pyronaridine/Artesunate (PRD/ASN) is manufactured by Shin Poong Pharmaceutical Company (Seoul, Korea) and consist of artesunate, a short acting artemisinin derivative with half-life of less than 2hrs (WHO, 2006) and PRD, a long acting drug with half- life of 12-17 days (Wattanavijitkul, 2010). This combination drug has low cost, long shelf-life, simplified once-daily dosing regimen, good tolerability and safety profile at all dose levels (Looareesuwan *et al*., 1996; Ringwald *et al*., 1997). Pyramax (PRD/ASN), remains active against wide spread CQ and SP resistant *Plasmodium falciparum* (Ramharter *et al*., 2008). Pyronaridine has not been commercially available as monotherapy or extensively deployed in Africa. However, the *in vitro* sensitivity to this drug decreased in China between 1988 and 1995suggesting the emergence of PRD resistance (Yang *et al*., 1997). Pyronaridine resistance has previously been selected in *P. berghei* and *P. yoelii* (Peters and Robinson, 1992), however, the stability and molecular organization of the phenotype was not investigated.

Pyronaridine (also referred to as Malaridine®) was first synthesized in 1970 at the Institute of Chinese Parasitic Disease, Chinese Academy of Preventative Medicine and is a highly effective blood schizonticidal agent (Shao, 1990; Simon *et al*., 2012). Malaridine® has been used in China for the treatment of malaria as a single agent for the past 30years. The pyronaridine nucleus is based on mepacrine (a 9-aminoacridine) with the addition of an amodiaquine-like side chain (Chen and Fleckenstein, 2001). More recently interest has been renewed in pyronaridine as a possible partner for use in artemisinin-based combination therapy (ACT) for malaria treatment. Indeed, Pyramax, ASN/PRD combination was recently listed as WHO prequalified combination therapy (Vivas *et al*., 2008; Ramharten *et al*., 2008; MMV, 2010). Pyronaridine drug has been reported to be less toxic than CQ and highly effective against CQ-sensitive and resistant parasites both in rodent malarias *in vivo* and *Plasmodium falciparum in vitro* (Wu *et al*., 1988; Peters and Robinson, 1992; Vivas et al., 2008).

Pyramax is intended to delay resistance emergence but studies indicate that use of drug combination with mismatched pharmacokinetics does not prevent selection of resistance

against the long acting drug (Hastings *et al.*, 2002). Thus, this is a drawback especially in high transmission African settings, particularly for the long acting drug which is left trailing in sub-therapeutic doses within the body and provides strong selection pressure for fast resistance emergence (White and Pongtavornpinyo, 2003). Consequently, there is need to understand the PRD resistance, yet, to date there are no well characterized drug resistant parasite lines against pyronaridine.

Understanding the molecular basis associated with resistance forms a baseline for tracking or monitoring resistant mutant in natural infection, designing new therapeutic strategies and identification of new drug targets. To study mechanisms involved in resistance, one needs to obtain well-characterized drug-resistant strains. However, such strains are not generally available for most antimalarials.

Murine malaria *Plasmodium berghei* parasites have been used as surrogates for *Plasmodium falciparum* to study the mechanisms of drug resistance by inducing resistance *in vivo*. This approach has led to the selection of drug-resistant parasite lines and subsequent studies on mechanisms of drug resistance (Peters, 1987; Nzila and Mwai, 2010). In preliminary studies, piperaquine (PQ) resistant *P. berghei* were previously selected (Kiboi *et al.*, 2009). These parasites remained sensitive to PRD (Kimani *et al.,* 2014) thus indicating no similarity in resistance mechanisms, as a result the current study proposed to use Piperaquine (PQ) resistant line, which was sensitive to PRD as the starting parasite to induce PRD resistance. Earlier studies have shown that a resistant strain to one drug which show no cross resistance is more amenable to give rise to resistant lines to another drug compared with fully drug susceptible strains (Afonso *et al.*, 2006; Nzila and Mwai, 2010). The study investigated PRD resistance using *P. berghei* ANKA as a surrogate model to *P. falciparum* by first selecting PRD resistant lines, cloned by limiting dilution, then evaluated the *MDR-1* gene for possible association with PRD resistance. Information derived from this study forms a first step towards understanding the mechanism of PRD resistance in *P. falciparum*.

1.2 Statement of the problem

The PRD/ASN (Pyramax) is a low cost drug combination with a simplified once-daily dosing regimen, good tolerability and safety profile, at all dose levels (Looareesuwan *et al*., 1996; Ringwald *et al*., 1996). However, there's pharmacokinetic mismatch whereby Pyronaridine as a long acting drug, may be left trailing in sub-therapeutic doses within the body, which provide strong selection pressure for emergence of resistance (White and Pontavornpinyo, 2003). This is a drawback as long half-life of PRD predisposes the drug to rapid emergence of resistance particularly in high malaria transmission African settings. In these regions characterized with high malaria incidences, the antimalarial drugs are widely misused especially in tropics (White and Pongtavornpinyo, 2003).The strong selection pressure presented by long acting antimalarial drugs especially at sub-therapeutic levels shows that selection of re-infecting resistant parasites may occur rapidly especially in Sub-Saharan African regions. Therefore, there is need to understand mechanism of PRD resistance, however, to date there are no well characterized drug resistant parasite lines against PRD. To monitor emergency of resistance against PRD, markers coding for their resistance need to be identified and the mechanism of drug resistance elucidated. However lack of well characterized and stable resistant lines has hampered these studies. More importantly, selection of resistance *in vitro* using the human malaria parasite *Plasmodium falciparum* is a tedious process and difficult to establish stable phenotypes for molecular studies. The alternative approach of selecting drug resistant isolates is therefore needed. Murine malaria parasites act as surrogate models to bypass these limitations.

1.3 Justification of the study

To date, no gene has been associated with PRD resistance. Investigations have demonstrated that the mode action of PRD is likely to be similar to that of CQ and other quinolone drugs (MMV, 2010). Thus, this drug may share some of the resistance mechanisms associated with quinolines. Indeed, many studies that have examined the mechanism of resistance to CQ and other quinoline based drugs in *P. falciparum*, have implicated *PfMDR-1* as the transporter gene that mediates resistance. In addition, due to structural similarity to quinoline based drugs, it is logical to hypothesize that the same

transporter gene may contribute to PRD resistance perhaps to some extent. Therefore, this study investigated the role of *MDR-1* gene association or lack of association in PRD resistance in *P. berghei*. This is a first step towards understanding the mechanisms of resistance to pyronaridine drug in *Plasmodium falciparum*. In fact, understanding these mechanisms could lead to identification of single markers, for mapping and monitoring the emergence and spread of drug resistance. In addition, such studies could also contribute to clarifying the mode of action, which eventually could lead to the identifying of new drug targets.

1.4 Null Hypothesis

i. *In vivo* pyronaridine selection pressure does not induce stable 'resistant' *P. berghei* ANKA lines.

ii. Multidrug resistant-1 gene is not associated with pyronaridine resistance in *P. berghei* ANKA.

1.5.0 General Objective

To select for resistance and investigate the molecular basis of pyronaridine resistance in *P. berghei* ANKA isolates.

1.5.1 Specific Objectives

i. To select PRD 'resistant' *P. berghei* ANKA clones.

ii. To determine the stability and sensitivity profile of clone diluted pyronaridine 'resistant' *P. berghei* ANKA clones.

iii. To assess for point mutations in coding sequence of *MDR-1* gene in pyronaridine 'resistant' *P. berghei ANKA* clones.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global incidences of malaria

Malaria disease is caused by the protozoan parasite *Plasmodium (apicomplexan* parasite*)*, and is transmitted by an infected *Anopheline* mosquito vector (WHO, 2010). The five *Plasmodia* species affecting humans notably are *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* (WHO, 2002). This genus includes over one hundred species, able to infect reptiles, birds and mammals. Malaria constitutes a major global health problem in tropical regions of the world, with 200 to 350 million cases annually and mortality reaching 3 million, particularly among children in sub-Saharan Africa (Guerin et al., 2002; Cox-Singh *et al*., 2008). Among them, *P. falciparum* is the most prevalent malaria species worldwide, especially in Africa, causing the most severe form of the disease and being responsible for over 90% of the deaths (Snow *et al*., 2005). *Plasmodium vivax* is the second most common species, located mainly in Asia and South America, and can cause a relapsing form of malaria (Price *et al.,* 2007).

Malaria occurs in over 100 countries but is mainly confined to poorer, tropical areas of Africa, Asia and Latin America (Hartl, 2004; WHO, 2011). *Plasmodium falciparum* predominates in Africa, New Guinea, Haiti and the Dominican Republic (**Fig. 1**) and it accounts for 90% of deaths in Sub-Saharan Africa (WHO, 2002).

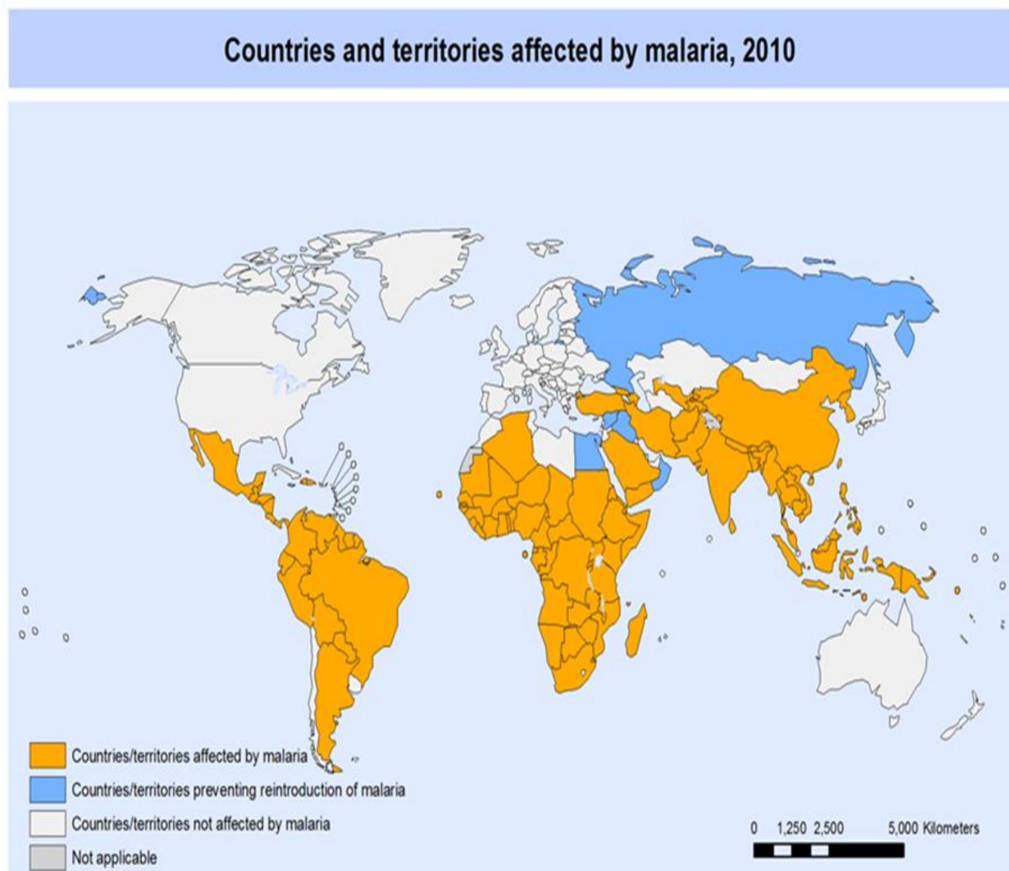

Figure 1. Global malaria distribution (WHO report, 2012). Malaria is endemic in Africa, Southeast Asia and South America.

Malaria is leading cause of death and disease in many developing countries, where young children and pregnant women are the groups most affected. In 2010, malaria caused an estimated 216 million clinical episodes, and 655,000 deaths. An estimated 91% of deaths in 2010 were in the African Region, followed by 6% in the South-East Asian Region and 3% in the Eastern Mediterranean Region (3%). About 86% of deaths globally were in children. A half of the world's populations live in areas at risk of malaria transmission in over 100 countries and territories (WHO, 2011).

2.2 Rodent malaria parasites

Rodent malaria is caused by four species, *Plasmodium chabaudi*, *Plasmodium berghei, Plasmodium vinckei* and *Plasmodium yoelii* (Smith and Parsons, 1996) which was isolated from wild thicket rats in Africa and have been adapted to grow in laboratory rodents (Carter and Diggs, 1977). *Plasmodium chabaudi chabaudi* establishes synchronous, chronic and recrudescing blood-stage infections in rodents, and exhibits many characteristics associated with the pathogenesis of human malaria, such as rosetting, sequestration and antigenic variation (Stephens *et al*., 2012). In research aimed at developing strategies for eradicating human malaria, the four murine malaria species are used as laboratory prototypes (Carlton *et al*., 2001). Rodent parasites often represent the only practical means towards *in vivo* experimentation (Janse and Waters, 1995). However in absence of sufficient similarity to human parasite, primate model exist but pharmacokinetic evaluations are much more expensive in monkey models that can sustain infection by *P. falciparum*. Housekeeping genes and biochemical processes are conserved between rodent and human parasites (Carlton *et al.,* 1998a).

Resistance in some rodent parasite has shown similarities to resistance in human parasites. For instance, atovaquone resistance in *P. berghei* and SP resistance in *P. chabaudi* have shown correlation to resistance in *P. falciparum* (Gervais *et al*., 1999; Carlton *et al*., 2001). However, CQ and ART resistance in *P. chabaudi* have no correlation to resistance in *P. falciparum* (Carlton *et al*., 1999; Afonso *et al*., 2006).

Plasmodium berghei was originally discovered by Vincke and Lips in 1948 and infects hamsters, rats and mice (Janse and Waters, 2006). At least five isolates (strains) of *P. berghei* have since been isolated namely, ANKA, SPII, WK6S, LUKA and K173 (Janse and Waters, 2006).

Generation of transgenic ANKA strain of *P. berghei* expressing exogenous green fluorescent protein (GFP) and/or luciferase has broadened possibilities for *in vivo* drug screening (de Koning-Ward *et al*., 2000; Janse *et al*., 2006). Green fluorescent protein has many potential applications in dissecting aspects of parasite biology and provides

new opportunities to study parasite-host cell interactions (Janse *et al*., 2006). Creation of a GFP fusion protein, in which the selectable marker gene and the gene encoding GFP are fused together is used as a means of observing transgenic parasites *in vivo* and selecting reversants that have lost the selectable markers (de Koning Ward *et al.,* 2000). 'Four reference' lines of the ANKA strain have been genetically transformed with reporter 10 genes GFP or fusion protein GFP-Luciferase namely (259cl2 (GFPcon); 354cl4; 507m6cl1; 676m1cl1) (Janse *et al.,* 2006).

All life cycle stages of the different isolates have a similar morphology and duration of development. The isolates also show a comparable sensitivity to antimalarial drugs and no variation in isoenzymes (Carlton *et al*., 2001; Janse and Waters, 2006). *Plasmodium berghei* has successfully been used in drug testing investigation (Ridley, 2002) and is probably the best practical model for experimental studies of human malaria drug resistance selection (Peters, 1999; Peters and Robinson, 2000; Xiao *et al*., 2004). Thus, in this study, *P. berghei* ANKA was used as experimental model to study molecular basis of pyronaridine resistance.

2.3 Human malaria parasites

The etiological agents in human malaria are four distinct species of *Plasmodium* parasites (Smith and Parsons, 1996; WHO, 2002). Among the human malaria parasites, *Plasmodium malariae* is milder than other types of malaria and commonly found in older red blood cells (Chiang *et al*., 2006). It is found in most endemic areas, throughout sub-Saharan Africa, but is much less common than the other species (Guerra *et al*., 2007). *Plasmodium ovale* is a rare parasite confined to tropical Africa and accounts for 10% of all malarial infections in Africa but is less common in other malarial endemic regions (Faye *et al*., 2002; Win *et al*., 2004). *Plasmodium vivax* is relatively non- lethal and has strong preferences for reticulocytes during invasions (Janse and Waters, 1995; Chiang *et al*., 2006). It causes up to 80 million cases per year with 15% in Africa and 85% outside Africa (Mendis *et al*., 2001). *Plasmodium falciparum* is the most common and primary cause of severe malaria in humans (Winstanley *et al*., 2004).

2.4 Life cycle of Plasmodium

Malaria parasites have complex life-cycles involving both an insect (vector) and a vertebrate (host). The life cycle of the malaria parasite is shown in **Fig. 2** (White, 2004). All malaria parasites have a universal life cycle. *Plasmodium* is transmitted to humans by the bite of an infected female *anopheline* mosquito (Beier, 1998; Frischknecht *et al*., 2004), which deposits malaria sporozoites from its salivary glands into the dermis of the host (Matsuoka *et al*., 2002)**.** Inoculated *Plasmodium* sporozoites have various fates; a proportion remains in the dermis after exhaustion of their motility, whilst others rapidly move through the blood stream into the liver to invade hepatocytes. It has been shown that a proportion of sporozoites invade the lymphatic vessels. It is notable that although the majority of lymphatic sporozoites are degraded there, recent studies have shown that some sporozoites may partially differentiate into exo-erythrocytic stages in the lymphatic vessels (Amin *et al*., 2006).

