INDUCEMENT OF *Plasmodium berghei* ANKA STRAIN RESISTANCE TO LUMEFANTRINE, PIPERAQUINE AND AMODIAQUINE IN A MOUSE MODEL.

Kiboi Daniel Muthui (BSc Hons)

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

2008

DECLARATION

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

Signed……..Date……..

Kiboi Daniel Muthui

This thesis has been submitted for examination with our approval as University Supervisors

1. Signed…….Date……

Dr Alexis Nzila

KEMRI/WELLCOME TRUST, KILIFI

2. Signed…….Date……

Dr Geoffrey M. Rukunga

KEMRI, NAIROBI

3. Signed……..Date……...

Dr Joseph Ng'ang'a

JKUAT, KENYA

DEDICATION

This thesis is dedicated to my parents James and Mary Kiboi and my brothers, Shadrack, late Hezron, Samuel, Charles and my sisters Phoebe and Mary.

ACKNOWLEDGEMENTS

Many of you, I have come across before and during these years of my MSc training, played various roles that have led to the successful completion of this thesis. My appreciation goes to you all for your smiles, those phone calls and emails, advice, your time, moral, financial as well as other material support and your prayers. While I cannot single out all of you, my sincere gratitude goes to:

My supervisors Drs Alexis, Geoffrey and Joseph:

Dr Alexis Nzila for accepting me to work within your group and for giving me a chance to find myself in this endless story. Your tireless efforts in turning ideas into scientific reality and gentle personality gave room to the realization of the goals in this thesis.

Dr Geoffrey Rukunga for accepting me to join the CTMDR during the research attachment. Your generosity, scientific advice and your concern over my progress are heartily appreciated.

Dr Joseph Ng'ang'a for your constructive criticisms, from the initial step of the baby proposal have gone along way. I can hardly overemphasize your role in shaping my way of scientific thinking.

Beatrice Irungu Your contributions, advice and continued support have been tremendous.

All staff at **KEMRI-CTMDR**- all the way from the center director, scientific and laboratory to the technical staff - thank you for all your support and friendship

All staff at **KEMRI Animal House** for maintaining the supply of the mice constant Keep up the good work.

My dad, mum and my siblings your Love and deep understanding have been the cornerstone foundation and a source of inspiration right from the beginning to the completion of this thesis.

Financial support: The work presented in this thesis received funding from Irish Health Research Board (HRB) of the Republic of Ireland and European & Developing Countries Clinical Trials Partnership (EDCTP). We were likely to suffer an irreversible monetary deficiency without you.

Above all, my gratitude goes to the **ALMIGHTY GOD** for making everything possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

2% RT ……2% relapse technique

4-DT……4-Day Suppressive Test

ACT…….Artemisinin Based Combination Therapy

AQ/ASN…….Amodiaquine-artesunate

AQ……....Amodiaquine

ATM ……..….Artemether

CDC ……Center for disease control and prevention

CM……...Cerebral malaria

CQ…….....Chloroquine

Crt …….Chloroquine-resistance transporter gene

DEAQ …….Desethylamodiaquine

DHA ……...Dihydroartemisinin

DHFR …….Dihydrofolate reductase

DHPS ……...Dihydropteroate synthase

DP…….drug pressure

EANMAT……...East Africa Network Monitoring Antimalarial Treatment

ED……...Effective dose

EDCTP…….European and Developing Countries Clinical Trials Partnership

EE….....exo-erythrocytic

FACS ……Fluorescence activated cell sorting

GFP …….....Green Fluorescent Protein

HRB……..Irish Health Research Board

HRP-II……..…..….. Histidine-rich protein 2

i.p ……...Intraperitoneal

IL-1……Interleukin-1

IL-12……Interleukin-12

INF-γ…….Interferon gamma

ITN……..Insecticide-Treated Nets

KEMRI ……...Kenya Medical Research Institute

LBW…….Low birth weights

LM/ATM ……..…Lumefantrine-artemether

LM……Lumefantrine

Mdr1…….multi-drug resistance-1

*Pf*ATPase 6 ……...*Plasmodium falciparum* calcium adenosine triphosphatase 6

*Pf*EMP ……...*Plasmodium falciparum* erythrocyte membrane protein

Pfmdr …..……*Plasmodium falciparum* multi-drug resistance 1 gene

PfMDR1 …….*Plasmodium falciparum* multi-drug resistance-1 transporter

PQ/DHA ……Piperaquine-dihydroartemisinin

PQ……...Piperaquine

PfCRT ……..*Plasmodium falciparum* chloroquine resistance transporter

PQP……....Piperaquine phosphate

PRBC……..Parasitized Red Blood Cell

PSG …….phosphate saline glucose buffer

QBC…….…Quantitative Buffy Coat

SP…….....Sulfadoxine-Pyrimethamine

ST …….serial technique

TNF…….Tumor necrosis factor

WHO……...World Heath Organization

ABSTRACT

The evolution of drug resistance in *Plasmodium falciparum* the species that causes the most virulent form of malaria in humans is a major public health problem. In response to increasing antimalarials resistance, old drug such as amodiaquine (AQ) have been resurrected while new once such as lumefantrine (LM) and Piperaquine (PQ) are being introduced. LM, PQ and AQ are potent partner drugs in artemether-lumefantrine (ATM/LM), piperaquine-dihydroartemisinin (PQ/DHA) and amodiaquine-artesunate (AQ/ASN) ACTs respectively. ATM, DHA and ASN are short acting drugs with a half-life of less than 2 hours. LM, PQ and AQ active derivative (N-desethylamodiaquine (DEAQ) are long acting drugs with a half-life 6, 19 and 14 days respectively. During elimination period the unprotected LM, PQ and DEAQ remain alone especially at sub-therapeutic concentrations. At this point, selection of re-infecting resistant parasites may occur rapidly especially in African regions with high malaria transmission. Markers coding for LM, PQ and AQ resistance are not clearly understood due to lack of well characterized resistant lines. The objective of the study was to induce and select *Plasmodium berghei* ANKA strains resistant to LM, PQ and AQ *in vivo*. *P. berghei* ANKA strain in mouse model was exposed to selection pressure from AQ, LM and PQ. Two methods were adopted: first the use of a single dose at every passage and second, the stepwise increase of drug pressure dose. Once every 7-10 days, parasitized erythrocytes were passed to the next group of naive mice. The level of resistance was assessed in the 4-DT at different intervals every 4^{th} or 8^{th} passage, the increase in ED_{50}, ED_{90} and ED_{99} was estimated graphically using version 5.5 of Statistica 2000 and indices of resistance, I_{50}, I_{90} and I_{99} calculated. Resistance was classified into four categories I_{99}

=1.0, sensitive, I_{99} =1.01-10.0, slight resistance, I_{99} =10.01-100.0, moderate resistance, and I_{99} = >100.0, high resistance. The ED_{50}, ED_{90} and ED_{99} of AQ against parent *P. berghei* ANKA strain was 0.95, 4.29 and 5.05 mg/kg.day respectively. Within thirty six passages the ED_{50}, ED_{90} and ED_{99} were 12.01, 19.13 and 20.73 mg/kg.day respectively. Slight AQ resistant line was selected with I_{50}, I_{90} and I_{99} of 12.64, 4.46 and 4.10 respectively. The ED_{50}, ED_{90} and ED_{99} of LM against parent *P. berghei* ANKA strain was 1.67, 3.93 and 4.48 mg/kg.day respectively. Within twenty eight passages, the ED_{50}, ED_{90} and ED_{99} were 9.76, 25.48 and 29.02 respectively. Slight LM resistant line was selected with I_{50}, I_{90} and I_{99} of 5.84, 6.48 and 6.48 respectively. The ED_{50}, ED_{90} and ED_{99} of PQ against parent *P. berghei* ANKA GFP was 1.30, 3.52 and 8.10 mg/kg.day respectively. Within twenty eight passages of selection pressure the ED_{50}, ED_{90} and ED_{99} was 122.00, 193.30 and 210.00 mg/kg.day respectively. Moderate PQ resistant line was selected with I_{50}, I_{90} and I_{99} of 93.85, 54.91 and 25.93 respectively, the line exhibited relative stability with I_{50}, I_{90} and I_{99} of 36.23, 42.76 and 20.85 after drug free five passages. Stable PQ resistant line was selected which could be used to study its mechanism of resistance. Further work is warrant for LM and AQ.