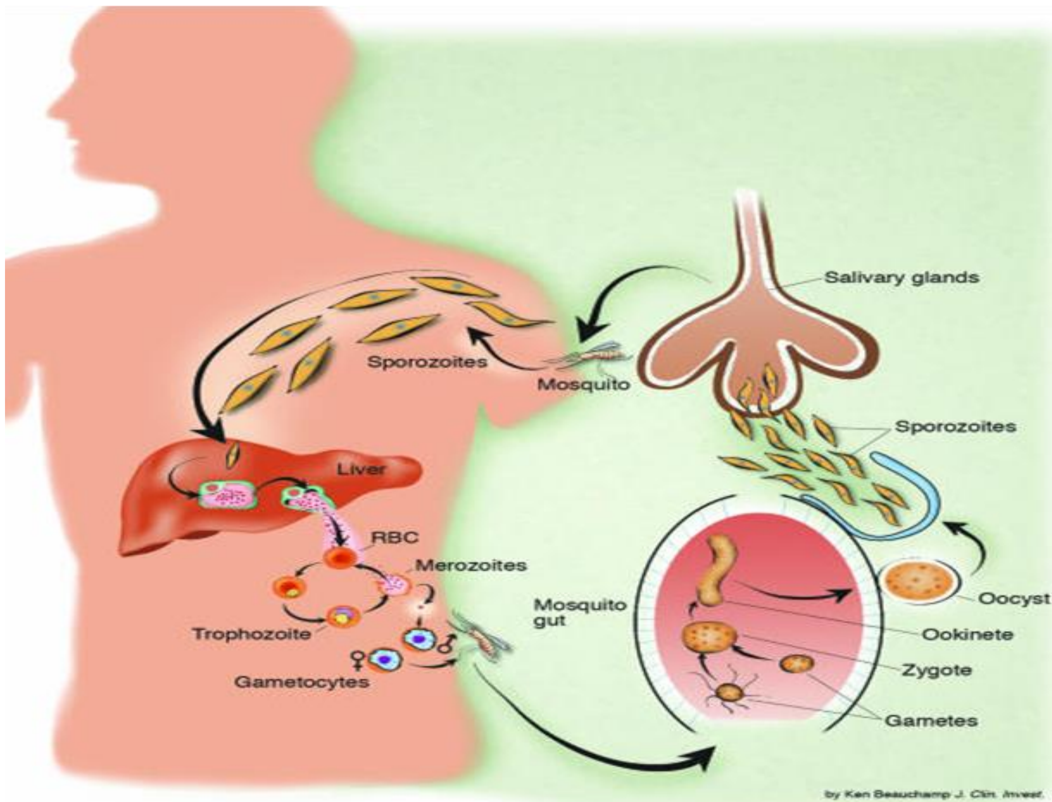

Figure 2: The life cycle of malaria parasite in human host and vector (insect). An infected female *Anopheles* mosquito feeds on host and injects sporozoites into the bloodstream that invade liver cells yielding merozoites. The blood stage of the infection comprises an asexual erythrocytic and the sexual forms (gametocytes). Gametocytes are ingested by mosquito where by fusion forms diploid zygote that is transformed to ookinete then oocyst in the basal epithelium of the midgut cell. The oocyst undergoes mitotic divisions to form sporoblasts which bud to sporozoites that migrate to salivary duct of the mosquito budding from sporoblasts. Inoculation of the sporozoites into a new host maintains the malaria life cycle.

The liver stage is an asymptomatic phase whereby the parasite undergoes silent logarithmic multiplication. In *P. ovale* and *P. vivax* infections, the latent liver stage (hypnozoite) can be prolonged. However, prolonged latent liver stage does not occur in *P. falciparum* infections. Rather, the liver stage lasts only about 6 days in *P. falciparum* infections, after which each sporozoite yields merozoites that invade and develop within RBCs, initiating the blood stage of the infection. The blood stage of the infection

comprises two distinct forms; an asexual erythrocytic form in which the parasites undergo repeated 48 hour cycles of multiplication, and the sexual forms (male and female), referred to as gametocytes. To undergo further development, the sexual forms must be ingested by mosquitoes (**Fig. 2**). During the asexual erythrocytic stage, the parasite undergoes complex transcriptional and morphological changes as it matures through the ring, trophozoite and schizont stages (Le Roch *et al.*, 2003; Bozdech *et al.*, 2003). For its survival, it requires haemoglobin as a food source, which it uses in complex metabolic pathways. At the end of 48 hours, each schizont bursts, releasing new merozoites, leading to an exponential increase in parasite levels. The asexual erythrocytic parasites are the pathogenic forms causing a febrile illness, which initially presents as non-specific symptoms, but which may develop into a severe disease, affecting different organ systems. Notably, these asexual intra-erythrocytic stages are main target of antimalarial drugs (Winstanley and Ward, 2006; Greenwood *et al.,* 2008). The genome of the malaria parasite is haploid, except briefly after fertilization.

Sexual reproduction in the *Plasmodium* parasite occurs in the *anopheles* mosquito. Sexual stage parasites (gametocytes) are non-pathogenic and are transmitted from the host to the *anopheles* vector during a blood meal. Ingestion of gametocytes by a mosquito activates the formation of gametes (gametogenesis) in the mosquito mid gut lumen, which fuse within minutes to form a diploid zygote. Soon after zygote formation, meiosis is executed and genetic recombination occurs. The zygote then transforms into a motile ookinete, which migrates to the basal epithelium of the midgut cell where it arrests and transforms into an oocyst. The oocyst undergoes several mitotic divisions to form sporoblasts. Sporozoite budding from sporoblasts takes 10-14 days after the blood meal, rendering this the longest developmental phase of the *Plasmodium* life cycle. The genetically distinct sporozoites actively egress the oocyst by proteolytic activity, entering the hemolymph, the circulatory system of the mosquito. When passing through the salivary glands, the sporozoites invade the acinar cell layer from the basal side, accumulating in the salivary duct of the mosquito. Inoculation of the sporozoites into a new host perpetuates the malaria life cycle (Matuschewski, 2006).

2.5 Malaria diagnosis

Early diagnosis and prompt treatment are fundamental components of the WHO global strategy for malaria control (WHO, 2010). Current confirmatory qualitative methods include parasitological confirmation by Microscopy, Rapid Diagnostic Test (RDTs) and Quantitative Buffy Coat (QBC) (WHO, 2010). In most malaria endemic countries of sub-Saharan Africa, the standard for laboratory confirmation of a clinical malaria diagnosis is a peripheral blood film, examined microscopically (Murray *et al.*, 2008; Batwala *et al.*, 2010). The quantitative buffy coat method (QBC^{TM}, Becton-Dickinson), uses micro-haematocrit tubes pre-coated with fluorescent acridine orange stain to highlight malaria parasites. Rapid Diagnostic Test (Antigen detection tests) is a third diagnostic approach involving the rapid detection of parasite antigens using rapid immuno-chromatographic technique based on the detection of the Histidine-rich protein 2 (HRP-II) of *P. falciparum*. Compared with light microscopy and QBC, this test yields rapid and highly sensitive diagnosis of *P. falciparum* infection.

2.6 Clinical features and pathogenesis of malaria

Malaria parasites can develop a wide range of symptoms. Clinical features of malaria range from uncomplicated (no apparent illness), to the classic symptoms of malaria, to severe disease. The pathogenesis of malaria fever is due to the rupturing of schizonts which stimulate human mononuclear cells to release tumor necrosis factor (TNF) and other pyrogenic cytokines (Lou *et al.*, 2001).

Plasmodial glycosylphosphatidylinositols induce production of elevated levels of pro-inflammatory cytokines, TNF-α, IL-1, INF-γ and IL- 12 implicated in development of cerebral malaria (CM) (Angulo and Fresno, 2002). Certain features of severe malaria are common to all infected species, with hypoglycemia and lactic acidosis occurring in the terminal phase of avian, rodent, simian and human malaria (Hearn *et al.*, 2000; Angulo and Fresno, 2002). Morphing molecules, *Plasmodium falciparum* erythrocyte membrane protein-1(PfEMP-1) major export products from the parasite to the surface of the infected erythrocyte causes the infected erythrocyte to stick to the endothelial cells that line blood vessels (Webster and Hill, 2003). Sequestration occurs principally during the

second half of the intra-erythrocytic asexual growth phase of the parasite, following the adherence of mature parasites (Beeson *et al*., 2000) to endothelial cells through electron-dense knobs on the pRBC surface, hence, blocks cerebral capillaries causing CM (Deitsch and Hviid, 2004). Sequestration is thought to play a key role in some of the life threatening complications characteristic of severe malaria such as metabolic acidosis, seizures, and hypoglycemia (Bell and Molyneux, 2007).

Sequestration of parasitized erythrocytes also occurs in murine CM although in a less prominent fashion than in humans (Hearn *et al*., 2000). *Plasmodium falciparum* sequestration is a distinctive pathologic feature that often leads to fatal human cerebral malaria. Sequestration of parasitized erythrocytes causes clogging of the minute capillaries, venules, and arterioles culminating in damage of vital organs like the brain, kidneys, lungs, liver, and gastrointestinal tract (Hearn *et al*., 2000).

2.7 Interventions used for malaria control.

The main pillars of the current efforts to control malaria as recommended by WHO include, long-lasting insecticidal nets (LLINs), indoor-residual spraying programs (IRS), chemoprophylaxis and vaccine development (WHO, 2010). However, improved diagnostics and usage of effective chemotherapy remains a principal mode of malaria control (WHO, 2010). As a last resort, intermittent preventive treatment with antimalarial drugs has been used to reduce the impact of malaria infection in the foetus during pregnancy (WHO, 2008a).

2.7.1 Vector control

Vector control remains the most generally effective measure to prevent malaria transmission, and as such it is one of the four basic technical elements of the global malaria control strategy which include; the Indoor residual spraying (IRS) with insecticides and use of Insecticide-treated nets (ITNs) (Lengeler, 2004). The widespread insecticide resistance raises concerns for vector control implementation and sustainability particularly for the control of the main vector of human malaria.

2.7.2 Vaccine development

Although research has greatly advanced understanding of malaria biology and epidemiology, a safe and effective malaria vaccine for use in humans remains elusive (Breman and Plowe, 2009). However, new vaccines that prevent malaria infection have been recently reported. These vaccines known as *RTS, S/AS01B* and *RTS, S/AS20B* are under clinical trials, thus, still not commercially available (Bejon *et al*., 2008; Kester *et al*., 2009).

2.7.3 Malaria chemotherapy

Antimalarial drugs can be classified and evaluated based on the stages of the malaria life cycle that they target, their molecular targets, chemical structure, half-life or cost (Daily, 2006; Schlitzer, 2007). Due to variation in stage specific biology, antimalarial drugs may act on different stages of the malaria parasite. For instance, tissue schizonticides kill hepatic schizonts and prevent invasion of erythrocytes, thereby preventing the establishment of clinical malaria. Hypnozoiticides kill persistent intra-hepatic stages (occurring in *P. vivax* and *P. ovale* infections) thus prevent relapses from these dormant stages of infection. Of the antimalarials in clinical use, only primaquine is available for the elimination of hypnozoites (Daily, 2006). Gametocytocides eliminate the intraerythrocytioc sexual forms (gametocytes), and thus prevent human to human transmission (Schlitzer, 2007). Antifolates attack all growing stages of the malaria parasite (Chiang *et al*., 2006).

Only artemisinin and its derivatives and primaquine (Chiang *et al*., 2006) have reported gametocytocidal activity. Most antimalarials in clinical use are blood schizonticides, which work by eliminating the asexual erythrocytic parasite form (Schlitzer, 2007). As there are no dormant hepatic stages in *P. falciparum* malaria, blood schizonticides are sufficient to manage infections.

Based on chemical structure, antimalarial drugs are classified as; aminoquinolines, aryl-alcohols, quinoline alcohols, antifolates, antibiotics and artemisinins (Robert *et al*., 2001)

2.7. 3.1 Aminoquinolines

Aminoquinolines (Quinolines) drugs used in the clinical management of *falciparum* malaria can be categorized into two main subgroups; a) 4-aminoquinolines which include chloroquine (CQ), amodiaquine (AQ) and piperaquine (PQ) and b) 8-Aminoquinolines include three members, primaquine, tafenoquine and pamaquine.

The main antimalarial drugs in this group are CQ and AQ. Amodiaquine is structurally related to CQ and has been in use for more than 70 years (Stepniewska and White, 2008). The antimalarial activity of AQ is exerted by the primary metabolite, monodesethylamodiaquine. Based on structural similarity, the 4-aminoquinoline drugs are hypothesized to act by inhibiting heme detoxification, and have been shown to accumulate within the digestive vacuole (DV) and to bind to heme *in vitro* (Foley and Tilley, 1998; Hayeshi *et al*., 2008).

Chloroquine has one of the longest half-lives among antimalarials with approximately 60 days, which provides a chemoprophylactic effect during the drug elimination phase but also exposes the parasites to an extended time period during which chloroquine has fallen below the therapeutic concentration, which may select for drug-resistant parasites (Stepniewska and White, 2008). Chloroquine mode of action is based on its accumulation in the food vacuole of the parasites. This interferes with heme polymerization (Biagini *et al*., 2003).

Chloroquine is hypothesized to leads to parasite death by heme poisoning (Wistanley and Ward, 2006). Polymorphisms in *PfCRT* have been demonstrated to be the main CQ resistance determinant (Sidhu *et al*., 2002). In some parasite strains PfMDR-1 can also modulate the degree of CQ resistance (Barnes *et al.,* 1992), indicating that some alleles and overexpression of PfMDR-1 may increase the concentration of CQ within the DV by active transport. Interestingly, studies have demonstrated linkage disequilibrium between *PfMDR-1* and *PfCRT* alleles in CQ resistant parasites in Southeast Asia and Africa, suggesting a functional interaction of both proteins (Hastings, 2006; Osman *et al.,* 2007)

The 8-aminoquinoline family of drugs includes three members, primaquine, tafenoquine and pamaquine (Sweeney *et al*., 2008). Primaquine is the only tissue schizonticide (exoerythrocytic) drug used for prophylaxis against all types of malaria. However, Primaquine has been shown to cause adverse reactions. Although the mechanism remains unclear, the drug is suspected to inhibit vesicular transport (Hiebsch *et al*., 1991) and the parasite enzyme dihydroorotate dehydrogenase (Ittarat *et al*., 1994).

2.7.3.2 Quinoline alcohols

Quinoline Alcohols include quinine (QN) and mefloquine (MFQ). Quinine, an aryl-amino alcohol, forms one of the oldest antimalarial agents and has been used by the native population of Peru for centuries in the form of pulverized bark of the cinchona tree to treat fevers and chills. In 1820, the active alkaloid from the bark was isolated and named quinine (Butler *et al*., 2010). Quinine is now used to treat severe cases of malaria and, as a second line treatment, in combination with antibiotics to treat resistant malaria. The molecular mechanism by which quinine acts against *P. falciparum* is only partially understood. Similar to CQ, quinine has been demonstrated to accumulate in the parasite's DV and can inhibit the detoxification of heme, an essential process within the parasite (Fitch, 2004).

Mefloquine is a 4-methanolquinoline with a long half-life of 14–18 days and was first introduced in the 1970s (Zhang *et al*., 1999; Stepniewska and White, 2008).The drug emerged as a successor to CQ in the 1980s but resistance emerged at the border between Thailand and Cambodia within a few years owing to widespread use of quinine (Duraisingh and Cowman, 2005). It has small therapeutic range and is less potent than CQ owing to relatively weak interaction with free heme (Winstanley *et al*., 2004). Mefloquine remains a drug of choice for prophylaxis before traveling to malaria areas where CQ-resistant *P. falciparum* exists (Baird, 2005). Resistance to MFQ is mediated by amplification of *PfMDR-1*, leading to overexpression of this resident DV membrane transporter (Cowman *et al*., 1994). Although the exact mechanism of action remains unclear, *in vitro* experiments demonstrate that MFQ can bind to heme and exert some antimalarial activity by inhibiting heme detoxification (Eastman and Fidock, 2009). However, studies on transgenic parasites expressing different *PfMDR-1* copy numbers,

observed a reduced parasite susceptibility to mefloquine with increased PfMDR-1 mediated import into the DV (Rohrbach *et al.,* 2006; Sidhu *et al.,* 2006), suggesting a primary mode of action outside of the DV (Foley and Tilley, 1998). Additionally, it has been shown that MFQ inhibits the import of other solutes into the DV and might therefore also target the PfMDR-1 transport function itself (Rohrbach *et al.,* 2006).

2.7.3.3 Aryl-alcohols

Common aryl-alcohols antimalarial drugs are lumefantrine (LM), pyronaridine (PRD) and halofantrine (HF). Lumefantrine is a synthetic fluorene antimalarial compound also chemically related to MFQ (Ezzet *et al*., 2000). Despite differences in the ring structure and side-chain substituents aryl-alcohols share the basic chemical characteristic, a hydroxyl group near the ring hypothesized to confer the antimalarial activity (Basco and Ringwald, 1998). Lumefantrine was first synthesized at the Academy of Military Medical Sciences, Beijing, China and has undergone preliminary clinical studies in China (Basco and Ringwald, 1998). It has an elimination half-life of up to 6 days in malaria patients with absorption being enhanced by co-administration with fat (Toovey and Jamieson, 2004). Lumefantrine is remarkably well tolerated with generally mild– nausea, abdominal discomfort, headache and dizziness (WHO, 2006). Lumefantrine is used only in combination with artemether for the treatment of uncomplicated multi-resistant *falciparum* malaria (Mutabingwa, 2005). Polymorphisms in *PfMDR-1*, particularly the variant N86, and amplification of the encoding gene (*PfMDR-1*) have been associated with reduced susceptibility to LM in Africa and Asia (Sisowath *et al.,* 2005; Price *et al.,* 2006; Sisowath *et al*., 2007). Halofantrine on the other hand is effective against CQ-resistant malaria but cardio-toxicity has limited its use as a therapeutic agent (Robert *et al.,* 2001).

Pyronaridine is an acridine derivative and a synthetic drug widely used in China (Robert *et al*., 2001). The Chinese oral formulation is reported to be effective and well tolerated but has a low oral bioavailability contributing to high cost of the treatment (WHO, 2006). Despite extensive reports of its highly potent anti-malarial activity, few studies have been conducted to determine the drug's mechanism of action. However, studies have shown that PRD does not cause the formation of a protein-DNA complex *in situ*

and thus does not appear to target the malaria parasite DNA topoisomerase II as earlier reported (Auparakkitanon *et al.*, 2003). Latest studies hypothesized that the primary mode of action of PRD is similar to that of CQ, namely inhibition of haematin formation, enhanced haematin induced red blood cell lysis and interference with glutathione- dependent heme degradation (Auparakkitanon *et al.,* 2006).

2.7.3.4 Folate antagonists

These compounds inhibit the synthesis of tetrahydrofolate co-factors essential in the synthesis of the pyrimidine deoxythymidylate for parasitic DNA (Gregson and Plowe, 2005). There are two groups of antifolates: (i) the dihydropteroate synthase (DHPS) inhibitors (type-I antifolates) that are sulphonamides and sulfones like sulfadoxine, sulfalene and dapsone, respectively and (ii) the dihydrofolate reductase (DHFR) inhibitors (type-II antifolates) that are diaminopyrimidines and biguanides like pyrimethamine, trimethoprim and proguanil, chlorproguanil, respectively (Bloland, 2001).

In response to development of emergence of CQ resistance, drug combination sulfadoxine–pyrimethamine (Fansidar) was introduced. Unfortunately, resistance due to point mutations in both target enzymes emerged quickly afterwards (Uhlemann and Krishna, 2005). As a result, sulfadoxine–pyrimethamine is now primarily used as intermittent preventative malaria treatment during pregnancy and, to a lesser extent, for the treatment of malaria infection. The two other antifolate drugs, dapsone and proguanil, were also administered as a combination, Lapdap™. Unfortunately dapsone causes hemolysis in Glucose-6-Phosphate deficiency deficient patients, and therefore this combination therapy is no longer recommended (Luzzatto, 2010).

In addition, aminopterin and methotrexate are also potent antifolates although they inhibit both human and parasite DHFR enzyme and are common cancer therapeutic agents (Bertino *et al.*, 1996). Methotrexate is only a slight modification of the dihydrofolate the normal substrate of DHFR, hence competes effectively with the substrate in the DHFR active site (Nduati *et al.*, 2005). These drug are however not

common antimalarial drugs due to their narrow therapeutic indices and life-threatening toxicity to the human host (Nzila, 2006).

2.7.3.5 Bisquinolines

Bisquinolines are compounds with two quinoline nuclei bound by a covalent aliphatic or aromatic link. Several of these compounds, including piperaquine, were identified as promising candidates during drug screening programs in the 1960s by Shanghai Pharmaceutical Industry Research Institute in China and Rhone Poulenc in France (Hung *et al.*, 2004). The drug has a long half-life of 17–25 days (Tarning *et al.*, 2004). The bis-4-aminoquinoline includes piperaquine (PQ) which, has structural similarities with CQ, although the exact mechanism of action is unclear, studies have shown that PQ accumulates in the DV and that it is a potent inhibitor of heme polymerization (Warhurst *et al.*, 2007). Intensive PQ monotherapy has led to the emergence of resistant *P. falciparum* strain; however, PQ is currently employed as a partner drug in an artemisinin-based combination therapy (Davis *et al.*, 2005).

2.7.3.6 Other antimalarial drugs

Atovaquone is a lipophilic hydroxynaphthoquinone analog structurally related to ubiquinol and is used for treatment of apicomplexan parasites, including *Plasmodium, Toxoplasma, Theileria* and *Babesia* (Srivastava *et al.,* 1997). It has a blood plasma half-life of 2–3 days (Hughes *et al.,* 1991; Hudson *et al.,* 1991). Molecular evidence exists that atovaquone specifically targets the cytochrome bc1 complex, located in the inner mitochondrial membrane, thereby inhibiting the respiratory chain. In *P. falciparum* blood stage parasites the respiratory chain is required for the regeneration of ubiquinone, the electron acceptor for dihydroorotate dehydrogenase, which is an essential enzyme for pyrimidine biosynthesis (Painter *et al.,* 2007). Atovaquone is currently used in combination with proguanil (Malarone), mainly as a prophylactic medication for tourists, due to the high costs and high incidences of resistance, which is conferred by single nucleotide polymorphisms in the cytochrome b gene (Gil *et al.,* 2003).

Antibiotics also play a major role in the treatment and prophylaxis of malaria. Tetracycline and chloramphenicol are well tolerated and effective as antimalarial drugs.

Tetracycline and its analogues doxycycline, azithromycin, clindamycin are used as prophylactic agents against *P. falciparum*; that are known to block translation by binding reversibly to the 30S subunit and distorting it in such a way that the anticodons of the charged tRNAs cannot align properly with the codons of the mRNA (Bloland, 2001; Robert *et al*., 2001; Chiang *et al*., 2006).

2.7.3.7 Artemisinin derivatives

Artemisinin also known as Qinghaosu, an active constituent of *Artemisia annua* (Chinese sweet wormwood), has been used as herbal remedy in china for many centuries. The artemisinin drugs (including its derivatives), now considered as the most important new antimalarials used for the treatment of severe malaria (Chiang *et al*., 2006; Eastman and Fidock, 2009), are also fast acting against multidrug-resistant *P. falciparum* strain. The various artemisinin derivatives include dihydroartemisinin (DHA), artemether, arteether, (oil soluble ethers), artesunate (water-soluble hemisuccinate) and artelinic acid. They are all cyclic endoperoxides or sesquiterpene lactone compounds and are well-tolerated and rapidly eliminated drugs (O'Neill and Posner, 2004). The exact mode and site of action of ART compounds is still unclear. Recent *in vivo* and *in vitro* studies have suggested that ART may specifically target essential *Plasmodial* PfATPase, a *P. falciparum* SERCA type Ca^{2+} dependent ATPase localized in the endoplasmic reticulum (Biagini *et al*., 2003; Eckstein-Ludwig *et al*., 2003; Meissner *et al*., 2007). Artemisinins are also thought to exert their activity through interaction with heme preventing detoxification of heme by polymerization into hemozoin (Robert and Meunier, 1998; O'Neill and Posner, 2004).

2.7.4 Combinational therapy

For half a century, CQ was the mainstay of uncomplicated malaria therapy. The eventual appearance and spread of CQ resistance led to the introduction of SP which soon met with rapid development of resistance, rendering it increasingly ineffective in many endemic areas (Wongsrichanalai *et al*., 2002). To date, *P. falciparum* clinical treatment failure has been reported for all the common classes of antimalarial drugs apart from artemisinin compounds, although there are recent reports of reduced artemisinin sensitivity in Cambodia (Noedl *et al*., 2008; Dondorp *et al*., 2009;

Cheeseman *et al*., 2012). To reduce the pace of selection of resistance, WHO recommends that all antimalarial therapies be deployed as combinations that include an artemisinin derivative as one of the partner drugs, an approach referred to as Artemisinin Combination Therapies (ACTs). The introduction of ACT in malaria endemic countries together with other malaria control interventions is reported to have contributed to the recent declines in malaria mortality and morbidity observed in some settings (WHO, 2009). Several different forms of ACTs have been evaluated, including; artesunate-amodiaquine (Brasseur *et al*., 2007), artemether-lumefantrine (Yeka *et al*., 2008), artesunate-mefloquine (Ashley *et al*., 2008) dihydroartemisinin-piperaquine (Bassat *et al*., 2009) and now the recently WHO prequalified combination therapy artesunate-pyronaridine, PRD/ASN (Ramharten *et al*., 2008). More recently, interest has been renewed in pyronaridine as a possible partner for use in artemisinin-based combination therapy (ACT) for malaria treatment. Hence, the study presented here was devoted to investigate association of point mutation in coding region of *MDR-1* gene with reduced susceptibility to PRD *in vivo.*

2.8 Global distribution of resistance

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today. Drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. Drug resistance has also played a significant role in the occurrence and severity of epidemics in some parts of the world (WHO, 2001). The introduction of CQ in the 1940s had a huge impact on global health, however, today resistance to the drug has been observed in every region where *P. falciparum* occurs (**Fig. 3**).

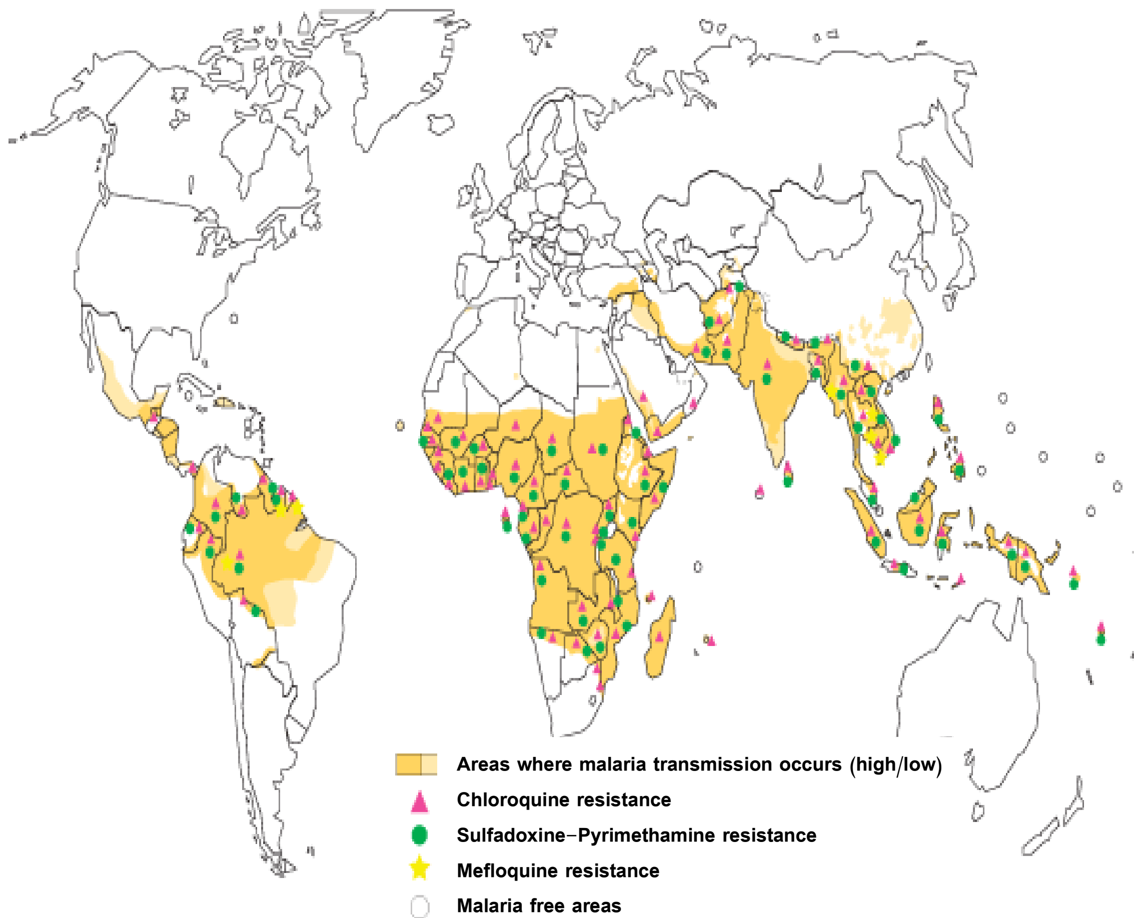

Figure 3: Distribution and epidemiology of drug resistant *P. falciparum* malaria (WHO, 2005).The map shows the resistance to antimalarial drugs developed in Southeast Asia, parts of South America and largely in Sub-Saharan Africa.

Resistance developed from several independent foci, and over the next 20 years gradually spread throughout South America and Southeast Asia arriving in East Africa in the late 1970s. Chloroquine resistance has since spread across all of Sub-Saharan Africa. As a result, many countries switched their first-line antimalarial drug to SP; however, resistance to SP grew and spread quickly, especially in Southeast Asia, South America and more recently in many areas of Africa (**Fig. 3**). This loss of effectiveness of the newer antimalarial drugs has also occurred at an alarming rate. For example, resistance to MFQ was reported as early as five years after its introduction as a prophylactic treatment in parts of Thailand, whereas resistance to atovaquone was even more rapid, emerging in the same year as its launch (Wongsrichanalai *et al*., 2002).

Initiatives have been introduced to stem the number of drug-resistance mediated clinical failures, such as the introduction of artemisinin derivative based combination therapy (ACTs) and the deployment of new drugs (e.g. LapDap; Chlorproguanil/dapsone). However it is clear that a better understanding of resistance mechanisms to currently used drugs is required to support the development of such strategies and help prevent the development of resistance to new therapies.

2.9 Drug resistance in malaria

Antimalarial drug resistance has been defined as “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject (WHO, 1965). Most often, the term resistance refers to therapeutic failure after administration of a standard dose of an antimalarial drug, as defined in the WHO standard *in vivo* test protocol (Talisuna *et al*., 2004). Previously, the evolution of antimalarial drug resistance was considered to be a single step process, whereby parasites that are fully sensitive to drug become fully resistant, thus leading to a dichotomous classification of clinical malaria infections i.e. any clinical infection classified as ‘resistant’ or ‘sensitive’ to an antimalarial at therapeutic doses. However, the use of such a strict and generalized dichotomy in classification of resistance has been questioned (Hastings and Watkins, 2006). This is because whilst such a strict distinction between sensitivity and resistance may be appropriate for antimalarial drugs to which resistance arises in a single step (e.g. Atovaquone to which resistance arises through a single mutation in the cytochrome *b* gene (Srivastava *et al*., 1999), this dichotomy does not take into account the important intermediate stage of drug tolerance. Drug tolerance is now considered a biologically important stage in the step-wise evolution of malaria drug resistance. Importantly, studies suggest that the drug elimination profile is an important parameter that determines the evolution of tolerance and ultimately resistance (Srivastava *et al*., 1999; Nzila *et al*., 2000)

2.9.1 Spread of drug resistance

The emergence of resistance in *Plasmodium* depends on multiple factors; the mutation rate of the parasite, the fitness costs associated with the resistance mutations, the overall

parasite load, the strength of drug selection, and the treatment compliance (Petersen *et al.,* 2011). The evolution of drug resistance mutations in itself is not sufficient for the spread of malaria drug resistance. Rather, the survival and subsequent multiplication of drug resistant parasites after drug treatment increases the likelihood of transmission of resistant sexual stages (gametocytes) to the next host, therefore increasing the likelihood of the spread of resistance. Notably, some studies demonstrated that gametocyte carriage and infectivity to mosquitoes was consistently higher in patients infected with drug resistant parasites than in those infected with drug sensitive parasites (Barnes and White, 2005). Therefore, although elimination of the asexual blood stages is the focus of treatment in individual symptomatic patients, at a population level, reducing the post-treatment carriage of sexual stages (gametocytes) is necessary to limit the transmission of resistant malaria parasites to new hosts. Interestingly, individuals living in regions where malaria is endemic develop an acquired partial immunity to malaria, which protects them against severe illness without eliminating chronic, mild and asymptomatic infections. The level of acquired immunity is lower for individuals living in low transmission settings compared to those living in high transmission settings (Chiyaka *et al*., 2009). This acquired partial immunity can affect malaria transmission to mosquitoes and drug resistance. Therefore, it would be expected that selective pressure would be lower in high transmission settings because infections from asymptomatic carriers have a higher transmission advantage than those from patients carrying resistant infections. Clearly, the spread of resistance is a highly dynamic process. Furthermore, because humans infected with *P. falciparum* often carry several distinct infections (different parasite clones), the intra-host dynamics involved after treatment may also affect the transmission of drug resistant parasites (Wargo *et al*., 2007). Importantly, studies based on mathematical models have demonstrated that treatment does not always eliminate infection in a population (Färnert *et al*., 2009; Chiyaka *et al*., 2009). In addition, drug resistance may enhance transmission if drug selection pressure diminishes the viability of sensitive gametocytes in a polyclonal infection, increasing the propensity for transmitting drug-resistant parasites (Hastings, 2006). Therefore, it has been argued that treatment with gametocytocidal drugs such as artemisinins can prevent the spread of malaria drug resistance (Barnes and White, 2005). In addition, it has been shown that

resistance spreads faster in regions with better access to drugs, and with increasing drug use (Ord *et al*., 2007). Again, spread of resistant parasites is also affected by the impact of antimalarials on the gametocytes, which are the transmissible stages of the parasite. Artemisinins have been shown to decrease the number of gametocytes carried by a patient, thereby reducing transmission (Okell *et al*., 2008). Therefore, in endemic settings it is important to consider not only the ability of antimalarial to cure clinical malaria, but also their effect on the transmission of drug resistant phenotype at a population level.

2.10 Drug resistance genes and resistance mechanisms in malaria parasites

Efficient drug treatment involves the targeting of an essential biological process, which differs from or is non-existent in the host. *Plasmodium* is highly adapted to its unique living environments and many genes show little or no sequence similarity to other genes encoding characterized proteins of malaria parasites (Gardner *et al*., 2002). This creates a number of putative drug targets; however, with little information to evaluate the functionality or essential nature of these determinants, target validation becomes a challenge. Additionally, the field is hampered by scarce or inefficient genetic tools for the analysis and identification of candidate genes in this haploid organism (Meissner *et al*., 2007). Linkage studies of three available experimental genetic crosses, investigation of field isolates and examination of candidate genes have led to the identification of the *CRT*, *MDR-1*, *DHFR*, *DHPS* genes responsible for resistance to the most important antimalarial drugs (Nagesha *et al*., 2001; Osman *et al*., 2007). These have been implemented as molecular markers to screen for the emergence of resistance and assess its spread, as means to inform rational drug policy decisions.

2.10.1 Multi drug resistant gene 1

The *Plasmodium falciparum* multidrug resistance transporter (PfMDR-1) is a *P. falciparum* ortholog of mammalian *P*-glycoproteins that mediate verapamil reversible multidrug resistance in mammalian cancer cells (Reed *et al*., 2000; Qi *et al*., 2004). The *PfMDR-1* gene is located on chromosome 5 and encodes a 162-kDa ABC type transporter, which localizes to the membrane of the digestive vacuole and consists of

two homologous halves, each with six predicted trans-membrane domains and a conserved nucleotide binding domain (Peel *et al.,* 2001; Valderramos and Fidock, 2006). The predicted structure of PfMDR-1protein and the nucleotide binding domains and known polymorphisms are shown in **Figure 4**. Polymorphisms occur at five amino acid positions; (N86Y, Y184F, S1034C, N1042D and D1246Y) (Foote *et al*., 1990).