CHAPTER ONE: INTRODUCTION

1.1 Background of the study.

Malaria is a febrile illness characterized by fever, malaise, cough, nausea and diarrhea. It is caused by erythrophagous parasitic protozoan belonging to genus *Plasmodium* (Smith and Parsons, 1996). It is a major public health problem in more than 90 countries affecting 40% of the world population (WHO, 2001). An estimated 1-2 million deaths occur annually and 90 % of the total deaths are in sub-Saharan Africa (Goodman and Mills, 1999). In Kenya, 34,000 deaths occur annually (WHO, 2006).

Four identified species cause the disease in human namely *P. falciparum*, *P. vivax, P. ovale* and *P. malariae* (Smith and Parsons, 1996; WHO, 2002). *P. falciparum*, most virulent accounts for majority of morbidity and mortality (Barry, 2005). The striking feature of *P. falciparum* under transmission conditions in Africa is that almost everyone develops a new infection every year and experiences a disease in their lives (Hviid, 2005).

Over the last two decades, malaria prevalence has increased and distribution expanded due to the spread of multi-drug resistant *P. falciparum* (Goodman and Mills, 1999; White, 2004). The expansion of drug resistance has rendered most first-line treatment ineffective, facilitated the transmission of drug resistant strains and increased the ease with which other antimalarial drugs are selected for resistance (Goodman and Mills, 1999; Bjorkman and Bhattarai, 2005).

For over 50 years, chloroquine (CQ) was the mainstay of malaria treatment and control, providing effective, affordable and widely available therapy across Africa (Wellems and Plowe, 2001). The progressive failure of CQ led to promotion of

sulfadoxine/pyrimethamine (SP) as the first line drug (Trape, 2001; Hyde, 2005b). However its resistance emerged and spread quickly reducing its therapeutic life (Gregson and Plowe, 2005). Effective, affordable first line treatment and strategies against resistance are therefore important if the fight against malaria is to succeed (WHO, 2006).

In response to increasing resistance, Artemisinin based Combination Therapies (ACTs) have been proposed to be the next strategy for the control of malaria (WHO, 2003, Yeung *et al*., 2004). In most ACTs, the artemisinin derivative is a short half-life drug while the partner drug is a long half-life drug (WHO, 2006). The weak link in the combinations is the period which the unprotected long-half-life drug remains alone, particularly at sub-therapeutic levels (White and Pongtavornpinyo, 2003). At this drug concentration selection of re-infecting resistant parasites may occur therefore promoting constant malaria transmission (Hastings *et al*., 2002). In low malaria transmission settings, this constitutes a minor problem but in high transmission areas the success of the ACT remains a big challenge (Sisowath *et al*., 2005).

A combination of lumefantrine (LM) and artemether (ATM) has been adapted as a first line treatment in many African countries (Mutabingwa, 2005). LM/ATM has mismatched pharmacokinetics, ATM is very short acting drugs with a half life of 2 hours while LM is long acting drugs with a half life of 6 days (Ezzet *et al*., 2000; WHO, 2006). Studies indicate that use of drugs combinations with mismatched pharmacokinetics does not prevent selection of the resistance against the long acting drug (Hastings *et al*., 2002).

Studies in Tanzania and Uganda also show that parasites carrying *P. falciparum* multi-drug resistance 1 gene (*pfmdr1*) Y86N allele tolerates residual LM following ATM/LM treatment (Sisowath *et al*., 2005; Dokomajilar *et al*., 2006). The tolerance

suggests the existence of strong selection pressure for LM resistance *in vivo* (Sisowath *et al*., 2005).

Another combination, Dihydroartemisinin (DHA) and piperaquine (PQ), currently under clinical trial in Africa also has mismatched pharmacokinetics (Yeung *et al*., 2004; Tarning *et al*., 2005). DHA is a short acting drug, half life of 45 minutes while PQ is a long acting drug with half life of 15-20 days (Hung *et al*., 2004; WHO, 2006). The selection pressure for resistance would therefore be exerted primarily by PQ (Basco and Ringwald, 2003; White, 2004). In China, the use of PQ as a monotherapy resulted in rapidly emergence of PQ resistant parasites (Hien *et al*., 2004). In an African settings, resistance to PQ may emerge rapidly especially due to hyper transmission of malaria and extensive use of the ACT.

With the loss of CQ and SP to resistance coupled with high costs of ACTs especially LM/ATM, use of quinoline amodiaquine (AQ) as a monotherapy has been on the increase (WHO, 2006). AQ remains the only cheap alternative drug in malaria endemic countries (Gorissen *et al*., 2000). However, several reports indicate a reduction of AQ activity against CQ resistant isolates of *P. falciparum* (EANMAT, 2003).

PQ, LM and AQ like CQ inhibit heme polymerization as their principal mechanism of action (Robert *et al*., 2001). This similarity is a strong predisposing factor in selection of LM, PQ and AQ resistance. Identifying genes involved in LM, PQ and AQ resistance is important for understanding and predicting how resistance to a given drug arises. It also has practical value in enabling diagnostic molecular methods be developed for identifying resistant parasites in clinical and field isolates. The difficulty is absence of well characterized LM, PQ and AQ resistant lines.

Drug resistant lines can either be induced *in vitro* using *Plasmodium falciparum* or *in vivo* using rodent malaria models. However, selection of resistance *in vitro* is a long term process and the stability of resistant phenotypes is difficult to establish. Drug resistance studies indicate that resistance can be easily achieved *in vivo*, using rodent malaria models (Peters, 1999; Xiao *et al.*, 2004).

Drug resistant rodent malaria model have successfully been used in predicting mechanism of resistance of different antimalarials. Atovaquone resistance in *P. berghei* and SP resistance in *P. chabaudi* has shown correlation to resistance in *P. falciparum* (Gervais *et al*., 1999; Carlton *et al*., 2001). However, CQ resistant *P. chabaudi* and artemisinin resistant *P. c. chabaudi* have no correlation to resistance in *P. falciparum* (Carlton *et al*., 1998b; Afonso *et al*., 2006).

Plasmodium berghei ANKA is a rodent malaria parasite that serves as an excellent model for biological investigations of human malaria parasites (Janse and Waters, 1995). *P. berghei* ANKA has been used successfully in testing antimalarials activity and especially for drugs that targets heme, (Ridley, 2002) such as the AQ, LM and PQ, therefore, *P. berghei* ANKA serves as an excellent model in the study.

The study presents *in vivo* selection of *P. berghei* ANKA strain resistance to AQ, LM and PQ by continuous exposure of the parasites to these drugs and also assessment of stability of the resistant lines induced. *P. berghei* ANKA strain expressing Green Fluorescent Protein (GFP) was used to select PQ resistant line while *P. berghei* ANKA expressing fusion protein GFP-luciferase was used to select AQ and LM resistant lines.

1.2 Justification

Attempts have been made to identify markers coding for LM, PQ and AQ resistance. Many studies have relied on human clinical *P. falciparum* isolates especially in area where these drugs have been used extensively. However, lack of highly and well characterized resistant strains have hindered these studies. This scientific research was an attempt to induce *P. berghei* ANKA resistance to LM, PQ and AQ and also assess the stability of the resistant phenotypes that could be used to study mechanism of resistance of these drugs. The study also attempts to forecast the level of risk of the selection of resistance that may follow deployment of LM, PQ and AQ among human populations.

Understanding the mechanisms of resistance of these drugs is critical since this information could be used to design strategies of extending their therapeutic lifetime. In addition, it could also lead to design of new combination therapies and drugs with high activity. Furthermore, the induced resistant lines will provide the platform to studies on cross-resistance with other related antimalarials.

CHAPTER TWO: LITERATURE REVIEW

2.1 Global incidences of malaria

Malaria remains a major global health problem throughout Africa, Oceania, Asia and Latin America, covering over 90 countries (WHO, 2006; Breman *et al*., 2006). At least 40% of the world's populations live in areas of stable malaria transmission where functional immunity is acquired from about the age of five (Goodman and Mills, 1999).