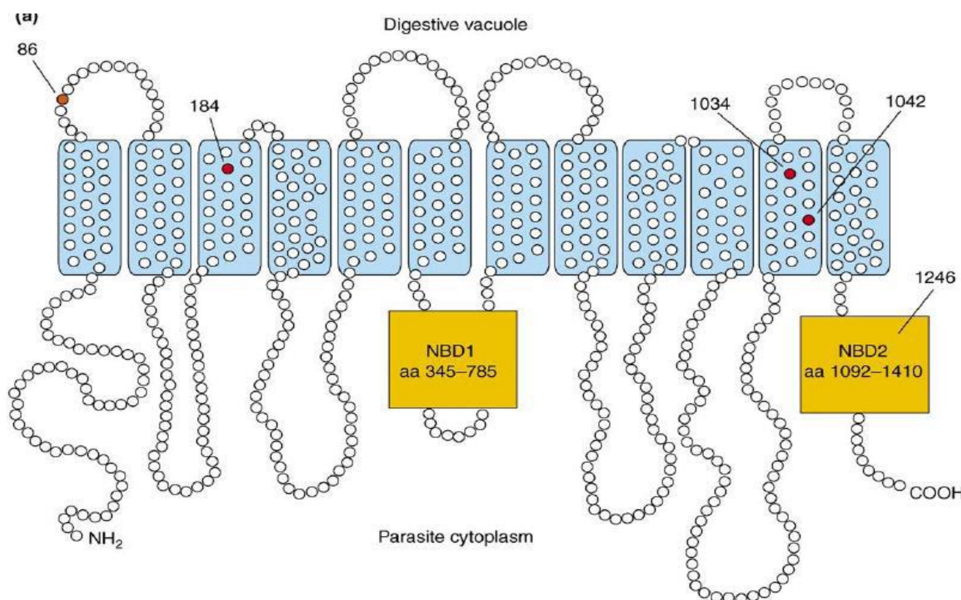

Figure 4: Predicted structure of PfMDR-1. The assembly of multi-drug resistance gene 1 consists of two homologous halves, each with six predicted trans-membrane domains ( ) and a conserved nucleotide binding domain ( ) with NH_2 and COOH terminal. The (**o**) represents amino acids while ( ) denotes five positions where polymorphisms occurs in multi-drug resistance gene 1.

PfMDR-1 is thought to play an ancillary role in CQ resistance, although the evidence has sometimes been conflicting. Indeed, although some studies reported an association between the *PfMDR-1* N86Y mutation and CQ resistance (Nagesha *et al*., 2001; Babiker *et al*., 2001; Mita *et al*., 2006), many studies have shown that *PfMDR-1* mutation status on its own is not sufficient to confer CQ resistance in a PfCRT wild type background (Sidhu *et al*., 2002; Sisowath *et al*., 2007; Beez *et al*., 2010). However, some studies have demonstrated that the *PfMDR-1* N86Y mutation may increase *in*

vitro CQ resistance in isolates already carrying the mutant *PfCRT* 76T allele (Durand *et al*., 2001; Valderramos and Fidock, 2006), demonstrating that PfMDR-1 indeed plays a role in modulating the level of CQ resistance. It has also been suggested that *PfMDR-1* polymorphisms may play a compensatory role in CQ resistant parasites (Duraisingh and Refour, 2005), or that they may modulate the degree to which CQ and other drugs or solutes accumulate in the food vacuole, by interacting with PfCRT and possibly other transporters (Reed *et al*., 2000; Sidhu *et al*., 2005; Rohrbach *et al*., 2006). The *PfMDR-1* point mutations alter the degree of parasite resistance to other major quinoline and quinoline-methanol like drugs including mefloquine, halofantrine, quinine and artemisinin. Notably, most studies have reported an inverse relationship between CQ resistance and resistance to these drugs (MFQ, LM), which are also thought to act on the parasite food vacuole (Duraisingh and Cowman, 2005; Rohrbach *et al*., 2006).

Several studies have linked *PfMDR-1* over-expression with drug resistance in *falciparum*, similarly to the multidrug resistance mechanism observed in mammalian cancer cells. For example *PfMDR-1* transcript levels increased after treatment with CQ, MFQ and QN (all quinoline based drugs), but not after treatment with pyrimethamine (an antifolate) (Myrick *et al*., 2003). This suggested that induction of *PfMDR-1* was specifically associated with the quinoline class of antimalarial drugs. Mechanisms other than *PfMDR-1* polymorphisms may also play a role in mediating altered drug responses in parasites. This study, selected resistance of PRD in *P. berghei* ANKA isolates, then investigated possible role of *MDR-1* gene which mostly associated with development of resistance in quinolone based antimalarial drugs. Further studies to identify possible gene targets that may be associated with PRD resistance are required.

2.10.2 Chloroquine resistance gene

Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT) is a member of the drug/metabolite super family of transporters (Sanchez *et al*., 2007). The *PfCRT* gene resides in chromosome 7 and encodes a 45-kDa protein with ten predicted trans-membrane domains, located on the membrane of the parasite digestive vacuole (Cooper *et al*., 2002; Goldberg, 2005). Polymorphisms in *PfCRT* are a key determinant of CQ-resistance both *in vivo* and *in vitro* (Fidock *et al*., 2000; Djimde *et al*., 2001). Of all the

mutations, the *PfCRT* K76T mutation is the most critical determinant of CQ resistance *in vitro* and *in vivo*, making it a useful marker of CQ resistance (Fidock *et al*., 2000; Djimde *et al*., 2001; Sidhu *et al*., 2002; Lakshmanan *et al*., 2005).

Several mechanisms are proposed to account for the exact mechanism by which mutated *PfCRT* mediates reduced concentration of CQ in the food vacuole of CQ resistant parasites. It was initially postulated that deprotonated CQ passively leaks out of the food vacuole along an electrochemical gradient, the mutated *PfCRT* acting as a channel (Warhurst *et al*., 2002). It has also been proposed that mutated *PfCRT* modulates drug accumulation indirectly by altering the pH of the DV either alone (Bennett *et al*., 2004) or through interaction with other transporters (such as the V-type H+ ATPase (PVP2), PfMDR-1 or the sodium hydrogen exchanger (PfNHE) (Zhang *et al*., 2002; Nessler *et al*., 2004). It has also been proposed to have a role in the transport of amino acids and small peptides within the digestive vacuole, although it is not clear how this might influence CQ action (Zhang *et al*., 2002; Zhang, *et al*., 2004). It has also been postulated that PfCRT may be an energy dependent CQ transporter which actively pumps CQ out of the food vacuole (Sanchez *et al*., 2007; Lehane and Kirk, 2008). The CRT transporter can also significantly influences parasite *in vitro* susceptibility to quinine, desethylamodiaquine, halofantrine and artemisinin (Wongsrichanalai *et al*., 2002).

2.10.3 Dihydrofolate reductase mutations

A bifunctional enzyme in the folate biosynthesis pathway, dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a well-defined target of traditional antimalarial drugs such as pyrimethamine and cycloguanil (Fohl and Roos, 2003; WHO, 2008b). DHFR catalyzes the production of THF from DHF while TS is in charge of transferring a methyl-group from N5, N10-methylene-tetrahydrofolate to dUMP thereby generating dTMP and THF. Important polymorphisms in *PfDHFR*, conferring resistance to pyrimethamine, are S108D and I164L, with N51I and C59R further modulating the strength of resistance, in addition to amplification of *PfDHFR* (Wernsdorfer and Noedl, 2003).

2.10.4 Dihydropteroate synthase mutations

PfDHPS is involved in producing a folate precursor and is inhibited by the sulfur-based drugs sulfadoxine and dapsone (Gregson and Plowe, 2005). Resistance to the sulfonamides and sulfones often administered in synergistic combination with antifols also results from sequential acquisition of mutations in the *DHPS* gene (White, 2004). Resistance to sulfadoxine is strongly associated with amino acids S436A, A437G, K540E, A581G and A613T/S having enhancing effects (Wernsdorfer and Noedl, 2003; Anderson and Roper, 2005; Khattak *et al*., 2013).

2.11 Strategies against drug resistance

Over the past few years, 13 countries in Africa have changed their national policies to require the use of more effective antimalarial treatments. World Health Organization recommends first, the use of ACTs which are highly efficacious and promises to delay the emergence of resistance where monotherapies are failing, (WHO, 2003) and secondly, use of molecular markers to predict emergence of resistance.

2.11.1 Artemisinin based combination therapies (ACTs)

One strategy for reducing the prevalence of malaria is the use of drug combinations, which is thought to protect each drug from the development of resistance and reduce the overall transmission of malaria (White, 2001). Artemisinin Combinational therapy using carefully matched drugs is now the recommended strategy both for clinical care and avoidance of drug resistance (Yeung *et al*., 2004). ACTs consisting of an artemisinin derivative, characterized with short elimination half-life and long elimination half-life partner drug (WHO, 2006). The artemisinin derivatives cause a rapid and effective reduction in parasite biomass as well as gametocyte carriage, while the partner drug, which has a longer duration of action, achieves effective clinical and parasitological cure. The reasoning is that, if two drugs have independent mechanisms of action, then mutations that confer resistance to each drug will only rarely co-exist in the same parasite (White, 2004). However, the weak link in the combination is the period during which the unprotected partner drug remains alone during its elimination period, particularly at sub-therapeutic concentrations (White, 2004; Sisowath *et al*., 2005). At

this drug concentration selection of re-infecting resistant parasites may occur (Hastings *et al*., 2002).

Since 2001, more than 60 countries have officially adopted artemisinin-based combination therapy (ACT) for the treatment of *falciparum* malaria (Nosten and White, 2007; Eastman and Fidock, 2009). Several different forms of ACT have been evaluated, including artesunate- sulphadoxine-pyrimethamine, artesunate-amodiaquine (Brasseur *et al*., 2007), artemether-lumefantrine (Staedke *et al*., 2008), artesunate-mefloquine (Ashley *et al*., 2006), artesunate-chlorproguanil-dapsone, artesunate-atovaquone-proguanil, dihydroartemisinin-piperaquine (Bassat *et al*., 2009) and artesunate-pyronaridine (Ramharter *et al*., 2008). However, clinical failures or at least longer parasite clearance times have been described in Cambodia (Noedl *et al*., 2008; Carrara *et al*., 2009; Dondorp *et al*., 2009; Rogers *et al*., 2009).

Pyramax®, a combination of artesunate (ASN)-pyronaridine (PRD) which was recently prequalified by WHO, is a prospective alternative for malaria treatment in African setting where malaria incidences are high. Pyronaridine (Malaridine®) was first synthesized in China and introduced for the treatment of malaria as a single agent for over 30 years in certain malaria prone regions of China (Shao, 1990). Recently, interest has been renewed in pyronaridine as a possible partner for use in Artemisinin-based Combination Therapy (ACT) for malaria treatment. Consequently, if strategy is to be devised to extend the expedient therapeutic lifetime of Pyramax®, there is a necessity to comprehend the molecular mechanisms of PRD resistance. However, a major breakthrough towards understanding the mechanism of resistance would be the development of stable resistant phenotypes.

2.12.2 Monitoring emergence of resistance

Predicting the fate of antimalarial drugs in the future is necessary for planning malaria control and instituting strategies which might delay the emergence of resistance (White and Pongtavornpinyo, 2003). Although removing antimalarial drug pressure may or may not compromise the fitness of resistant parasites, rotating the limited number of safe, effective and affordable antimalarial drugs could be considered (Plowe, 2003). Studies in Malawi show that cessation of CQ use was followed by re-emergence and

predominance of CQ-sensitive *P. falciparum* (Plowe, 2003). Monitoring the emergence and development of resistance is therefore one of the most promising options against complete loss of antimalarials to resistance (WHO, 2003). Molecular markers can provide direct and convincing evidence of selection for resistant parasites to antimalarial drug treatment (WHO, 2001). However, only few molecular markers of resistance are currently available, hence limiting detailed studies on the mechanisms of resistance (Plowe, 2003).

2.12 Selection of drug resistant parasites

Drug resistance can be selected without mosquito passage (Peters, 1999) by-passing the sporozoite-induced infection to induce resistance comparable to human malaria infection (Hayton *et al*., 2002; White, 2004). Drug resistant lines can be induced *in vitro* using *P. falciparum* or *in vivo* using murine malarial models (Carlton *et al*., 2001). However, selection of resistance *in vitro* is a long process, expensive and the stability of resistant phenotypes is intricate to establish (Nzila and Mwai, 2010). *In vivo* models of rodent malaria offer a practical and informative means of evaluating the activity and potential resistance of novel antimalarials and antimalarial combination. For instance, previous studies have shown that resistance can be easily achieved *in vivo* using rodent malaria models (Hunt *et al*., 2007; Kiboi *et al*., 2009) by continuous CQ pressure (Cooper *et al*., 2002) and early work on sulfadoxine, pyrimethamine and sulfadoxine/pyrimethamine (SP) resistance was also carried out with *P. berghei* (Peters, 1975), by exposing parasites in mice to increasing doses of these drugs. Although some mechanism of drug resistance between *P. falciparum* and murine malaria does not correlate (Cravo et al., 2001; Afonso *et al*., 2006; Hunt *et al*., 2007) other mechanisms are similar. Mechanism of resistance to mefloquine in *P. berghei* and *P. chabaudi* (Gervais *et al*., 1999; Cravo *et al*., 2003), atovaquone resistance in *P. berghei* and *P. yoelii* (Syafruddin *et al*., 1999) and antifolates resistance in *P. chabaudi* (Culleton *et al*., 2005). This shows that murine parasites are invaluable tools in elucidating mechanism of resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was carried out at the Centre for Traditional Medicine and Drug Research (CTMDR) and Centre for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute (KEMRI) Headquarters, Nairobi, Kenya.

3.2 Parasites and test compounds

Transgenic ANKA *P. berghei* resistant parasites to Piperaquine expressing Green Florescence Protein (GFP) line maintained by serial passage of blood from mouse to mouse at KEMRI animal house was used.

Pyronaridine was a gift from Professor Steve Ward, Liverpool School of Tropical Medicine, Liverpool, UK, courtesy of Dr. Nzila Alexis, KEMRI-Kenya. The drug was stored at 4^0C and on the day of administration, pyronaridine phosphate was freshly prepared by solubilizing it in solution consisting 70% Tween-80 (d=1.08gml^{-1}) and 30% ethanol (d=0.81gml^{-1}) and diluted tenfold with double distilled water.

3.3 Handling of experimental animals and ethical consideration

Handling of animals was done in accordance with Care and Use of Laboratory Animals manual, KEMRI guidelines. Permission to use laboratory mice was sought from the KEMRI-ACUC. A total of 300 male Swiss albino mice weighing 20±2g random-bred at KEMRI, Nairobi, Kenya were used in this study. The experimental duration for determination of effective doses was 4 days. The animals were maintained in the animal house in standard Macrolon type II cages with air temperature of 22ºC and 50-70% relative humidity, clearly labeled with experimental details and fed on commercial rodent food and water *ad libitum*. The needle size for cardiac puncture and intra-peritoneal infection used was 26G×5/8″. The drug was administered orally by using ball-tipped mouse gavage needles. After the experiment, mice were sacrificed by euthanizing using sodium pentobarbitone. The euthanized mice were then placed in

biohazard disposable autoclave bags and autoclaved at 121ºC for 15 minutes before incineration to destroy all infectious agents and to avoid environmental contamination. Ethical approval to carry out this study was granted by KEMRI.

3.4 Preparation of inoculums

Parasitized red blood cells (PRBCs) were collected from donor mice with a rising parasitaemia of 5-10% and according to the level of parasitaemia blood diluted with phosphate saline glucose (PSG) buffer to reach approximately 2×10^7 PRBCs per 200µl of the inoculum (**appendix 1**). The infected erythrocytes in 200 µl inoculum were found to represent a parasite load equivalent to standard parasite density for Swiss albino mice weighing 20±2g (Fidock *et al*., 2004**)**. This allows accurate estimation of parasitaemia on D_4 post infection.

3.5 Determination of 50% and 90% effective doses (ED_{50} and ED_{90})

The ED_{50} and ED_{90} was determined using standard 4-Day Suppressive Test (4-DT) as described by Peters *et al*., 1975. Four serial drug dilutions (5.0, 2.5, 1.25, 0.625mgkg^{-1} respectively) were used to determine effective doses against parent strain. Male Swiss albino mice, 5 mice per dose group in four different doses and 5 mice in the control group were inoculated intra-peritoneally each with 2×10^7 PRBC in 200µl inoculum on day zero (D_0). Drug was administered orally at 4 hrs, 24 hrs, 48 hrs and 72 hrs post infection. Thin blood films were prepared from tail snips on day four (D4) post infection, fixed in absolute methyl alcohol and stained for 10 min with freshly prepared 10% (v/v) Giemsa solution. Parasitaemia was determined by microscopic examination of Giemsa-stained blood films taken on day 4. Microscopic counts of blood films from each mouse were processed using MICROSOFT® Excel (Microsoft Corp.) Percentage (%) chemosuppression (parasite reduction) of each dose was determined as described by Tona *et al*., 2001;

[(A-B)/A] x 100] = % Chemosuppression

Where A is the mean parasitaemia in the negative control group and B is the parasitaemia in the test group. The 50% and 90% effective doses (ED_{50} and ED_{90}) were estimated graphically using linear regression, version 5.5 of Statistica 2000.

3.6 Drug selection experiment

After establishment of ED_{50} and $ED_{90,}$ the study adopted stepwise increase of drug pressure dose to generate Pyronaridine resistance (Serial Technique). Acquisition of resistance was assessed at appropriate intervals using standard 4-DT to confirm the response levels of the parasites to the compound. Standard 4-Day Suppressive Test (4-DT) permits the measurement of the ED_{50} and ED_{90}, as well as the `index of resistance' at the 50% and 90% levels (I_{50} and I_{90}).

The I_{50}/I_{90} was calculated using the formula: $I_{50}/I_{90} = ED_{50}/ED_{90}$ of resistant line/ ED_{50}/ED_{90} of parent strain (Merkli and Richle, 1980; Xiao *et al*., 2004).

3.7 Cryopreservation of P. *berghei* ANKA parasites

At each passage, 500-600µl of *P. berghei* ANKA PRBC in 50µl of heparin was collected by cardiac-puncture from a test group mouse with a parasitaemia of 2-5%. The blood was mixed with 500-600µl of a glycerol/PSG solution (20% glycerol; v/v), the suspension was then transferred to 2 cryotubes, 500µl per vial. The vials were left for 5-15min at 4°C and then stored at -80ºC.

3.8 Drug tests and cloning of parasite

To generate a genetically homogenous resistant parasite at least two selected generations of drug resistant parasite were cloned by limiting dilution cloning (Janse and Waters, 2002). Briefly, a mouse with parasitaemia between 0.5 and 1% was selected as donor mouse. From the donor mouse, 5µl of infected blood was collected from tail blood in 1µl of heparin and diluted in 1ml of 1×PBS. The number of infected erythrocytes was estimated in 200µl of diluted blood and parasitaemia per 1µl of the cell suspension calculated. The cell suspension was then diluted further with 1×PBS to an estimated final concentration of 2parasites/200µl PBS. Fifteen mice were intravenously injected each with 200µl per mouse of diluted cell suspension. Cloning was deemed successful

when 20-50% of the inoculated mice became positive and showed a parasitaemia of between 0.3- 1% at day 8 post-infection.

3.9 Stability tests

The stability of PRD resistant line was evaluated by measuring drug responses after making 5 drug free passages followed by measurement of ED_{90} in the 4DT. Also by freeze-thawing of parasites from -80°C stored for a period of four weeks followed by measurement of ED_{90} in the 4DT.

3.10 Extraction of genomic DNA

Parasitized blood cells were harvested from mice under general anaesthesia, when trophozoite stages were most prevalent into PBS (pH 7.2). In preparing genomic DNA, mouse white blood cells was removed by successive filtration of infected blood using Plasmodipur filters (Euro-Diagnostica) as described by Janse and Waters, 2002. Briefly, packed cells were re-suspended in 5 volumes of cold (4 °C) 1 × erythrocyte Lysis buffer (ammonium chloride solution) for 15–30 minutes, before spinning at 800xg for 8 min to pellet the parasite. Genomic DNA was extracted using the commercially available QiAamp DNA Blood Kit (Qiagen) following manufacturer's instruction.

3.11 Amplification and analysis of multi-drug resistant gene 1

To amplify coding region of multi-drug resistant gene 1 (4260 base pairs), 1 µl of genomic DNA from each sample was used as template in 25µl PCR reactions. The other reagent in the reaction mixture were; Master mix consisting of 1X PCR buffer, 2m $MMgCl_2$, 10Mm dNTPs,10pmoles of forward 26base pairs (5'-GTCTAAATGTTGTAATTTGTTGTCCT-3') and reverse 24base pairs (5'-TTCACGCTATAAAAGTACAGACTA-3') primers and 0.025U DreamTaq DNA polymerase. The reaction mixture was run in thermo-cycler (Applied Biosystems) using the following optimized PCR protocol: Initial denaturation for 95°C for 5 minutes, Denaturation for 95°C for 1min, followed by annealing at 50 °C for 30 seconds and extension for 68 °C for 5 minutes for 35cycle followed by finals single extension step at

72 °C for 10 minutes (This is the temperature needed for optimal activity for most polymerase used in Polymerase Chain Reaction).

Products of PCR amplification were analyzed using gel electrophoresis system. In general, 5ul of PCR products were run in a 1 % Agarose gel for 30-35 minutes after which the gel was stained in Ethidium bromide (EtBR) solution for 15 minutes. The 1kb gene ruler was run alongside the *MDR-1* gene amplicon. To visualize PCR bands, UV trans-illuminator (Gel Doc 2000 BIORAD, Milan Italy) was used. Gene sequence of the whole coding region of about 4260bp was obtained from http://plasmodb.org. Accession No. PBANKA_123780. Primers for amplification and sequencing were described by Kiboi *et al*., 2014 (**Appendix 3**). PCR products were purified using GeneJet™ PCR purification kit (Thermo scientific™) and then sequenced based in BigDye v3.1 using a 3730xlsequencer.

3.12 Data analysis

The data for the 4-day suppressive test was recorded in MICROSOFT® Excel (Microsoft) spreadsheets and used within version 5.5 of Statistica 2000 (Statistica 5.5 Statsoft Inc. 2000) to estimate the ED_{50} and ED_{90} in $mgkg^{-1}$. The I_{50} and I_{90} were calculated and resistance grouped into four categories namely; I_{90}=1.0, sensitive, I_{90}=1.01-10.0, slight resistance, I_{90}=10.01-100.0, moderate resistance and I_{90}= >100.0, high resistance (Merkli and Richle, 1980). Contigs were assembled and edited using Genetyx version 8.0 software and gene sequences of both drug selected and unselected clone were compiled and analyzed using Pairwise Sequence Alignment tool available at European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) website (http://www.ebi.ac.uk/Tools/psa/) **(Appendix 4).**

CHAPTER FOUR

RESULTS

4.1 Effective dosages of Parent strain

Before drug exposure, effective doses of Pyronaridine that reduces parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) determined in the standard 4-Day Suppressive Test (4-DT) for the parent line were found to be 1.83mgkg^{-1} and 4.79mgkg^{-1} respectively **(Table 1 and Fig. 5).**

Table 1: *In vivo* activity of PRD against parent strain before drug pressure.

In vivo activity of PRD against parent strain before drug pressure. The table shows the 4-DT results used to derive the effective doses (ED_{50} and ED_{90}) of the parent strain before selection of pyronaridine resistance.

No. of cages	PRD Dosage (mgkg^{-1}.day)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	5	0.38	12.56	87.44
2	2.5	1.00	32.95	67.05
3	1.25	1.73	56.90	43.10
4	0.625	2.12	69.70	30.30
Control	Tween-80 in 3% ethanol solution	3.05	100	No activity

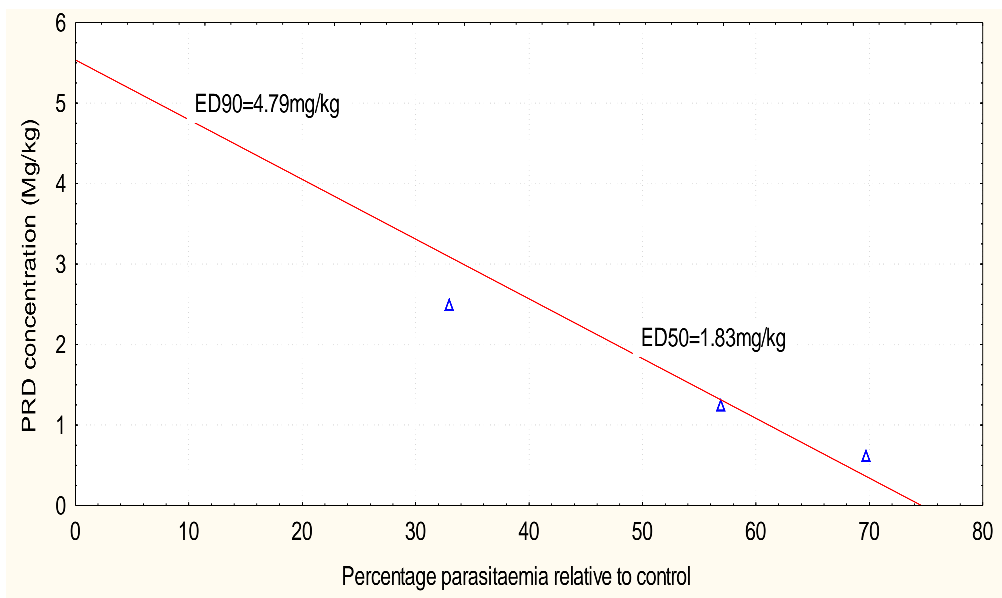

Figure 5: Graph showing determination of effective doses (ED_{50}/ED_{90}). The graph indicates determination of effective doses (ED_{50} and ED_{90}) *in vivo* activity of PRD against *P. berghei* ANKA before drug pressure.

4.2 Acquisition of pyronaridine resistance

The dose equivalent to ED_{90} was selected as starting drug pressure for selection of PRD resistance. After reviving parasites and making two passages, 4 male Swiss albino mice were inoculated and the parasitaemia at D_3 post infection (p.i) was estimated to be 5.77% before subjecting the mice to 5mgkg^{-1}as starting drug pressure. After 3 days post administration of drug, the parasitaemia reduced to 1.2% before it rose to 10.75% 11days post infection, this was recorded as passage 0. The mouse with high parasitaemia was chosen as donor for parasites passage to 3 mice (passage 1) and after evaluating level of parasite density, the mice were subjected to selected high dose. The dose range for the mice in 5 passages was increased to 25mgkg^{-1} before the stability was assessed. At every passage, 3 male Swiss mice were infected with *P. berghei* and after attainment of >2% parasitaemia, the mice were treated orally with drug pressure dose of PRD. The doses were increased gradually from 5mgkg^{-1} to highest 100mgkg^{-1} depending on the growth of the parasites. During the development of resistance, the susceptibility to PRD changed markedly (**Fig. 6**).

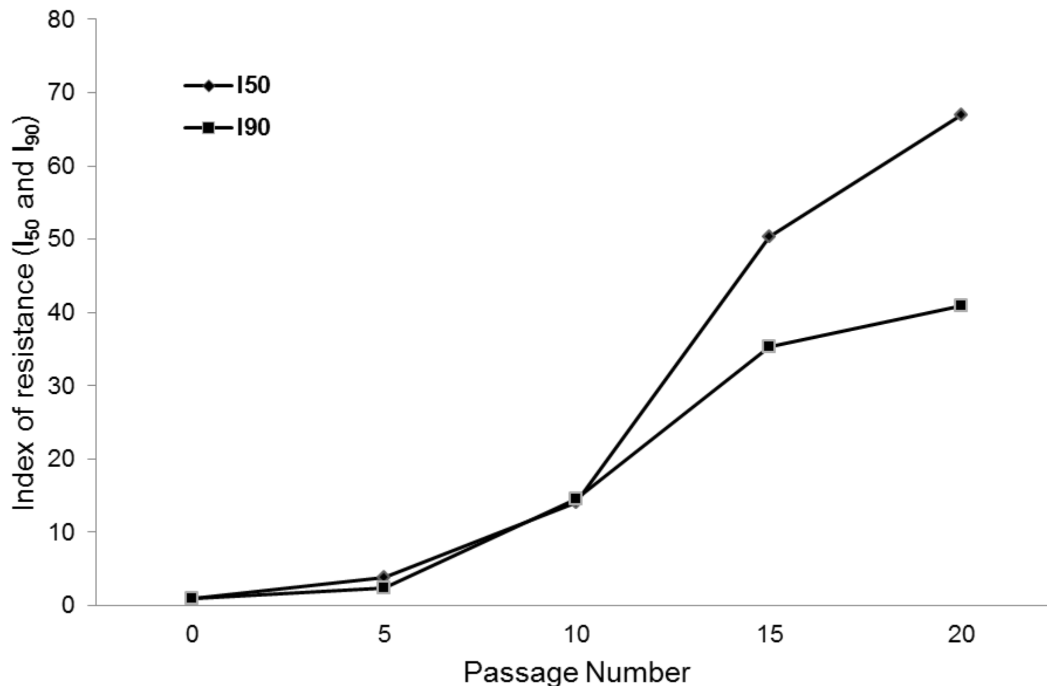

Figure 6: Graph showing increase in level of resistance. Level of resistance was assessed after every five (5) passage during the selection of pyronaridine resistance for over a period of 6 months. The index of resistance (I_{50} and I_{90}) indicates the degree of parasites resistance to pyronaridine and is clearly the ratio of the ED_{50} or ED_{90} of the resistant line to that of the parent strain.

From the first and subsequent passages, the drug pressure dose was increased by dose ranging from 5-10mgkg^{-1} depending on the growth of the parasites. The interval between subsequent passages of PRBCs for the first nine consecutive passages after drug pressure was average of eleven days, as the parasites acquired resistance, interval reduced to average of seven days between subsequent passages throughout the experiment. After twenty drug pressure passages the ED_{50} and ED_{90} increased by 66 and 40 folds respectively (**Table 2**). The generated resistant parasites were scored as PRD^R line.

Table 2: Summary of results during selection of pyronaridine resistance *in vivo*.

Selection of Pyronaridine resistance in *Plasmodium berghei* ANKA strain using serial technique. Data are presented as effective doses that suppressed parasitaemia by 50% and 90% (ED_{50}, ED_{90}) and as 50% and 90% indices of resistance (I_{50} and I_{90}, defined as the ratio of the ED_{50} or ED_{90} of the resistant line to that of the parent strain).

Passage Number	ED_{50} (mgkg-1)	I_{50}	ED90(mgkg-1)	I_{90}
Parent parasite	1.83	1.00	4.79	1.00
5th	7.12	3.90	11.38	2.38
10th	25.82	14.11	69.43	14.49
15th	92.05	50.3	168.98	35.27
20th	122.49	66.93	195.98	40.91
Limiting dilution cloning of 20th passage of DP	145.51	79.51	193.10	40.31
Drug free passages after dilution cloning of 20th passage of DP	107.50	58.74	146.10	30.50
One month of cryopreservation dilution clones resistant parasites	73.48	40.15	107.10	22.36

The dilution cloned resistant parasite after 20 passages of selection pressure of pyronaridine resistance was used to analyze the gene polymorphism in the targeted Multi-drug resistance gene.

4.3 Drug sensitivity profile test of pyronaridine resistant clone

Pyronaridine resistant (PRD^R) clone diluted parasite was evaluated at $50mgkg^{-1}$ administered for 4 successive days in groups of five mice along the untreated controls

(both sensitive and starting parasite) and in addition this parasite were submitted to half of the dose (25mgkg^{-1}) for four consecutive days (**Fig. 7**). As anticipated, starting parasite and sensitive were cleared by 25mgkg^{-1} while PRD^{R} clone exhibited growth in presence of 50mgkg^{-1}. Under PRD treatment, no parasites were detected in mice infected with sensitive parasite over the 15 day p.i follow up period. The study concluded that PDR^{R} was successfully cloned and this clone was selected for isolation of genomic DNA for *MDR –1* gene targeting.

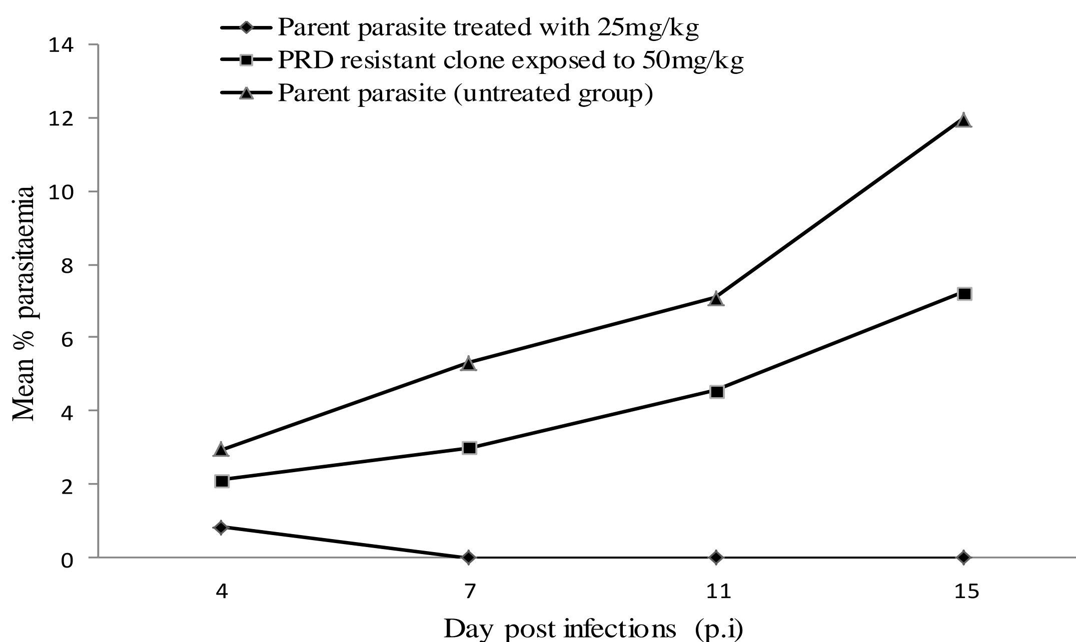

Figure 7: Graph showing drug sensitivity profiles test of PRD^{R} clone. The drug response of the parent strain (starting parasite) and resistant clone to PRD and development of parasitaemia in untreated mice in a group of mice taken on day 4, 7, 11 and 15 post infection after 20th passage of drug pressure. Parasitaemias were assessed after four days post-infection and mice were treated using a 4-day test (4-DT).

4.4 Stability test

After dilution cloning, the effective doses determined were ED_{50}=145.51mgkg^{-1}, ED_{90} =193.10mgkg^{-1}. However, after cryopreservation and revival of the parasite, this resistance decreased, with ED_{50} and ED_{90} of 73.48 mgkg^{-1} and 107.10mgkg^{-1} respectively. Growing them in absence of drug for five passages, the effective doses

were determined to be ED_{50}=107.50mgkg^{-1} and ED_{90}=146.1mgkg^{-1} respectively (**Table 2**). These suggest that PRD resistance develops rapidly as long as the selection pressure was maintained. Despite decrease in ED_{50} and ED_{90}, the study concluded that the parasites retained resistance as I_{90} was over 40 times compared to I_{90} of parent strain (Table 2). From these results, stable pyronaridine resistant *P. berghei* line was selected and could be used for elucidation of markers associated with pyronaridine resistance.

4.5 Analysis of PCR amplified MDR-1 gene and Sequencing

The coding region of *MDR-1* gene (4260bps) of both the parent and resistant line was PCR amplified and purified before sequencing. The amplicons were run in a gel and visualized under Ultra-Violet trans-illuminator. The banding patterns indicated that the gene amplified was between 4kb and 5kb (**Fig. 8**), therefore, this shown that the target gene was amplified (approximated 4260bp).

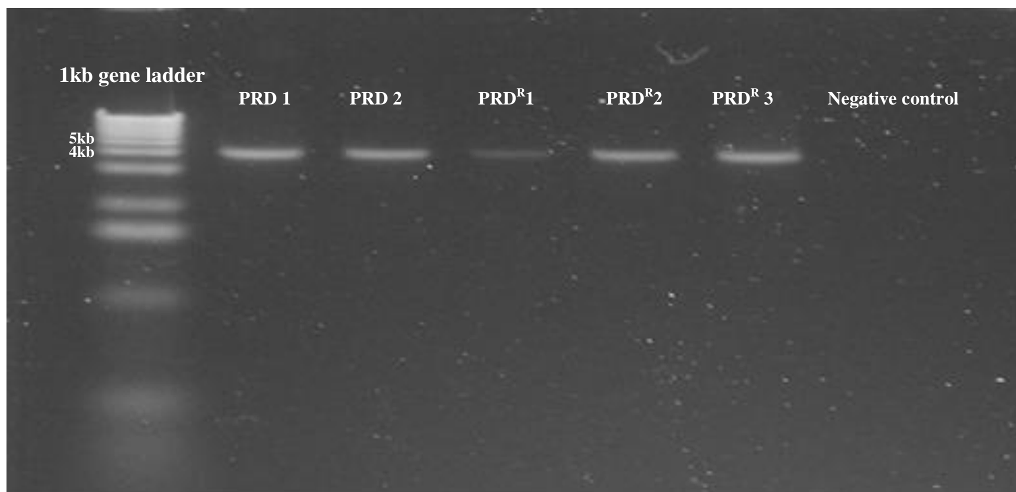

Figure 8: Gel photo showing analysis of PCR amplified MDR-*1* gene (4260base pairs). Lane one in gel photo is 1kb gene ruler used to estimate the size of amplified target gene, lanes PRD 1 and PRD 2 represent amplified gene from starting parasite while lanes PRD^R 1, PRD^R 2 and PRD^R 3 is amplified *MDR-1* gene from 20th drug pressured parasites (Clone diluted resistant line). The last lane is negative control.