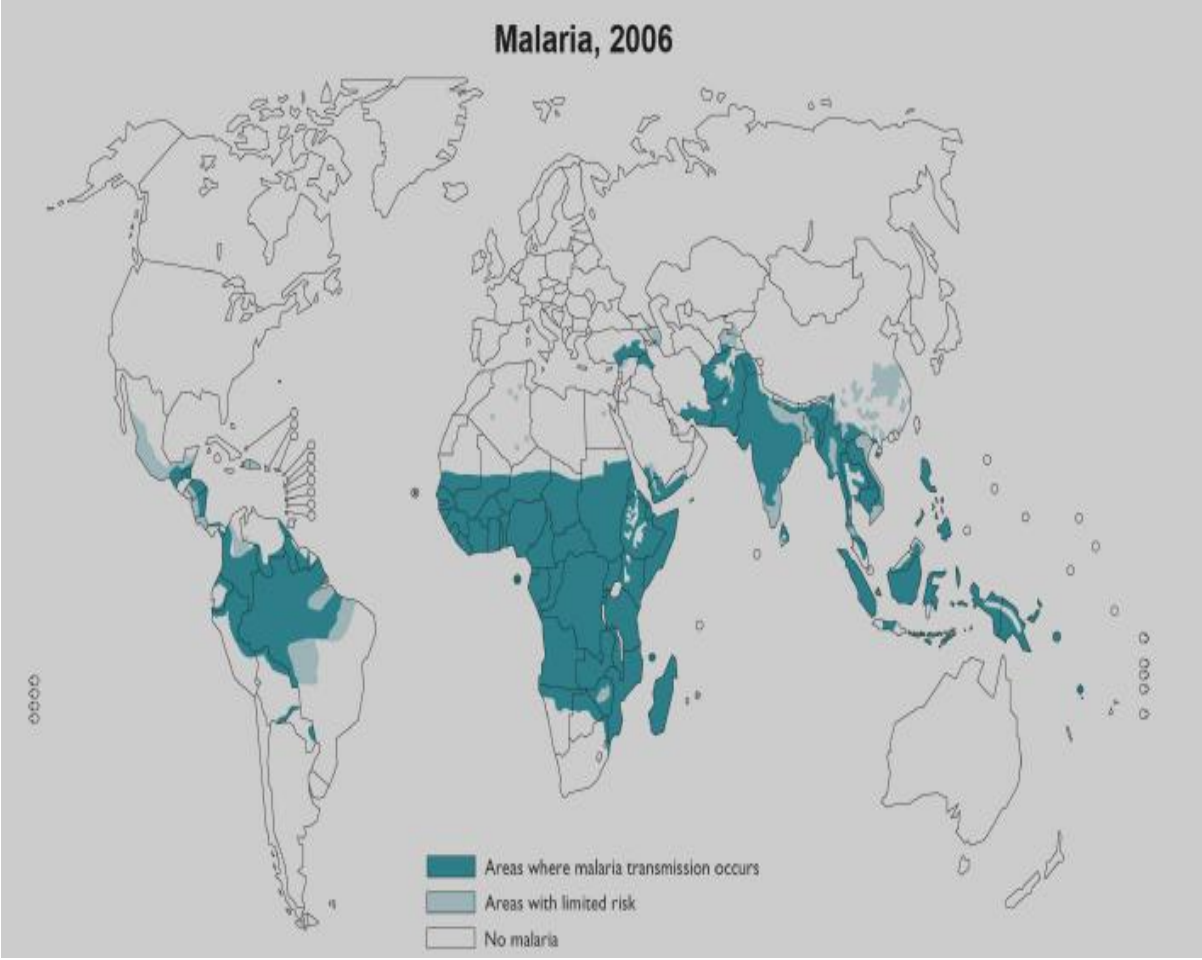


Fig. 1: The Global distribution of malaria (WHO 2006).

Malaria causes 500 million acute clinical cases and approximately 3 million deaths every year (WHO 2006). The disease burden is concentrated in young children

and pregnant women with children experiencing an estimated six episodes of malaria per year (WHO, 2003, Breman *et al*., 2006). A number of indirect negative effects by malaria on health and development such as impaired cognitive functions and increased susceptibility to other diseases e.g., HIV incidences are also increasing (Bjorkman and Bhattarai, 2005). Those at greatest risk of malaria are the poor and marginalized populations (WHO, 2002).

Malaria burden is not uniformly distributed and over 90% of the disease burden is in sub-Saharan Africa (WHO, 2003). The geographical distribution of the four human malaria parasite species is variable and dependent on season, endemicity and vector distribution (WHO, 2006). In sub-Saharan Africa, around 750,000 children under five each year die of malaria, accounting for over 10% of the total disease burden measured in disability adjusted life years (Goodman and Mills, 1999). Malaria economic burden accounts for a reduction of 1.3% in the annual economic growth rate in malaria endemic countries (Goodman and Mills, 1999; WHO, 2001).

Majority of infections and almost all deaths in Africa are caused by *P. falciparum*, the most dangerous of the four human malaria parasites (WHO, 2006). In addition, the most effective malaria vector, the mosquito *Anopheles gambiae* is widespread in Africa and difficult to control (WHO, 2003). Most of the remaining burden is distributed between the Indian sub-continent, Southeast Asia and Oceania, and the South America (WHO, 2002).

2.2 Human malaria species.

The apicomplexan *Plasmodium* species infect many animal species including primates, lizards and birds (Barry, 2005). The etiological agents of human malaria are

recognized as four distinct species of *Plasmodium* namely *Plasmodium falciparum, P. vivax, P. ovale and P. malariae* (Smith and Parsons, 1996; WHO, 2002).

P. falciparum is the most common and the primary cause of severe malaria in humans (Winstanley *et al*., 2004). *P. ovale* and *P. vivax* cause benign and relapsing tertian malaria (Win *et al*., 2004).

P. vivax with the widest geographical distribution throughout the world is relatively non-lethal and has strong preference for reticulocytes during invasion (Janse and Waters, 1995; Chiang *et al*., 2006). *P. vivax* causes up to 80 million cases per year with the 15% in Africa and 85% outside Africa (Mendis *et al*., 2001). The prevalence of *falciparum and vivax* malaria is approximately equal in the Indian subcontinent, eastern Asia, Oceania, and South America (WHO, 2001).

P. malariae is found in most endemic areas, throughout Sub-Saharan Africa, but is much less common than the other species (Guerra *et al*., 2007). *P. malariae* is milder than other types of malaria and commonly found in older red blood cells (Chiang *et al*., 2006). *P. ovale* is a rare parasite, mainly confined to tropical Africa and accounts for 10 % of all malaria infections in Africa but is less common in other malaria-endemic regions (Faye *et al*., 2002; Win *et al*., 2004).

2.3 Rodent malaria parasites

In research aimed at developing strategies for eradicating human malaria, rodent malaria parasites species are used as laboratory models (Carlton *et al*., 2001). There are four African rodent malaria parasites namely *Plasmodium berghei, P. yoelii, P. chabaudi* and *P. vinckei* (Smith and Parsons, 1996).

Rodent parasites often represent the only practical means towards *in vivo* experimentation (Janse and Waters, 1995). Housekeeping genes and biochemical processes are conserved between rodent and human parasites (Carlton *et al*., 1998a). Molecular basis of resistance in some drug resistant rodent parasites has shown similarities to resistance in human parasite. Atovaquone resistance in *P. berghei* and SP resistance in *P. chabaudi* has shown correlation to resistance in *P. falciparum* (Gervais *et al*., 1999; Carlton *et al*., 2001). However, CQ resistant *P. chabaudi* and artemisinin resistant *P. c. chabaudi* have no correlation to resistance in *P. falciparum* (Carlton *et al*., 1998b; Afonso *et al*., 2006).

P. 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, SP11, NK65, LUKA and K173 (Janse and Waters, 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).

Generation of transgenic ANKA strain of *P. berghei* expressing exogenous proteins green fluorescent protein (GFP) and/or luciferase has broadened possibilities for *in vivo* drug screening (de Koning-Ward *et al*., 2000; Franke-Fayard *et al*., 2006). *P. berghei* GFP lines strongly express GFP detectable by fluorescent microscopy and fluorescence activated cell sorting (FACS) (de Koning-Ward *et al*., 1998). 'Four reference' lines of the ANKA strain have been genetically transformed with reporter

genes GFP or fusion protein GFP-Luciferase namely (259cl2 (GFPcon); 354cl4; 507m6cl1; 676m1cl1) (Janse *et al*., 2006).

P. berghei has successfully been used in drug testing investigations (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).

The other rodent parasites play invaluable role in malaria research. *P. chabaudi* shows antigenic variation during long lasting, non-lethal, infections *in vivo* hence a useful model in mechanisms of drug resistance and antigenic variation (Janse and Waters, 2006). *P. berghei* infections are rapidly lethal to laboratory rodents hampering studies on the *in vivo* generation and selection of antigenic variants (Janse and Waters, 1995). *P. yoelii*, which is extensively used in studies on the biology of liver stage and blood stage antigens and their role in immunity and vaccine development (Janse and Waters, 2006).

2.4 Lifecycle of plasmodium

The malaria parasite has a complex life cycle (Fig. 2) involving development in female anopheline mosquitoes and in humans presenting a major obstacle in the design of control measures (CDC, 2007).

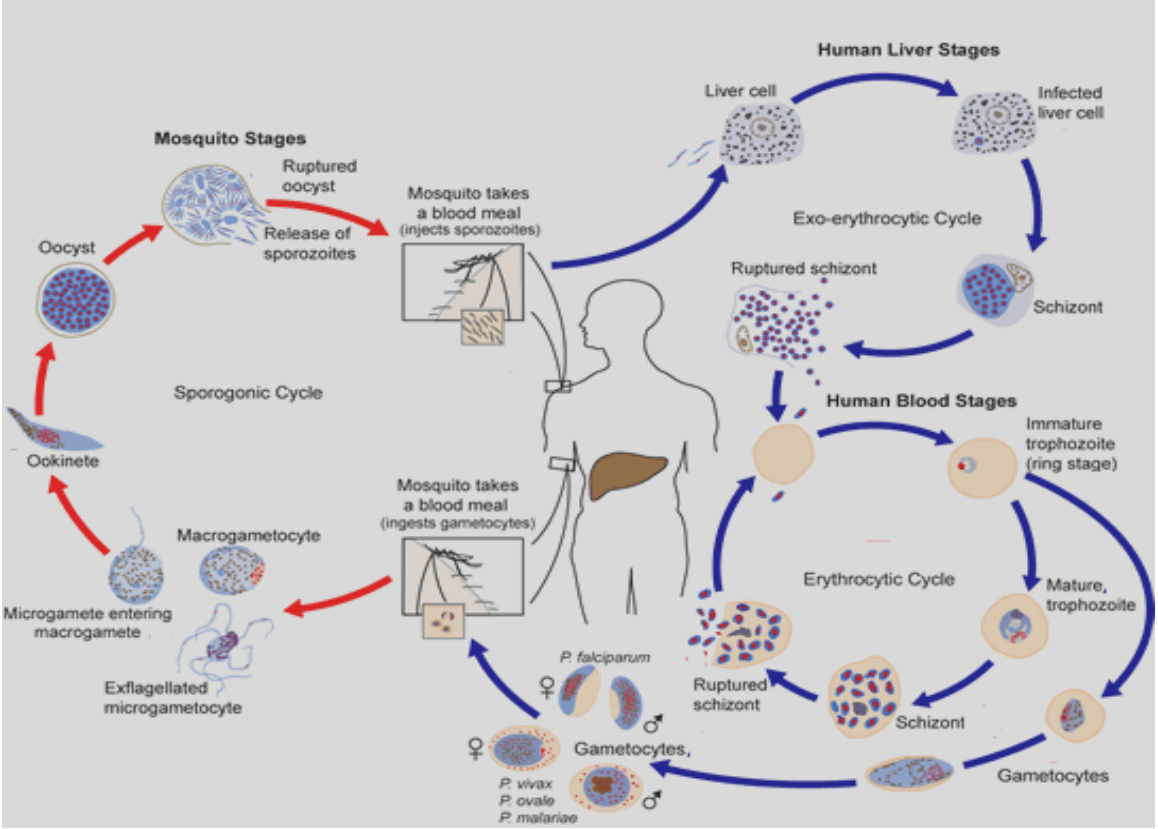


Fig. 2: Lifecycle of malaria parasite. Source: (CDC, 2007).

Infection is initiated after malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (Fig 2). Sporozoites infect hepatocytes, mature into schizonts and then rupture to release merozoites, which infect erythrocytes. The parasites undergo asexual multiplication (erythrocytic schizogony), after which erythrocytes burst and releases new merozoites capable of re-initiating new erythrocytic schizogony. Some parasites in newly infected erythrocytes differentiate into male (microgametocytes) and female (macrogametocytes) which an *Anopheles* mosquito ingests during a blood meal (Barry, 2005). The gametes fuse to form a zygote which then differentiates into a motile and elongated ookinetes. Ookinetes invade the midgut wall of the mosquito, develop into oocysts that grow and ruptures to release sporozoites. The sporozoites migrate to the salivary glands ready to initiate new infection (Fig 2).

Despite the similarities in the lifecycles between malaria parasites, *P. berghei* show specific features such as strong preference for invading reticulocytes and asynchronicity of blood stage development in natural infection (Carlton *et al*., 2001; Janse and Waters, 2006). The asexual cycle of the haploid blood stages is short (22-23 h) and the period of development of gametocytes is also short (26h). In addition, the exo-erythrocytic (EE) development is short with the sporozoites developing into mature EE schizonts within 43-52 h after invasion of parenchymal cells (Janse and Waters, 1995).

2.5 Diagnosis

Diagnosis of malaria is based on light microscopy, fluorescent microscopy acridine orange (AO) stained thick blood smears and Quantitative Buffy Coat (QBC) (WHO, 2001). Other approaches are rapid diagnostic test based on lactate dehydrogenase (pLDH) and the detection of the histidine-rich protein 2 (HRP-II) of *P. falciparum* (WHO, 2006). However, light microscopy is the gold standard for species-specific diagnosis and quantification of parasite density following drug treatment (WHO, 2001).

2.6 Pathogenesis and Clinical Manifestations of Malaria

Clinical manifestations of malaria range from mild uncomplicated 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 proinflammatory 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). Adherence of parasitized erythrocytes to the endothelium blocks cerebral capillaries causing CM (Deitsch and Hviid, 2004). CM is characterized by a coma situation accompanied by metabolic acidosis, seizures, and hypoglycemia (Angulo and Fresno, 2002). Sequestration of parasitized erythrocytes also occurs in murine CM although in a less prominent fashion than in humans (Hearn *et al*., 2000).

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 Control of malaria

Strategies against malaria infection and disease require integrated approaches, such as vaccine development, use of chemotherapeutic agents and chemoprophylaxis and control of the mosquito vector using insecticides (Webster and Hill, 2005; Winstanley *et al*., 2004; Breman *et al*., 2006).

2.7.1 Vector control

The principal objective of vector control is the reduction of *Anopheles* breeding using insecticide and repellent application hence reducing the levels of transmission. The use of chemicals has not been successful across Africa and Latin America (Breman *et al*., 2006), due to the emergence of mosquito strains resistant to the commonly used

insecticides. Furthermore, insecticides have detrimental side effects to the environment, thus there is a pressing need to seek other alternatives.

2.7.2 Insecticide-Treated Nets (ITN)

Control has traditionally relied on reduction of the female A*nopheline mosquito* through clearing of breeding sites and use of insecticides however the vector is highly efficient and widespread (WHO, 2003). Use of nets for protection against nuisance insects started in historical times (Lengeler, 2004).

In the 1980s, Insecticide-treated nets (ITNs) were introduced greatly increasing their effectiveness (Lengeler *et al*., 1998). ITNs are a low-cost and highly effective method of reducing the incidence of malaria (D'Alessandro, 2001). They have been shown to substantially reduce child mortality in malaria-endemic areas of Africa (WHO, 2003). In sub-Saharan Africa several studies show that ITNs reduce morbidity and mortality in pregnant women (Gamble *et al*., 2007). In an area of intense malaria transmission in Kenya, the ITNs reduced parasitaemia during pregnancy by 38 % and the risk of low birth weight by 23% (Phillips-Howard *et al*., 2003).

ITNs reduce the pressure on antimalarials drugs which is important in view of the increasing drug-resistant falciparum malaria parasites (Lengeler, 2004). However, the long term use of ITNs would delay the acquisition of immunity which plays an important role against malaria infections (Gamble *et al*., 2007). In addition, though such nets provide partial protection against biting and reduces the number of infective malaria parasites in mosquitoes, disease management still relies heavily on chemotherapy (White, 2004).