After the PCR amplification, the coding sequence of 4260bp was sequenced and assessed for point mutation (**Appendix 4**). Evaluation of sequence variation in *MDR-1 gene* found no point mutation.

CHAPTER FIVE

DISCUSSION

The biggest challenge to disease control using drug treatment is genetic elasticity and high frequency of human malaria parasite *Plasmodium falciparum* to develop resistance to structurally and mechanistically related and unrelated drugs (accelerated resistance to multiple drugs, ARMD) (Rathod *et al*., 1997). Accelerated resistance to multiple drugs is characterized by the ability of a strain to generate a drug-resistant clone when put under drug pressure. This results from the high mutation rate during parasite multiplication. To study molecular basis of resistance, one needs to obtain well-characterized drug-resistant strains. However, such strains are not generally available for most antimalarial drugs. Murine malaria—*Plasmodium berghei, Plasmodium chabaudi, Plasmodium yoelii* and *Plasmodium vinckei*—have been used as surrogates for *Plasmodium falciparum* to study the mechanisms of drug resistance by inducing resistance *in vivo* (Peters, 1987). This approach has led to the selection of drug-resistant parasite lines and subsequent studies on mechanisms of drug resistance (Nzila and Mwai, 2010).

Therefore, this study reports the selection of resistance to PRD in *P. berghei* as preliminary step towards evaluation of the molecular basis underlying PRD resistance. In this regard, the research work investigated the role of multidrug resistance gene -1 in modulating PRD resistance which is known to be associated with reduced quinoline susceptibility (Fidock *et al*., 2000; Henry *et al*., 2009). The results obtained in this study present evidence that the *PfMDR-1* gene point mutation may not linked to PRD resistance.

5.1 Selection of Pyronaridine resistance

In this study, resistance to Pyronaridine was first selected, then genetic variation of multi-drug resistance gene in resistant line evaluated. The study showed that PRD resistance in *P. berghei* ANKA GFP can be selected relatively easily by starting with Piperaquine resistant *P. berghei* clone earlier selected by Kiboi *et al*., 2009.

Initial studies on PRD resistance *in vivo* found that resistance in *P. berghei* (ANKA) developed gradually when PRD was administered at 4 $mgkg^{-1}$, with no detectable resistance within several passages (Shao and Ye, 1986). Peters *et al*., were able to derive pyronaridine-resistant *P. berghei* and *P. yoelii* strains by *in vivo* serial passage, applying drug at 3 or 10 $mgkg^{-1}$ (Peters and Robinson, 1992). However, resistance development was slow and was more difficult to achieve with the higher dose (Peters and Robinson, 1992). Interestingly, this study attained maximum level of resistance (>40 times the ED_{90} of parent strain) by applying higher doses ranging from 5$mgkg^{-1}$ to 100$mgkg^{-1}$ for over a period of 180 days. In fact, this shows that it is more effective to select PRD resistance by maintaining high continuous drug pressure. This is due to genetic potentiation of the parasites to generate mutations in response to drug treatment (called the accelerated resistance to multiple drugs phenotype (Rathod, 1997) which might have occurred during generation of Pyronaridine-resistant line.

Early reports using murine malaria indicated that a strain resistant to one drug was more amenable to give rise to resistant lines to another drug, compared with strains that are fully drug susceptible. For instance, Pyrimethamine- and chloroquine-resistant parasites were more easily generated from drug-resistant strains than from drug-susceptible ones in *P. chabaudi* and *P. vinckei* (Afonso *et al*., 2006; Nzila and Mwai, 2010). Evidence that the same phenomenon prevails in *P. falciparum* has been provided by Rathod's group (Rathod *et al*., 1997).

5.2 Stability of pyronaridine resistant line

The stability of pyronaridine resistance after dilution cloning was studied by drug free passages and freeze-thawing. When the drug was removed for 5 passages after 20th passage of selection pressure, the level of resistance decreased although the index of resistance (>40 times the ED_{50}) still remained high. This was coherent with other studies on PRD resistance stability, in *P. berghei* RP and of *P. berghei* (ANKA), where sensitivity started to return after making a number drug-free passages, after which the resistance remained stable (Peters and Robinson, 1999; Xiao *et al*., 2004).

After making 5-drug free passages and upon revival of the parasites after one month of cryopreservation at -80°C, as shown by this data, there was a marginal decrease in resistance. This decrease in ED_{50}/ED_{90} recorded upon revival of the strains is common and indicates that some of the mechanisms of PRD resistance are outcome of epigenetic changes such as gene amplification, protein over expression and protein modifications. Observation from previous PRD stability studies on *P. berghei* RP and *P. berghei* ANKA have shown that the sensitivity started to return after making a number of drug-free passages, after which the resistance remained stable (Peters and Robinson, 1999; Xiao *et al*., 2004). Despite decrease in resistance, the study concluded that the parasites retained resistance as I_{50}/I_{90} compared to I_{50}/I_{90} of parent strain remained high.

5.3 Analysis of MDR-1 gene in pyronaridine 'resistant' clone

The mechanism by which resistance to Pyronaridine develops is unknown, but may be due to a direct effect on the Pyronaridine mode of action or modulation of targeted gene (Simon *et al*., 2012). Cross resistance with Chloroquine *in vitro* appears to be incomplete and inconsistent, whereas, *in vivo* Pyronaridine retains activity against Chloroquine-resistant strains (Basco and Le Bras, 1994). Wu *et al*., 1988, described an increase in the number of food vacuoles in trophozoites from a Pyronaridine-resistant *P. berghei* RP line, some of which were fusing. There was also a marked reduction in the digestive food vesicles containing malaria pigment granules for both trophozoites and schizonts and typical hemozoin grains were not formed in the Pyronaridine-resistant parasites (Wu, 1988; Auparakkitanon *et al*., 2006). These and other ultra-structural differences suggested that resistance may be due to a direct effect of Pyronaridine drug on the target.

In another study, Li *et al*., 1995, found over-expression of a 54 kDa protein in a Pyronaridine-resistant strain of *P. berghei* ANKA. The protein was localized mainly in the cytoplasm of erythrocytic stage trophozoites, schizonts and merozoites and less commonly in the cytoplasm of infected erythrocytes (Li *et al*., 1995). Interestingly, a 54 kDa protein was also expressed in chloroquine-resistant *P. berghei* ANKA suggesting a common effect, though whether this was related to resistance development is unknown (Li *et al*., 1995).

Reliable molecular markers of resistance play a vital, sentinel role in the surveillance of drug efficacy (Uhlemann *et al*., 2005). For example, screening for the *PfCRT* K76T mutation, which is strongly associated with CQ resistance *in vitro* and with CQ treatment failure in clinical settings, has documented the rapid worldwide dissemination of CQ resistance and high-lighted the need for alternative first-line drugs in Africa (Fidock *et al*., 2000; Sidhu *et al*., 2002).

Structurally related compounds to pyronaridine, such as amodiaquine, demonstrated slight resistance against PQ-resistant line, additionally, the AQ resistant line has shown resistance to pyronaridine (Langat *et al*., 2012). These studies suggest that the PRD resistance may share similar mechanism of resistance, thus, the PRD resistance may be related to other antimalarial drugs resistance. The *P. falciparum* multidrug resistance gene has been implicated in altering parasite susceptibility to a variety of currently available antimalarial drugs. Point mutations in *PfMDR-1* have been associated with changes in parasite susceptibility to chloroquine, quinine, mefloquine, and Artemesinin derivatives in both laboratory lines and clinical isolates, but these mutations have limited use as molecular markers (Uhlemann and Krishna, 2005; Duraisingh and Cowman, 2005; Woodrow and Krishna, 2006). The analysis of data from this study did not link *PbMDR-1* polymorphisms with modulation of PRD resistance. In recent *in vitro* studies on PRD resistance, there was absence of mutation in *MDR-1* gene involved in quinoline resistance in *P. falciparum* (Pradines *et al*., 2010), therefore, *MDR-1* gene polymorphism is suggested not to be linked to PRD resistance.

In general, malaria parasite can employ a range of mechanisms to overcome drug challenge, in addition to structural modification of the target protein. These include amplification of the gene encoding the target or copy number or its upregulation during transcription or translation, or by compromising the drug itself by inactivation or sequestration. For instance, evidence suggests that PfMDR-1 is phosphorylated (Lim and Cowman, 1993). Mammalian MDR transporter homologs, when phosphorylated display altered transport activity (Lim and Cowman, 1993). Thus, the phosphorylation status of PfMDR-1 may produce a similar alteration of transport in resistant parasites. This suggest that PRD resistance may not be linked to polymorphisms in *MDR-1* gene

as found in this study, nevertheless, other mechanism of resistance related to *MDR-1* gene and other possible candidate genes should be explored.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

6.1.1 Selection of pyronaridine resistance

The ED_{50} and ED_{90} of PRD against parent *P. berghei* ANKA strain was 1.83 and 4.79mgkg^{-1} respectively.

After dilution cloning of 20th drug pressure passage, the effective doses (ED_{50} and ED_{90}) determined were 145.51 and 193.10mgkg^{-1} respectively, yielding I_{50} of 79.51 and I_{90} of 40.31. Such value of $I_{50/90}$ was suggestive that the starting parasites acquired resistance after twenty drug pressure passages.

6.1.2 Mechanism of pyronaridine resistance

This study reports the experimental selection of a stable pyronaridine-resistant *P. berghei* line by drug pressure. The study is highly pertinent for further experimental studies on pyronaridine resistance since artesunate-pyronaridine may be soon deployed extensively in Africa and elsewhere**.**

The results showed that the alignment of multi-drug resistant gene nucleotide sequences of both parent and resistant parasites were similar, thus *PbMDR-1* gene is not associated with PRD resistance in *Plasmodium berghei*. However, other mechanism of *MDR-1* gene alteration or additional genes may be involved in modulating PRD resistance.

This parasite can be used to explore other genes that mediate PRD antimalarial drug resistance by using high throughput comparative genomic studies based on genome-wide approaches. If relevant, the resulting information may be used to devise strategies to prevent or monitor PRD resistance in human malaria.

6.2 Recommendations

To select highly PRD resistant lines (I_{90} of >100) and to maintain the resistance already obtained, drug selection pressure should be continued.

Studies should be done to investigate cross resistance of PRD resistant line with other antimalarial drugs.

Studies should also be initiated to investigate whether the stability of PRD resistant phenotypes will be maintained once the resistant line is cryopreserved and drug free passages over 3months.

Finally, following investigation of whole coding region of *MDR-1* gene, other ways at which *MDR-1* gene alteration or additional genes are involved in modulation of PRD reduced susceptibility should be extensively explored. Use of modern genome-wide studies can be used to elucidate mechanisms that are involved in the Pyronaridine resistance.

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APPENDICES

Appendix 1: Preparation of buffers and solutions.

Preparation of drug solutions

30ml (30%) of ethanol (d=0.81gml^{-1}) was added to 70ml (70%) of tween-80 (d=1.08gml^{-1}), then this solution was diluted 10 fold with double distilled water.

Staining buffer

1.5grams of potassium di-hydrogen phosphate and 7.5grams of disodium hydrogen phosphate was weighed and then dissolved in 1.5litres of doubled deionized water. The solution was thoroughly mixed and then made up to 2.5litres distilled water.

Preparation of Giemsa stain (500ml stock solution)

3grams of Giemsa stain was weighed and then dissolved in 300ml of methyl-alcohol and 200ml glycerol. This was then mixed thoroughly for 1 hour and then filtered using a Filter paper, Whatman no.1. The stock solution was Stored in the dark at 4°C. For fresh working solution, 1 ml of stock solution was made up to 10ml using staining buffer.

Phosphate Saline Glucose (PSG) buffer

The following salt was weighed accurately; 5.392 grams of Di-sodium hydrogen phosphate Na_2HPO4 (anhydrous). 40.312 grams of Potassium di-hydrogen phosphate, KH_2PO4 (anhydrous). 1.7grams of sodium chloride, (NaCl). 10 grams of D-glucose. The salts were dissolved in 1litre of double deionized, then mixed thoroughly and sterilize by autoclaving at 121^0C for 15 minutes and store in dark at 4^oC.

Appendix 2: Results of 4-Day Suppressive Test during inducement of pyronaridine resistance.

Table 1: *In vivo* activity of PRD against *P .berghei* ANKA GFP after 5th passage of drug pressure.

No. of cages	Dosage($mgkg^{-1}$.day)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	10	0.74	36.88	63.13
2	5	1.05	52.50	47.50
3	2.5	2.04	102.13	-2.13
4	1.25	2.08	103.75	-3.75
Control	7% Tween-80 in 3% ethanol solution	2.00	-	-

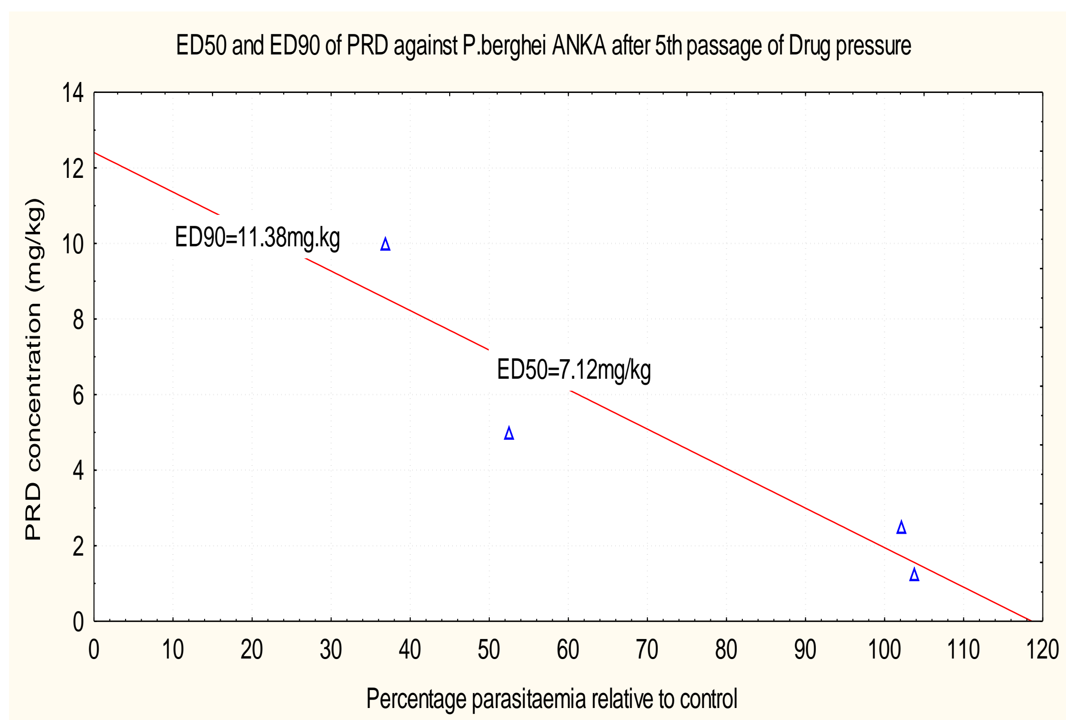


Table 2; *In vivo* activity of PRD against *P. berghei* ANKA GFP after 10th passage of drug pressure.

No. of cages	**Dosage ($mgkg^{-1}$.day)**	**Average parasitaemia**	**%parasitaemia relative to control**	**% Activity**
1	50	1.36	28.55	71.45
2	25	2.38	49.97	50.03
3	12.5	2.83	59.48	40.52
4	6.25	3.37	70.75	29.25
Control	7% Tween-80 in 3%ethanol solution	4.76	-	-

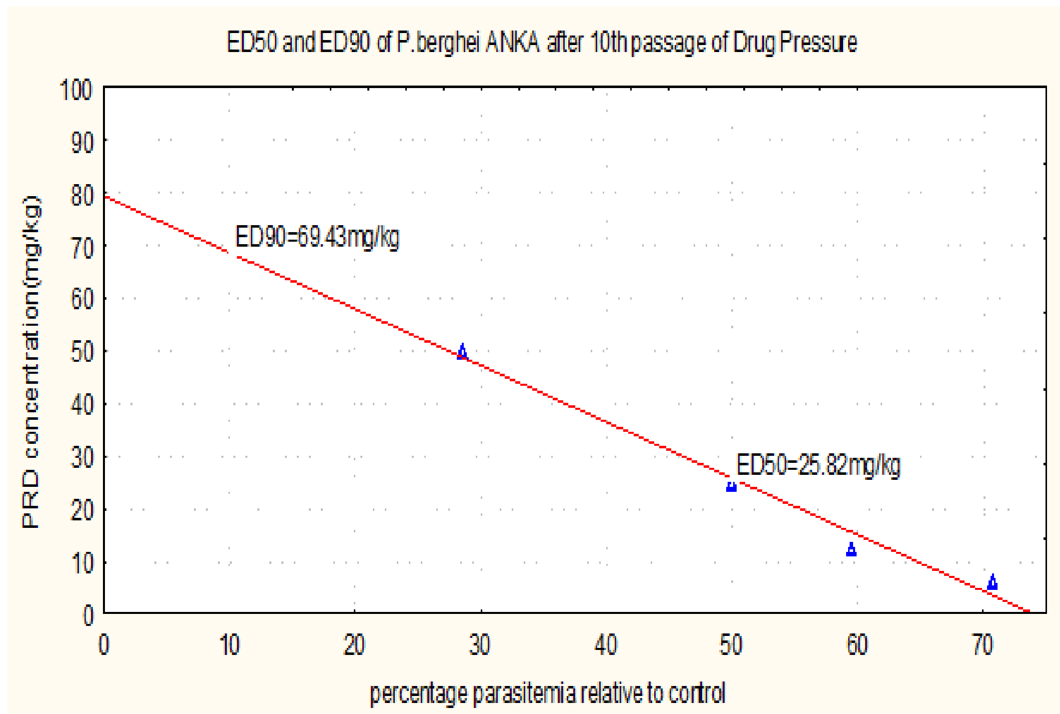


Table 3; *In vivo* activity of PRD against PQ^R P .berghei ANKA GFP after 15th passage of drug pressure.