2.7.3 Vaccine development

A malaria vaccine would provide a much-needed way of reducing the disease burden. However an effective vaccine has remained elusive (Webster and Hill, 2003). The diversity of parasite's antigens expressed at different stages of the life cycle and immune evasion by intrinsic antigenic variation has hindered development of a vaccine (Florens *et al*., 2002). Majority of vaccine candidates are based on a limited number of parasite antigens with no guarantee that any will give a high level of sustained protection (Webster and Hill, 2003).

Advances in genomics have aided proteomics research in identifying potential vaccine candidates such as *var* genes encoding *P. falciparum* erythrocyte membrane protein (PfEMP), and *rifin*, *stevor* and *clag* gene products given their prominent role in malarial pathogenesis (Hoffman *et al*., 2002; Webster and Hill, 2003). A promising subunit vaccine, RTS, S/AS02A based on pre-erythrocytic antigen also induces partial protection in young African children against falciparum infection and a range of clinical illness (Alonso *et al*., 2004).

Although the generation of initial vaccines will reduce the malaria burden, it will not render other control measures such as chemotherapy redundant (Moorthy *et al*., 2004). Moreover, with a safe, effective and affordable vaccine being several years away (Greenwood, 2005), chemotherapy remains the most important means of controlling malaria (Ridley, 2002; White, 2004).

2.7.4 Chemotherapy

The current available antimalarial agents can be classified according to therapeutic action against the different life cycle stages of the parasites and according to their chemical structure (Winstanley *et al*., 2004, Biagini *et al*., 2005).

The main life cycle targets are the trophozoites and schizonts in the red blood cells, the schizonts in the liver and finally the gametocytes in red blood cells (Chiang *et al*., 2006). The anti-trophozoite/schizont drugs are mefloquine, chloroquine, quinine, halofantrine, sulfadoxine, dapsone, amodiaquine, atovaquone and artemisinin (Robert *et al*., 2001). The liver schizont drugs are primaquine, lumefantrine and pyrimethamine while the anti-gametocyte drugs are chloroquine, artemisinin, amodiaquine, and quinine. Primaquine is effective against both gametocytes and hypnozoites (Vangapandu *et al*., 2006).

The chemical structure classifications are aminoquinolines, aryl-alcohols, quinoline alcohols, antifolates and artemisinins (Robert *et al*., 2001).

2.7.4.1 Aminoquinolines

2.7.4.1.1 4-Aminoquinolines

CQ and AQ are the main 4-aminoquinolines and most successful compounds for the treatment and prophylaxis of malaria. They are easily synthesized, cheap and generally well tolerated (Robert *et al*., 2001).

The 4-aminoquinolines accumulate to high concentrations within the acidic food vacuole of *Plasmodium*. The primary driving force for drug accumulation is binding to ferriproptoporphyrin IX (heme), a by-product of hemoglobin degradation (Sullivan Jr *et al*., 1996). The drug-heme complex is essential for drug activity, retains its cytotoxicity

and accumulates to a level capable of killing the parasite (Winstanley *et al*., 2004). The drug-heme complexes prevent the detoxification of heme by polymerization into malaria pigment (hemozoin). Other proposed mechanisms of action of CQ and other aminoquinolines include intercalation with parasite DNA, impairment of lysosome function, inhibition of heme-dependent parasite protein synthesis (Robert *et al*., 2001; Vangapandu *et al*., 2006).

AQ is a synthetic antimalarial compound with a pharmacokinetic properties and mode of action similar to that of CQ (Li *et al*., 2002; Warhurst *et al*., 2003). The global use of AQ has declined owing to its association with hepatotoxicity and occurrence of agranulocytosis (Biagini *et al*., 2005). However, some countries have continued to use AQ in the therapeutic management of uncomplicated malaria with no reports of severe adverse effects (WHO, 2001).

AQ is pro-drug which is rapidly metabolized in the liver to N-desethylamodiaquine (DEAQ) within 6–12 hrs. DEAQ has a higher concentration time profile and remains in the plasma for 10–14 days and is responsible for most of the antimalarial activity (Li *et al*., 2002; Mariga *et al*., 2004). AQ is more potent than chloroquine *in vitro* reflecting increased potential for complexation with heme (Winstanley *et al*., 2004). Because AQ retains a high degree of efficacy against all but the most highly chloroquine-resistant strains, there has been a recent increase in its use (Li *et al*., 2002; WHO, 2006). Although cross resistance with CQ exist, the utility of AQ in combination with other antimalarial drugs is being studied in parts of Africa (Winstanley *et al*., 2004).

AQ is still widely used as monotherapy, providing continued selection pressure for resistance and may continue to worsen despite deployment of the corresponding ACTs (WHO, 2006). In addition, the presence of low-level AQ resistance in the East Africa region provides a clear warning of the existing dangers in widespread use of AQ monotherapy.

2.7.4.1.2 8-Aminoquinolines

Primaquine and Tefanoquine are the main 8-aminoquinolines and have gametocytocidal activity against all human malarial parasite species (Vangapandu *et al*., 2006). Primaquine is mainly used against hypnozoites responsible for the relapsing forms of *P. vivax* and *P. ovale* and interferes with the mitochondrial function of *Plasmodium* (Baird, 2005). Primaquine is currently used for hepatic malarial chemoprophylaxis to eliminate *P. falciparum* at the early stage of infection (Chiang *et al*., 2006). Despite its good oral absorption, serious toxicity is a major problem in patients with glucose-6-phosphate dehydrogenase deficiency (Robert *et al*., 2001).

Tefanoquine is a primaquine analog with a longer elimination half-life of 14 days compared to 4 hours for primaquine (Chiang *et al*., 2006). Tefanoquine has a larger therapeutic index than primaquine and hence a useful molecule for chemoprophylaxis of *P. falciparum* and for prevention of relapses of *vivax* malaria (Robert *et al*., 2001).

2.7.4.2 Quinoline methanol

Quinoline antimalarials and related aryl alcohols owe their origins to quinine (Ridley, 2002). Quinine the active ingredient of cinchona bark has the longest period of effective use but there is a decrease in clinical response against *P. falciparum* in some areas (Robert *et al*., 2001). The liabilities of quinine are associated with toxicity (such as

tinnitus, nausea, and dizziness) and a three daily dosage administration over 7 days required results in poor compliance (Baird, 2005).

Mefloquine is structurally related to quinine and its long half-life of 14–21 days has probably contributed to the rapid development of resistance (Robert *et al.*, 2001). Mefloquine 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 chloroquine 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 chloroquine-resistant *P. falciparum* exists (Baird, 2005).

2.7.4.3 Antifolates

The understanding of the role of folate derivatives in humans led to identification and development of antifolates as therapeutic agents (Nzila, 2006). Antifolates attack all growing stages of the malaria parasite (Chiang *et al.*, 2006). Fully reduced folate cofactors are essential for the key one-carbon transfer reactions in nucleotide biosynthesis and metabolism of some amino-acids (Hyde, 2005a).

The type-I antifolates such as sulfones and sulfonamides like sulfadoxine and dapsone, respectively inhibit dihydropteroate synthase (DHPS). The type-II antifolates, such as pyrimethamine and proguanil inhibit dihydrofolate reductase (DHFR) (Nzila, 2006). These compounds inhibit the synthesis of tetrahydrofolate co-factors essential in the synthesis of the pyrimidine deoxythymidylate for parasitic DNA (Gregson and Plowe, 2005). The most common antifolates are sulfadoxine, pyrimethamine, dapsone and

biguanides cycloguanil and chlorcycloguanil the active metabolites of proguanil and chlorproguanil, respectively (Robert *et al.*, 2001; WHO, 2006).

The combination of DHPS and DHFR inhibitors is synergistic hence their use in combination in the treatment of malaria (Nzila, 2006). Combination of sulfadoxine-pyrimethamine is currently the only single-dose long-acting antimalarial drug that has ideal properties such as low cost, documented safety and ease of use (WHO, 2001; Robert *et al*., 2001). Unfortunately resistance to SP is widespread in high-transmission areas of Africa due to their long half life of 4 days (Winstanley *et al*., 2004; WHO, 2006).