No. of cages	Dosage ($mgkg^{-1}$.day)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	100	2.02	44.56	55.44
2	50	3.57	78.68	21.32
3	25	3.67	80.96	19.04
4	12.5	4.05	89.34	10.66
Control	7% Tween-80 in 3%ethanol solution	4.53	-	-

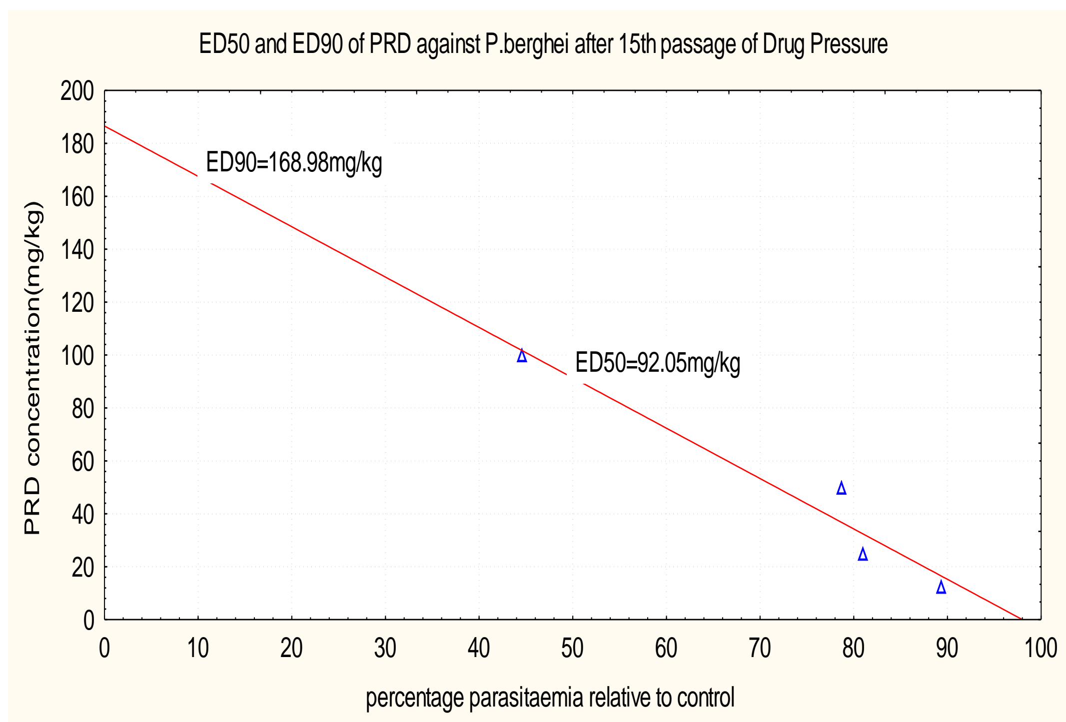


Table 4; *In vivo* activity of PRD against *P .berghei* ANKA GFP after 20th passage of drug pressure.

No. of cages	Dosage($mgkg^{-1}$.day)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	100	1.89	71.52	28.48
2	50	2.11	79.85	20.15
3	25	2.77	104.95	-4.95
4	12.5	3.01	113.91	-13.91
Control	7%Tween-80 in 3%ethanol solution	2.64	-	-

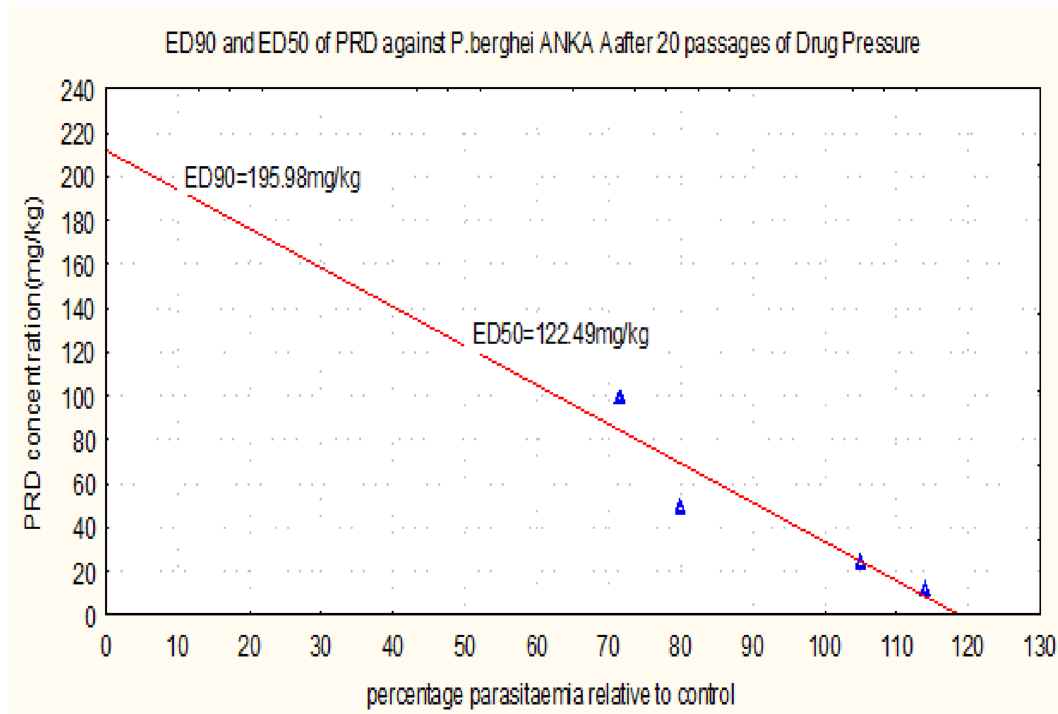


Table 5; *In vivo* activity of PRD against *P. berghei* ANKA GFP after dilution cloning of 20th drug selection passage.

No. of cages	Dosage ($mgkg^{-1}$.day)	Average parasitaemia	% parasitaemia relative to control	% Activity
1	100	1.64	93.75	6.25
2	50	2.08	119.34	-19.34
3	25	2.74	157.16	-57.16
4	12.5	2.77	158.88	-58.88
Control	Tween-80 in 3% ethanol solution	1.75	-	-

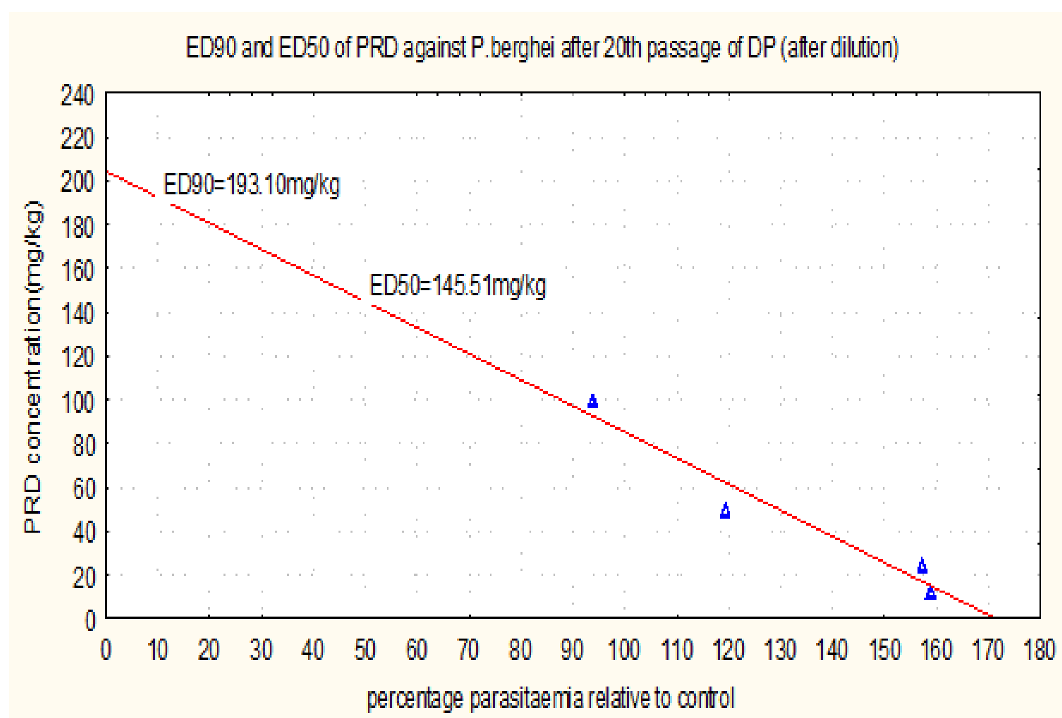


Table 6; *In vivo* activity of PRD against *P.berghei* ANKA GFP after withdrawal of PRD for 5 passages after 20th passage of drug pressure.

No. of cages	Dosage ($mgkg^{-1}.day$)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	100	1.45	72.01	27.99
2	50	1.77	88.23	11.77
3	25	2.77	137.94	-37.94
4	12.5	2.98	148.01	-48.01
Control	7%Tween-80 in 3% ethanol solution	2.01	-	-

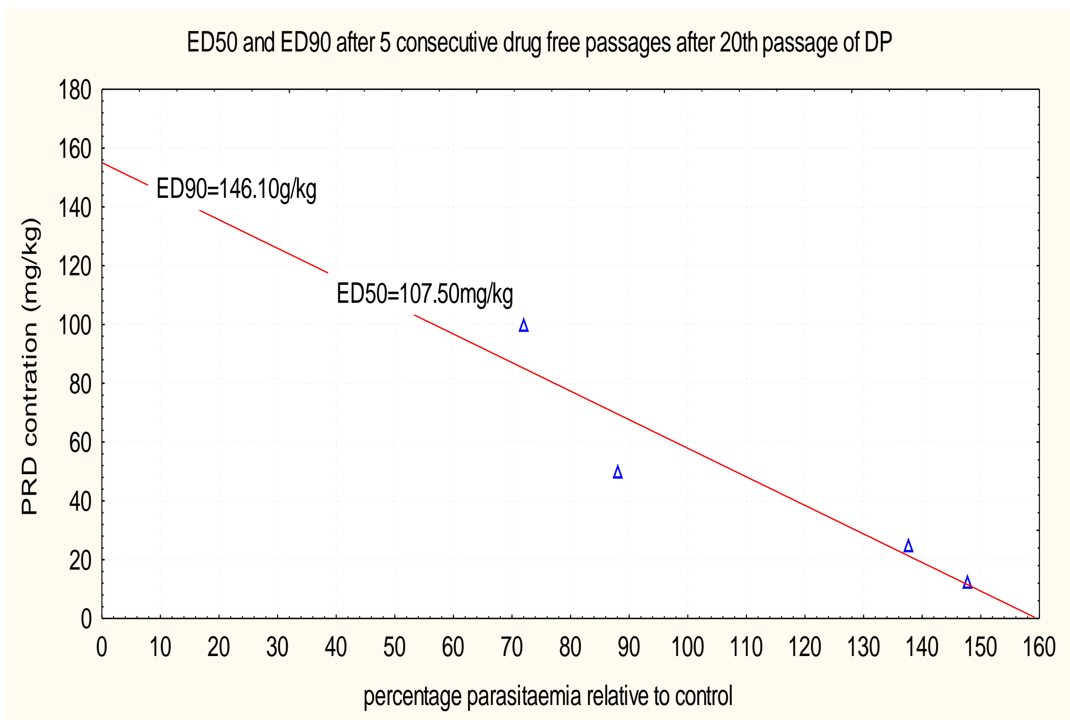
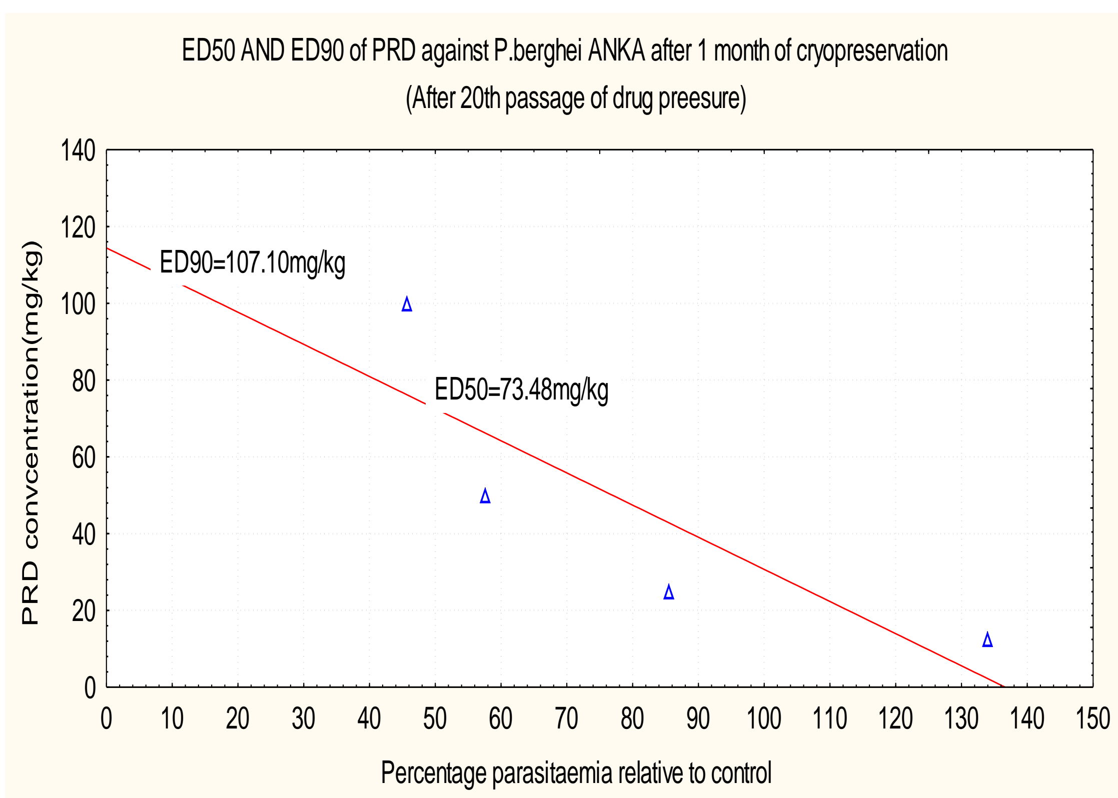


Table 7; *In vivo* activity of PRD against PRD^{R} *P. berghei* ANKA GFP isolate after one month of cryopreservation of 20th passage of drug pressure.

No. of cages	Dosage ($mgkg^{1}.day$)	Average parasitaemia	%parasitaemia relative to control	% Activity
1	100	1.31	45.67	54.33
2	50	1.65	57.57	42.43
3	25	2.44	85.51	14.49
4	12.5	3.83	133.95	-33.95
Control	Tween-80 in 3% ethanol solution	2.86	-	-



Appendix 3: Primers for amplifying and sequencing of *PbMDR-1* gene.

Primer Name:	Pbmdr-1:PCR and Sequencing Primer (5' to 3'):
Pbmdr1-1f UTR	GTCTAAATGTTGTAATTTGTTGTCCT
Pbmdr1-1r	CAGTATCATTCACACTTTCTCC
Pbmdr1-2f	GTGCAACTATATCAGGAGCTTCG
Pbmdr1-2r	CACTTTCTCCACAATAACTTGCTACA
Pbmdr1-3f	GCAGCTCTATATGTAATAAAAGGGTC
Pbmdr1-3r	GTCGACAGCTGGTTTTCTG
Pbmdr1-4f	CTTTGAATTACGGTAGTGGCT
Pbmdr1-4r	TCGCTAGTTGTATTCCTCTTAGA
Pbmdr1-5f	TGGAGTAGTTAGTCAAGATCCT
Pbmdr1-5r	GTGCCTTGTTCAACTATTACAC
Pbmdr1-6f	TCAAATAGAGATCAAGAATCAACAGG
Pbmdr1-6r	GGATATAAACCACCTGCCACT
Pbmdr1-7f	GCCAAGTAAACCATCATTCTTCA
Pbmdr1-7r	TCGCGTTGTAATGGTATATGCT
Pbmdr1-8f	GGATTTTTATCGTCGCATATTAACAG
Pbmdr1-8r	TAGCTTTATCTGCATCTCCTTTGAAG
Pbmdr1-9f	TGCAATAGATTATGACAGTAAAGGGG
Pbmdr1-9r	ATCTTTCAAATCGTAGAATCGCAT
Pbmdr1-10f	CTTCAAAGGAGATGCAGATAAAGCTA
Pbmdr1-10r	GATTCAATAAATTCGTCAATAGCAGC
Pbmdr1-11f	TGCAATAGTTAACCAAGAACCAATGT
Pbmdr1-11r UTR	CAATAGCCGATTAAAAGAAAAAACGA
Pbmdr1 f (UTR)	TTCACGCTATAAAAGTACAGACTA

Appendix 4: Pairwise alignment of parent and resistant clone *PbMDR-1* gene sequences.

The resistant parasites sequence is represented by EMBOSS_001 while the parent parasite sequence is represented by EMBOSS_002 in the following alignment.