Proguanil has been widely used for malaria prophylaxis however due to widespread drug selection pressure on the parasite and the subsequent development of resistance, its clinical response has deteriorated (Gregson and Plowe, 2005). A combination of chlorproguanil-dapsone (Lapdap) with similar mode of action as SP is more potent and more rapidly eliminated (<50hrs) hence lower selection pressure for resistance than SP (Nzila, 2006).

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).

2.7.4.4 Aryl-alcohols

Common aryl-alcohols antimalarials are lumefantrine, pyronaridine and halofantrine. Lumefantrine (LM) is a synthetic fluorene antimalarial compound also chemically related to mefloquine (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 *et al*., 1998). LM was first synthesized at the Academy of Military Medical Sciences, Beijing, China and has undergone preliminary clinical studies in China (Basco *et al*., 1998). LM 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). LM is remarkably well tolerated with generally mild–nausea, abdominal discomfort, headache and dizziness (WHO, 2006). LM is used only in combination with artemether for the treatment of uncomplicated multi-resistant falciparum malaria (Mutabingwa, 2005).

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). Halofantrine on the other hand is effective against CQ-resistant malaria but cardiotoxicity has limited its use as a therapeutic agent (Robert *et al*., 2001).

2.7.4.5 Bisquinolines

Bisquinolines are compounds with two quinoline nuclei bound by a covalent aliphatic or aromatic link (Davis *et al*., 2005). Piperaquine is a potent bisquinoline antimalarial available as piperaquine base (PQ) or as its water-soluble tetra-phosphate salt, piperaquine phosphate (PQP) (Davis *et al*., 2005). PQP was first synthesized in

1960s by Shanghai Pharmaceutical Industry Research Institute in China and Rhone Poulenc in France (Hung *et al*., 2004). In China PQ replaced CQ as the first-line treatment for *P. falciparum* malaria from 1978 until the emergence of resistance in the 1990s (Tarning *et al*., 2005). Despite its extensive use, only two pharmacokinetic studies have been published and preclinical and pharmacokinetic data on PQ are therefore limited (Hung *et al*., 2004).

PQ is active mainly on late stage trophozoites and its mechanism of action is similar to 4-aminoquinolines mainly interfering with the heme polymerization (Giao *et al*., 2004). The drug has a long half life of 17–25 days (Tarning *et al*., 2004). Because of its relatively low cost and good tolerability, PQ has enjoyed resurgence in clinical use as a coformulation with dihydroartemisinin in the product Artekin ® (Holleykin Pharmaceuticals Co. Ltd, Guangzhou, China) (Basco and Ringwald, 2003; WHO, 2006). However, the persistence of PQ at sub-therapeutic plasma concentration may drive selection of resistant parasite following re-infection especially in hyper-endemic areas (Hastings *et al*., 2002; Hung *et al*., 2004).

2.7.4.6 Artemisinin derivatives

Artemisinin was originally extracted from Qinghao plant (*Artemisia annua*; sweet wormwood), in China (Robert and Meunier, 1998). The artemisinin and its derivatives namely artesunate, artemether, arteether, artelinic acid and dihydroartemisinin are potent, well-tolerated and rapidly eliminated drugs (O'Neill and Posner, 2004). Artemisinins are all metabolized to dihydroartemisinin, the main active agent which act specifically on blood stage parasites and is effective against multi-drug-resistant *P. falciparum* strains (Ridley, 2002; Mutabingwa, 2005). Artemisinins inhibits an essential plasmodial calcium

adenosine triphosphatase 6 (*Pf*ATPase 6) (Eckstein-Ludwig *et al*., 2003). 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).

Artemisinins are appropriate choices for severe malaria but their main role in uncomplicated malaria is in combination therapy (WHO, 2001). Many public health strategies worldwide are now dependent on artemisinin group however its major disadvantage is its relatively high cost (WHO, 2003). There is no reported resistance to these compounds however, LM driven selection, *pfmdr1* Y86 N allele reduces sensitivity of *P. falciparum* to artemisinins *in vitro* (Toovey, 2006). Although, artemisinins are still highly active, their activity is limited by poor oral bioavailability, high recrudescence incidence and actions on blood stages parasites only (Chiang *et al*., 2006). They are therefore being used increasingly in combination with longer half-life drugs to reduce treatment time and increase individual compliance (Mutabingwa, 2005).

2.7.4.7 Combination therapy

Combined antimalarial therapies include old drugs in new combinations (CQ and SP), an old drug combined with a new drug (AQ and ASN), and new drugs in combination (LM and ATM) (WHO, 2006; Wiseman *et al*., 2006). Artemisinin based combination therapy has proved highly efficacious in Africa and is the therapy of choice for countries with failing monotherapies (Mutabingwa, 2005).

2.8 Drug resistance in malaria

Since the wide-scale deployment of antimalarial drugs in the 20th century, human malaria parasites have been under tremendous selection pressure to evolve mechanisms of resistance (White and Pongtavornpinyo, 2003).

Fig. 3: Distribution and epidemiology of drug resistant *P. falciparum* malaria (Wongsrichanalai *et al*., 2002).

The rapid progression of drug resistance across Africa has been the prime obstacle to management and control of malaria (Wongsrichanalai *et al*., 2002). Drug resistant *P. falciparum* was first reported in Thailand in 1961 (Anderson and Roper, 2005). To date, resistance *in vivo* has been reported against all antimalarial drugs except artemisinin and its derivatives (White, 2004).

2.8.1 Causes of resistance

Resistance can be attributed primarily to increased selection pressures on *P. falciparum* in particular, due to indiscriminate and incomplete drug use for self treatment (D'Alessandro and Buttiens, 2001). *P. falciparum* has high degree of plasticity and has developed resistance to antimalarial drugs that have been used extensively in the field (Rathod *et al*., 1997)**.** Repeated exposure of the parasite to a drug (drug pressure) is the key cause of emergence of resistance to most antimalarials (D'Alessandro and Buttiens, 2001).

Probability of emergence of resistance also depends on the level of host immunity (White, 2004), elimination half life (D'Alessandro and Buttiens, 2001), exposure of the parasite to sub-therapeutic drug levels (Yeung *et al*., 2004), increased plasticity of *P. falciparum* genomes and reduction in antimalarial susceptibility (Rathod *et al*., 1997). Young ring stages parasites and mature schizonts are resistant to most antimalarials except for the artemisinins derivatives (Hastings, 2004). Likelihood of transmission of resistant genes to the next host and the spread of resistance therefore increases (Yeung *et al*., 2004).

2.8.2 Spread of resistance

CQ and SP resistance in Africa appears to have been imported from South-East Asia (Hastings *et al*., 2002; Wongsrichanalai *et al*., 2002). *P. falciparum* from South-East Asia have been shown to have an increased propensity to develop drug resistance (Rathod *et al*., 1997). Interestingly, Africa, where most *P. falciparum* mortality occurs never developed its own CQ resistance despite wide-scale deployment of the drug (Wellems and Plowe, 2001; Trape, 2001). CQ resistance is not evenly distributed with

resistance having spread more rapidly in East than in West Africa (D'Alessandro and Buttiens, 2001; Labbe *et al*., 2003).

In Kenya, resistance to CQ was first detected in non-immune tourists in 1978 and in semi-immune Kenyans in 1982 (Wongsrichanalai *et al*., 2002). In the 1990s, the percentage of resistance increased to 85% in western Kenya (D'Alessandro and Buttiens, 2001). In 1998, SP replaced CQ for first-line treatment while AQ became the drug for second-line treatment (EANMAT, 2003).

Useful therapeutic life of SP was shorter than expected probably because of its prolonged half-life, causing a higher probability of selecting resistant strains (D'Alessandro and Buttiens, 2001). In East Africa, the current SP resistance levels are incompatible with effective malaria treatment and pose a real threat to malaria control (EANMAT, 2003). Imported drug resistant parasites may have a different antigen repertoire and thus are more virulent due to less strain specific protective immunity in the population (Bjorkman and Bhattarai, 2005).

Rapid spread of drug resistant parasites occurs through two mechanisms. First, extensive use of a drug leads to higher numbers of circulating gametocytes in the resistant infections than in the sensitive ones (White, 2002). Secondly, gametocytes carrying resistant genes produce higher densities of parasites (oocysts) in the mosquitoes than those carrying sensitive genes (Hastings, 2004). The spread of CQ-resistant parasites from Asia to Africa is enough proof that travelers spread drug-resistant parasites to different parts of the world (Anderson and Roper, 2005).

2.8.3 Genetic basis of drug resistance

Genetic events conferring antimalarial drug resistance are spontaneous, rare and are thought to be independent of the drug used (White, 2004). They are mutations in or changes in the copy number of genes encoding or relating to the drug's parasite target or influx/efflux pumps that affect intra-parasitic concentrations of the drug (Hyde, 2005b).

Research has identified the chloroquine resistance transporter (*CRT*) and the multi-drug resistance-1 (*MDR1*) transporter as key determinants to antimalarial drug resistance (Valderramos and Fidock, 2006). Although the target for the artemisinins has recently been identified (ATPase6), preliminary studies have not associated polymorphisms in the gene encoding this enzyme with reduced susceptibility to artemisinins (White, 2004). However, of particular concern is the fact that the *pfmdr1* Y86N allele reduces sensitivity to artemisinins *in vitro* (Toovey, 2006).

2.8.3.1 *crt gene*

The gene *crt* encodes a putative transporter *CRT* protein (Wellems and Plowe, 2001). The protein has Mw of 45-kDa, contains ten predicted transmembrane domains localized on the membrane of the digestive vacuole and is involved in drug flux and/or pH regulation (Valderramos and Fidock, 2006). The *CRT* transporter can also significantly influences parasite *in vitro* susceptibility to quinine, DEAQ (the primary metabolite of amodiaquine), halofantrine and artemisinin (Wongsrichanalai *et al*., 2002). Lys76Thr mutation in the *pfcrt* gene, the most common marker for CQ resistance has also been strongly associated with 4-aminoquinoline resistance such as AQ (Ochong *et al*., 2003; Holmgren *et al*., 2005). AQ is effective against some CQ-resistant strains of *P.*

falciparum (Mariga *et al*., 2004). Probably due to high absorption of AQ by some CQ-resistant parasite or mechanism of resistance is different from that of CQ resistance.

2.8.3.2 *mdr1 gene*

Multi-drug resistance 1 gene (*mdr1*) encodes a 162-kDa P-glycoprotein homologue-1(*Pgh*-1) that localizes on digestive vacuole membrane (Hyde, 2005b). The gene consists of two homologous halves, each with six predicted transmembrane domains and a conserved nucleotide binding domain (Valderramos and Fidock, 2006). In multi-drug resistance, both gene amplification and mutation of the *mdr* transporter genes may occur (Duraisingh and Cowman, 2005). *Pfmdr1* point mutations or changes in *pfmdr1* copy number might affect accumulation of drug in the digestive vacuole which could affect the *in vitro* potency of the drugs (Sidhu *et al*., 2006).

Multi-drug resistance occurs when parasites selected for resistance to one drug become resistant to a broad range of structurally unrelated drugs (Wongsrichanalai *et al*., 2002). Resistance to mefloquine and other structurally related arylaminoalcohols in *P. falciparum* results from amplifications (i.e., duplications, not mutations) in *Pfmdr1*, (Duraisingh and Cowman, 2005). *Pfmdr1* amplification has also been associated with an increased risk of failure of short term LM/ATM treatment (Sisowath *et al*., 2005). Significant selection for the *pfmdr1* Y86N polymorphism suggests that this mutation might be useful as a molecular marker of LM resistance (Sisowath *et al*., 2005). It is therefore important to monitor the efficacy of LM especially in high-transmission areas where drug selection pressure may be the greatest and where LM/ATM has been extensively used.

2.8.3.3 Dihydrofolate reductase (DHFR) mutations

P. falciparum resistance to antifols (pyrimethamine and cycloguanil) results from the sequential acquisition of mutations in DHFR (White, 2004). Each mutation confers a stepwise reduction in susceptibility. Four point mutations in DHFR underlie resistance to pyrimethamine (Hastings, 2004). A single point mutation at DHFR Ser108Asn causes pyrimethamine resistance with only moderate loss of susceptibility to chlorcycloguanil (Gregson and Plowe, 2005). The addition of Asn51Ile and/or Cys59Arg mutations confers higher levels resistance to pyrimethamine (Hastings, 2004). High-level resistance to both drugs is conferred by addition of fourth Ile164Leu mutation (White, 2004; Plowe, 2003).

2.8.3.4 Dihydropteroate synthase (DHPS) mutations

The *dhps* gene encodes the sulfonamides and sulfones target enzyme DHPS (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). Four point mutations are involved in DHPS and as with DHFR appear sequentially following introduction of treatment with SP (Anderson and Roper, 2005). DHPS mutations associated with decreased susceptibility to sulfonamides and sulfones include Ser436Ala, Ala437Gly, Ala581Gly, and Ser436Phe coupled with Ala613Thr/Ser (Plowe, 2003).

2.8.4 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. WHO recommends first, the use of ACTs which are highly efficacious and promises to delay the emergence

of resistance where monotherapies are failing, (WHO, 2003). Secondly, use of molecular markers to predict emergence of resistance.

2.8.4.1 Artemisinin based combination therapies (ACTs)

ACTs comprise an artemisinin derivative with very short elimination half-life and long elimination half-life partner drug (WHO, 2006). The rationale is that, if two drugs have independent mechanisms of action, then mutations that confer resistance to each drug will only seldom co-exist in the same parasite (White, 2004).

So far only few countries have changed their first-line treatment to ACTs due to high costs and limited operational experience in Africa (WHO, 2003). African countries with the bulk of malaria mortality and morbidity are the least able to afford effective but expensive ACT (Watkins *et al*., 2005, WHO, 2006). Among the ACTs available are Dihydroartemisinin-piperaquine (DHA/PQ or Artekin®), Lumefantrine-Artemether (LM/ATM) or Co-Artem®), Artesunate-amodiaquine and artesunate-sulfadoxine/pyrimethamine, Artesunate-mefloquine (Mutabingwa, 2005; WHO, 2006).

LM/ATM is among the fixed-ratio combination therapy currently available. An advantage of this combination is that lumefantrine has never been used as a monotherapy for the treatment of malaria (Mutabingwa, 2005). LM/ATM has been intensively studied and successfully used (Alin *et al*., 1999). ATM significantly reduces initial parasite biomass with parasites surviving ATM action facing maximal LM concentrations therefore reducing transmissibility of parasites (Watkins *et al*., 2005). By this logic, most ACTs should improve both treatment cure rate and delay the emergence of drug resistance (Watkins *et al*., 2005). 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).

Recently, a co-formulated product of PQ and DHA has been shown to be safe, effective and acceptable in clinical trials (Hien *et al*., 2004). The cost PQ/DHA is half that of LM/ATM in Cambodia, China and Vietnam where it has been used extensively (Yeung *et al*., 2004). Although yet to be internationally approved its low price and high efficacy are very promising to developing countries (Giao *et al*., 2004; Yeung *et al*., 2004). The use of PQ/DHA is likely to increase in future, driving the selection pressure for PQ resistance.

2.8.4.2 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 reemergence and predominance of CQ-sensitive *P. falciparum* (Plowe, 2003). Monitoring the emergence and development of resistance is therefore 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 by antimalarial drug treatment (WHO, 2001). However, only few molecular markers of resistance are available namely SP, cycloguanil and CQ (Plowe,

2003). Markers for resistance to most other antimalarial drugs are lacking because molecular mechanisms of resistance are not yet clearly understood.

2.8.5 Selection of drug resistant parasites

Most antimalarial drugs inhibit the multiplication of sensitive malarial parasites but allow the drug-resistant mutants to survive and multiply (White, 2004). Drug resistant mutants can be selected without mosquito passages by exposing of large numbers of malaria parasites (*in vitro*, in animals models) to sub-therapeutic antimalarial drug concentrations (Peters, 1999; White, 2004). Resistance to one drug may also be selected for by another drug in which the mechanism of resistance is similar, a phenomenon known as cross-resistance (Hyde, 2005b). Drug pressure or continuous exposure of parasite to drugs especially long half-life drugs present a strong selective pressure for resistant strain (D'Alessandro and Buttiens, 2001; Hastings *et al*., 2002).