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EMBOSS_001         1 ATGGCGGAAGAAAAAAGTAATAATAATAGTATCAAACATGAAGTTGAGAA     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002         1 ATGGCGGAAGAAAAAAGTAATAATAATAGTATCAAACATGAAGTTGAGAA     50

EMBOSS_001        51 AGAGTTAAATAAGAAATCTACCGTTGAGCTGTTTAAAAAGATCAAGTCAC    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002        51 AGAGTTAAATAAGAAATCTACCGTTGAGCTGTTTAAAAAGATCAAGTCAC    100

EMBOSS_001       101 AAAAGATTCCATTGTTTTTGCCATTTCATTCATTACCATCCAAATATAAG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002       101 AAAAGATTCCATTGTTTTTGCCATTTCATTCATTACCATCCAAATATAAG    150

EMBOSS_001       151 AAATTATTAGTTGTATCTTTTATATGTGCAACTATATCAGGAGCTTCGTT    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002       151 AAATTATTAGTTGTATCTTTTATATGTGCAACTATATCAGGAGCTTCGTT    200

EMBOSS_001       201 GCCTATTTTTATTTCGGTGTTTGGTGTTACCATGGCAAATCTTAATATTG    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002       201 GCCTATTTTTATTTCGGTGTTTGGTGTTACCATGGCAAATCTTAATATTG    250

EMBOSS_001       251 GAGAAAGTGTGAATGATACTGTTTTAAAATTAATAATAGTTGGTATATGT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002       251 GAGAAAGTGTGAATGATACTGTTTTAAAATTAATAATAGTTGGTATATGT    300

EMBOSS_001       301 CAATTTATATTATCATCGATTTCAAGTTTATGCATGGATGTTGTTACTAC    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002       301 CAATTTATATTATCATCGATTTCAAGTTTATGCATGGATGTTGTTACTAC    350

EMBOSS_001       351 AAAAATTTTAAGAACATTAAAATTAAAATATTTAAAAAGTGTTTTTCATC    400
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EMBOSS_002       351 AAAAATTTTAAGAACATTAAAATTAAAATATTTAAAAAGTGTTTTTCATC    400

EMBOSS_001       401 AAGATGGAGAATTTCATGATAATAATCCAGGTTCTAAATTAACATCTGAT    450
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EMBOSS_001       451 TTAGATTTTTATTTAGAACAAGTAAATGCAGGAATAGGAACAAAATTTAT    500
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EMBOSS_002       451 TTAGATTTTTATTTAGAACAAGTAAATGCAGGAATAGGAACAAAATTTAT    500

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EMBOSS_002       501 TACAATATTTACATATAGTAGTTCATTTTTAGGTTTATATTTCTGGTCAT    550

EMBOSS_001       551 TATATAAAAATGTAAGATTAACATTATGTATTACATGCGTTTTCCCAGTA    600
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EMBOSS_001       601 ATATATATATGCAGCTCTATATGTAATAAAAGGGTCCGATTAAATAAAAA    650
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EMBOSS_001       801 AAATTTTATGGAATCACTACATATTGGTTTAATTAATGGATTTATATTAG    850
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EMBOSS_002       851 CTTCTTATGCTTTGGGATTTTGGTATGGTACTAGAATTATAATACATGAT    900

EMBOSS_001       901 ATTAAAACTTTGAATTACGGTAGTGGCTTCAATGGAAGCGCAGTTATATC    950
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
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EMBOSS_001      1101 AAAATTAGATGATATTAAAAAAATTGAATTTAAAAATGTAAAGTTTCATT   1150
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EMBOSS_001      1651 GATTTTGTAGCATCCTTACCAGATAAATATGACACTTTAGTAGGTTCTAG   1700
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EMBOSS_001      1951 ACAGGAAATGATGAAAACAAACAAGGTGCTATAAATAGTAATAACGGAAG   2000
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      1951 ACAGGAAATGATGAAAACAAACAAGGTGCTATAAATAGTAATAACGGAAG   2000

EMBOSS_001      2001 TGTAATAGTTGAACAAGGCACTCATGATAGTTTGATGAAAAATAAAAATG   2050
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2001 TGTAATAGTTGAACAAGGCACTCATGATAGTTTGATGAAAAATAAAAATG   2050

EMBOSS_001      2051 GTATTTACTATTCTATGATTCAAAACCAGAAAGTATCGTCAAGTGGAAAT   2100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2051 GTATTTACTATTCTATGATTCAAAACCAGAAAGTATCGTCAAGTGGAAAT   2100

EMBOSS_001      2101 GGTGAAAATGACTGTGATAATAATAGTAGTGTATATAAAGATTCTGATAC   2150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2101 GGTGAAAATGACTGTGATAATAATAGTAGTGTATATAAAGATTCTGATAC   2150

EMBOSS_001      2151 AGGTGCTGCTAAATCTGCTACTGATACAAATATGGATATTAACATAGATA   2200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2151 AGGTGCTGCTAAATCTGCTACTGATACAAATATGGATATTAACATAGATA   2200

EMBOSS_001      2201 AAGATTTCAATATTCGAAAAGAAAAAGAGATTGCAGATACTGATAAGCCA   2250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2201 AAGATTTCAATATTCGAAAAGAAAAAGAGATTGCAGATACTGATAAGCCA   2250

EMBOSS_001      2251 AGTAAACCATCATTCTTCAAAAGAATGTTTGGAAAGAAAGAGAAGAAACC   2300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2251 AGTAAACCATCATTCTTCAAAAGAATGTTTGGAAAGAAAGAGAAGAAACC   2300

EMBOSS_001      2301 TCCTAGCAATTTGAGTATGGTATATAAAGAAATGTTTTCTCACAAAAAAG   2350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2301 TCCTAGCAATTTGAGTATGGTATATAAAGAAATGTTTTCTCACAAAAAAG   2350

EMBOSS_001      2351 AGGTTTTTATTATACTTTTAAGTACTATAGTGGCAGGTGGTTTATATCCA   2400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2351 AGGTTTTTATTATACTTTTAAGTACTATAGTGGCAGGTGGTTTATATCCA   2400

EMBOSS_001      2401 TTGTTTGCCATATTATATGCAAAATATGTTGGAACATTATTTGATATCAC   2450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2401 TTGTTTGCCATATTATATGCAAAATATGTTGGAACATTATTTGATATCAC   2450

EMBOSS_001      2451 AAACATGGAACATAATTCAAATAAATATTCTCTCTATATATTACTTATTG   2500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2451 AAACATGGAACATAATTCAAATAAATATTCTCTCTATATATTACTTATTG   2500

EMBOSS_001      2501 CTTTATCTATGTTTATTTCTGAAACGTTAAAAAATTATTATAATAATTTA   2550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
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EMBOSS_002      2501 CTTTATCTATGTTTATTTCTGAAACGTTAAAAAATTATTATAATAATTTA   2550

EMBOSS_001      2551 ATTGGAGAAAAGGTTGAGAACAAATTTAAATATTTATTGTTTGAGAGTAT   2600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2551 ATTGGAGAAAAGGTTGAGAACAAATTTAAATATTTATTGTTTGAGAGTAT   2600

EMBOSS_001      2601 AATACATCAAGAAATTGGTTTTTTTGATAAAGATGAACATGCCCCTGGAT   2650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2601 AATACATCAAGAAATTGGTTTTTTTGATAAAGATGAACATGCCCCTGGAT   2650

EMBOSS_001      2651 TTTTATCGTCGCATATTAACAGAGATATACATTTGTTAAAAACTGGTTTA   2700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2651 TTTTATCGTCGCATATTAACAGAGATATACATTTGTTAAAAACTGGTTTA   2700

EMBOSS_001      2701 GTAAATAATATTGTAATATTTACGCATTTTATTATTTTGTTTATTATTAG   2750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2701 GTAAATAATATTGTAATATTTACGCATTTTATTATTTTGTTTATTATTAG   2750

EMBOSS_001      2751 TACGATTTTGTCATTTTATTTTTGCCCAATAATAGCAGGAGCTTTAACAT   2800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2751 TACGATTTTGTCATTTTATTTTTGCCCAATAATAGCAGGAGCTTTAACAT   2800

EMBOSS_001      2801 TAGCATATACCATTACAACGCGAACATTTGCTATAAGAACACGATTGCAA   2850
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2801 TAGCATATACCATTACAACGCGAACATTTGCTATAAGAACACGATTGCAA   2850

EMBOSS_001      2851 AAATCTAAAGAAATAGAGAGGATTGGAAGTAAAAGAGATGGACAGTTTTC   2900
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2851 AAATCTAAAGAAATAGAGAGGATTGGAAGTAAAAGAGATGGACAGTTTTC   2900

EMBOSS_001      2901 ATATACTAATGATGAAGAAATATTTAAAGACCCTAACTTTTTAATTCAAG   2950
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2901 ATATACTAATGATGAAGAAATATTTAAAGACCCTAACTTTTTAATTCAAG   2950

EMBOSS_001      2951 AAGCATTTTATAATATGCAAACAATTGTTACATATGGATTAGAAGATTAT   3000
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      2951 AAGCATTTTATAATATGCAAACAATTGTTACATATGGATTAGAAGATTAT   3000

EMBOSS_001      3001 TTTTGTAAATTAATAGAAAATGCAATAGATTATGACAGTAAAGGGGATAG   3050
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3001 TTTTGTAAATTAATAGAAAATGCAATAGATTATGACAGTAAAGGGGATAG   3050

EMBOSS_001      3051 AAGAAAAATGATAGTTAATTCATTATTATGGGGATTTAGTCAATGTACAC   3100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3051 AAGAAAAATGATAGTTAATTCATTATTATGGGGATTTAGTCAATGTACAC   3100

EMBOSS_001      3101 AATTATTTATTAATGCATTTGCTTATTGGTTAGGTTCTATTTTGATAGAT   3150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3101 AATTATTTATTAATGCATTTGCTTATTGGTTAGGTTCTATTTTGATAGAT   3150

EMBOSS_001      3151 CACCGTATTATAGAGGTTGATAATTTTATGAAATCTTTATTTACATTTAT   3200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3151 CACCGTATTATAGAGGTTGATAATTTTATGAAATCTTTATTTACATTTAT   3200

EMBOSS_001      3201 ATTTACTGGTAGTTATGGTGGTAAATTAATGTCCTTCAAAGGAGATGCAG   3250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3201 ATTTACTGGTAGTTATGGTGGTAAATTAATGTCCTTCAAAGGAGATGCAG   3250

EMBOSS_001      3251 ATAAAGCTAAAATAACATTTGAGAAATATTATCCTATAATGGTTAGAAAA   3300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3251 ATAAAGCTAAAATAACATTTGAGAAATATTATCCTATAATGGTTAGAAAA   3300

EMBOSS_001      3301 TCAAATATAGATGTAAGAGATGAAAGTGGTATAAGAATAAATGATCCAAA   3350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3301 TCAAATATAGATGTAAGAGATGAAAGTGGTATAAGAATAAATGATCCAAA   3350

EMBOSS_001      3351 TAAAATAGACGGAAAAATAGAAGTGAAGGATGTTAATTTTAGATATTTAT   3400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002      3351 TAAAATAGACGGAAAAATAGAAGTGAAGGATGTTAATTTTAGATATTTAT   3400

EMBOSS_001      3401 CTAGACCAAATGTACCAATATATAAAGATTTATCCTTTAGTTGTGATAGC   3450
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                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3401 CTAGACCAAATGTACCAATATATAAAGATTTATCCTTTAGTTGTGATAGC   3450

EMBOSS_001     3451 AAAAAAACAACAGCTATAGTTGGAGAAACTGGATGTGGTAAATCCACGAT   3500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3451 AAAAAAACAACAGCTATAGTTGGAGAAACTGGATGTGGTAAATCCACGAT   3500

EMBOSS_001     3501 TATGCATTTATTAATGCGATTCTACGATTTGAAAGATGACCACGTTTTAT   3550
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3501 TATGCATTTATTAATGCGATTCTACGATTTGAAAGATGACCACGTTTTAT   3550

EMBOSS_001     3551 TGGATAATCAACATATTGAAAAAGACAATAAAGATAAATCAAAGGATATA   3600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3551 TGGATAATCAACATATTGAAAAAGACAATAAAGATAAATCAAAGGATATA   3600

EMBOSS_001     3601 GAAATGCGTGATGCAACATCTATGAAAAATTTGAACGAATTGGGTAAAAA   3650
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3601 GAAATGCGTGATGCAACATCTATGAAAAATTTGAACGAATTGGGTAAAAA   3650

EMBOSS_001     3651 AAACGCAAATGAAGAATTTACTGTTTACAAAAATAGTGGCAAAATTTTAC   3700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3651 AAACGCAAATGAAGAATTTACTGTTTACAAAAATAGTGGCAAAATTTTAC   3700

EMBOSS_001     3701 TTGATGGTATAGATATTTGTGATTATAACTTAAAAGATCTAAGAGGATTA   3750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3701 TTGATGGTATAGATATTTGTGATTATAACTTAAAAGATCTAAGAGGATTA   3750

EMBOSS_001     3751 TTTGCAATAGTTAACCAAGAACCAATGTTGTTTAATATGTCTATTTATGA   3800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3751 TTTGCAATAGTTAACCAAGAACCAATGTTGTTTAATATGTCTATTTATGA   3800

EMBOSS_001     3801 AAATATAAAATTCGGTAAACAAGATGCAACATTAGATGATGTAAAAAGAG   3850
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3801 AAATATAAAATTCGGTAAACAAGATGCAACATTAGATGATGTAAAAAGAG   3850

EMBOSS_001     3851 TATGTAAATTTGCTGCTATTGACGAATTTATTGAATCATTACCAAATAAA   3900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3851 TATGTAAATTTGCTGCTATTGACGAATTTATTGAATCATTACCAAATAAA   3900

EMBOSS_001     3901 TATGATACTAACGTAGGACCTTATGGTAAAAGTTTATCAGGTGGTCAAAA   3950
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3901 TATGATACTAACGTAGGACCTTATGGTAAAAGTTTATCAGGTGGTCAAAA   3950

EMBOSS_001     3951 ACAACGAGTTGCTATTGCTAGAGCCTTATTAAGAGAACCTAAAATATTAT   4000
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     3951 ACAACGAGTTGCTATTGCTAGAGCCTTATTAAGAGAACCTAAAATATTAT   4000

EMBOSS_001     4001 TGTTAGACGAAGCTACTTCATCTCTTGATTCACACTCAGAAAAATTAATC   4050
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     4001 TGTTAGACGAAGCTACTTCATCTCTTGATTCACACTCAGAAAAATTAATC   4050

EMBOSS_001     4051 GAAAAAACTATTGTTGATATTAAAGATAAAGCTGACAAAACAATCATTAC   4100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     4051 GAAAAAACTATTGTTGATATTAAAGATAAAGCTGACAAAACAATCATTAC   4100

EMBOSS_001     4101 TATTGCTCACAGAATTGCATCTATTAAAAGATCAAATAAAATTGTAGTTT   4150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     4101 TATTGCTCACAGAATTGCATCTATTAAAAGATCAAATAAAATTGTAGTTT   4150

EMBOSS_001     4151 TTAATAACCCAGATAAAAAATGGATCTTTTGTTCAAGCCCAAAAAACACAT   4200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     4151 TTAATAACCCAGATAAAAAATGGATCTTTTGTTCAAGCCCAAAAAACACAT   4200

EMBOSS_001     4201 GACGAATTGATTCGTGATAAAGATAGTGTTTATACGAAATATGTCAAATT   4250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002     4201 GACGAATTGATTCGTGATAAAGATAGTGTTTATACGAAATATGTCAAATT   4250

EMBOSS_001     4251 AACTAAATAA     4260
                    ||||||||||
EMBOSS_002     4251 AACTAAATAA     4260
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Appendix 5: Ethical approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **February 26, 2013**

TO: **SHADRACK KIMANI,**
PRINCIPAL INVESTIGATOR

THRO': DR. JENNIFER ORWA,
THE ACTING DIRECTOR, CTMDR,
NAIROBI

RE: **SSC PROTOCOL NO. 2457 (*INITIAL SUBMISSION*): INVESTIGATION OF MOLECULAR BASIS OF PYRONARIDINE RESISTANCE IN *PLAMODIUM BERGHEI* ANKA ISOLATES**

This is to inform you that during the 212th meeting of the KEMRI/ERC meeting held on 26th February 2013, the above study was reviewed.

The Committee notes that the above referenced study aims to select pyronaridine resistant lines in *P. berghei* using piperaquine resistant clones as the starting parasite .

The above referenced is laboratory based with an animal component. There being no human contact no ethical issues arise the study is therefore **granted approval** for implementation effective this **26th day of February 2013,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **25th February 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **13th January 2014**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study. Kindly remit the letter of approval from the Animal Care and Use Committee for our records.

Yours sincerely,

DR. ELIZABETH BUKUSI
FOR: SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 6: Publication

academicJournals

Vol. 8(6), pp. 111-117, August, 2014
DOI: 10.5897/AJBR2014.0780
Article Number: E29B75C46711
ISSN 1996-0778
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http://www.academicjournals.org/AJBR

African Journal of Biochemistry Research

Full Length Research Paper

Plasmodium berghei ANKA: Selection of pyronaridine resistance in mouse model

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Received 19 May, 2014; Accepted 12 June, 2014

Pyronaridine is a partner drug in, Pyramax®, a combination of artesunate (ASN)-pyronaridine (PRD) which was recently prequalified by WHO drug as a potential alternative for treatment of malaria in African setting. Pyronaridine is a mannich base, with a long half-life, thus predisposed to resistance. In this study, we selected pyronaridine resistance by submitting *Plasmodium berghei* ANKA line *in vivo* to increasing pyronaridine concentration for 20 successive passages over a period of six months. The effective doses that reduce parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) determined in the standard four-day suppressive test for the parent line were 1.83 and 4.79 $mgkg^{-1}$, respectively. After 20 drug pressure passages, the ED_{50} and ED_{90} increased by 66 and 40 fold, respectively. After dilution cloning, the parasites were grown in the absence of drug for five passages and cryo-preserving them at -80°C for at least one month, the resistance phenotypes remained stable. Thus, the resistant phenotype line could be used to explore genetic determinants associated with pyronaridine resistance; therefore, this strain represents a vital tool to study the mechanisms of resistance.

Key words: Malaria, pyronaridine, Pyramax®, resistance, *Plasmodium berghei* ANKA.

INTRODUCTION

Malaria is a global public health concern. The emergence of resistance, particularly in *Plasmodium falciparum*, has been a major contributor to the global resurgence of malaria in the last three decades (Marsh, 1998). In reality, *P.*

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Abbreviations: ASN, Artesunate; **GFP,** green fluorescent protein; **PRDR;** pyronaridine resistant clone.