In-vivo models of rodent malaria offer the most practical and informative means of evaluating the activity and potential resistance hazard of novel antimalarials and antimalarial combinations (Stewart *et al*., 2004). Most widely used procedures to select for resistance are the `serial technique' (ST), in which drug selection pressure *in vivo* is gradually increased, and the `2% relapse technique' (2%RT), in which a single, large drug dose is applied at the time of each passage (Peters, 1999).

Early work on resistance to CQ was carried out with *P. berghei* RC. The level of drug resistance was evaluated by calculating the ED_{50}, (i.e) the dose of CQ that reduces parasitaemia by 50% after 4-DT (Peters *et al*., 1975). After an average of 60 passages of the parasite, the daily doses of CQ reached 100 mg/kg and the ED_{50} of the resistant strain was 60 times higher than that of the original strain (Coquelin *et al*., 1997; Peters, 1999).

In other studies, stable resistance to SP was also selected in *P. chabaudi* by single-step treatment in a clone that was already resistant to pyrimethamine (Hayton *et al*., 2002).

Drug resistant rodent malaria parasites have successfully been used in predicting mechanism of resistance of different antimalarials. *P. chabaudi* has been used to investigate the genetics of mefloquine resistance and SP resistance (Hayton *et al*., 2002; Cravo *et al*., 2003). Molecular analysis of these pyrimethamine resistant rodent parasite clones has shown that the mechanisms of pyrimethamine resistance are remarkably similar to those seen in *P. falciparum* (Walliker, 2005). In *P. berghei* and *P. yoelii*, atovaquone-resistant parasites have been selected in which point mutations in the gene encoding mitochondrial cytochrome b have been implicated (Carlton *et al*., 2001).

2.9 Hypothesis

LM, PQ and AQ selection pressure induces stable resistant *Plasmodium berghei* ANKA lines *in vivo*.

2.10 Objectives of the study

General objective

To induce and select *Plasmodium berghei* ANKA lines resistant to LM, PQ and AQ *in vivo.*

Specific objectives

1. To determine the effective doses (ED_{50}, ED_{90} and ED_{99}) of LM, PQ and AQ on *Plasmodium berghei* ANKA strain.

2. To select *Plasmodium berghei* ANKA line 'resistant' to LM, PQ and AQ

3. To determine the stability of the 'resistant' lines.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

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

3.2 Parasites

Transgenic ANKA strain of *P. berghei* expressing Green Fluorescent Protein (GFP) (*P. berghei* ANKA GFP) obtained from Swiss Tropical Institute courtesy of Dr S. Wittlin was used to select PQ resistant strains. Transgenic ANKA strain of *P. berghei* expressing fusion protein GFP-Luciferase (*P. berghei* ANKA GFP-Luciferase, reference line: 676m1cl1 (Janse *et al*., 2006) obtained from Leiden University Medical Center, Netherlands was used to select AQ and LM resistant lines.

Transgenic *P. berghei* lines that express fluorescent markers have been used for visualization and counting of parasites. GFP 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, provides a means to both observe transgenic parasites *in vivo* and to select for revertants that have lost the selectable marker (de Koning Ward *et al*., 2000).

3.3 Test compounds

Lumefantrine, Piperaquine phosphate and Amodiaquine dihydrochloride as the test drugs were obtained from Dr Alexis Nzila's Lab at KEMRI-Wellcome Trust, Kilifi, Kenya.

3.4 Handling of laboratory animals

Male, Swiss albino mice weighing 20±2g random-bred at KEMRI, Nairobi, Kenya were used in the study. The animals were housed in experimental room in the animal house in a standard Marrolon type II cages clearly labeled with experimental details. The mice were maintained in animal room at 22 ^{0}C and 60-70% relative humidity and fed on commercial rodent food and water *ad libitum*.

The needle size for cardiac puncture and intra-peritoneal infection was 26G×5/8″. After the experiment, mice were sacrificed by euthanizing in chloroform. 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 during incineration.

The study, SSC No: 1208 was approved on 27th February 2007 during 141st meeting of KEMRI/National Ethical Review Committee

3.5 Preparation of inoculum

Parasitized red blood cells (PRBC) were collected from donor mouse with a rising parasitaemia of 5-10% and according to the level of parasitaemia blood diluted with phosphate saline glucose (PSG) buffer (appendix 1) to reach approximately 2×10^7 PRBC per 0.2 ml of the inoculum.

3.6 Preparation of drug solutions

On the day of administration, each drug was freshly prepared by solubilizing in solution consisting 70% Tween-80 (d=1.08g/ml) and 30% ethanol (d=0.81g/ml) and diluted 10 fold with double distilled water.

3.7 Determination of 50% and 99% effective doses (ED_{50} and ED_{99})

The assay protocol was based on a 4-day suppressive test described by Peters *et al.*, 1975. For each test drug, 25 male Swiss albino mice with 5 mice per dose group in four different doses and 5 mice in the control group were intraperitoneally inoculated each with 2×10^7 PRBC in 0.2 ml inoculum on day zero (D_0). Drugs were administered orally (p.o) at 4 hrs, 24 hrs, 48 hrs and 72 hrs post infection. Thin blood films were prepared from tail snips on day four (D_4) post infection, fixed in methanol and stained for 20 min with freshly prepared 10% (wt/vol) Giemsa solution. Percentage (%) chemosuppression (parasite reduction) of each dose of each drug was calculated using the formula: (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. A dose response line was drawn and the ED_{50} and ED_{99} estimated from the line.

3.8 Drug pressure Assay

Ten Swiss albino mice for each of the test drug (20±2g) were intraperitoneally infected each with donor blood containing $2x10^7$ *P. berghei* ANKA PRBC contained in 0.2 ml inoculum on day zero (D_0). The randomly infected mice were then divided into a test and control group with five mice each. Parasitaemia was monitored until it rose to about 2-7 % after which test group mice were then treated with the drug at concentration equivalent to the drug ED_{99}. After treatment, parasitaemia was monitored to about 2-7 % when mouse was selected for donation of PRBC to the next naive group of 10 mice. The second test group mice were then treated with the same amount of the drug equivalent to

the drug ED_{99}. Level of resistance was initially assessed after every four passages (approximately 1 month) and thereafter after every eight passages (approximately 2 months) of drug pressure assay.

Stepwise increase of drug pressure dose was adopted after three months of drug selection pressure for both AQ and LM assays but from the first passage for PQ assay.

3.9 Cryopreservation of *P. berghei* ANKA PRBC

At each passage, 0.5-0.6 ml of *P. berghei* ANKA PRBC in 0.05 ml of heparin was collected by cardiac-puncture from a test group mouse with a parasitaemia of 2-7%. The blood was mixed with 0.5-0.6 ml of a glycerol/PSG solution (20% glycerol; v/v), the suspension was transferred to 2 cryotubes, 0.5ml per tube. The tubes were left for 5-15min at 4°C and the vials were then stored at -80ºC.

3.10 Assessing the level of resistance

Resistance is the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of an antimalarial drug in the dose normally recommended (WHO, 2006).

Level of resistance was assessed by determining new ED_{50} and ED_{99} based on a 4-day suppressive test described by Peters *et al*., 1975 (Section 3.7) after every 1 month and thereafter after every 2 months of drug pressure assay and calculating 50% (I_{50}) and 99% (I_{99}) indices of resistance.

I_{50} was calculated from the ratio of ED_{50} of resistant line to that of parent strain while I_{99} was calculated from the ratio of ED_{99} of resistant line to that of parent strain (Xiao *et al*., 2004).

3.11 Stability of resistance in absence of drug

The stability of resistant lines was assessed when I_{50} and I_{99} of 10 and above was recorded.

PQ resistant line from the 12^{th} passage were examined for their drug response by five serial passages without the drug pressure followed by determination of the ED_{50} and ED_{90} based on the Peters 4-Day Suppressive Test (Peters *et al*., 1975) (Section 3.7).

3.12 Statistical analysis

The data for the 4-day suppressive test were recorded in 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_{99} in mg/kg.day. I_{50} and I_{99} were calculated and resistance grouped into four categories namely I_{99}=1.0, sensitive, (2) I_{99}=1.01-10.0, slight resistance, (3) I_{99}=10.01-100.0, moderate resistance and (4) I_{99}= >100.0, high resistance (Merkli and Richle, 1980).

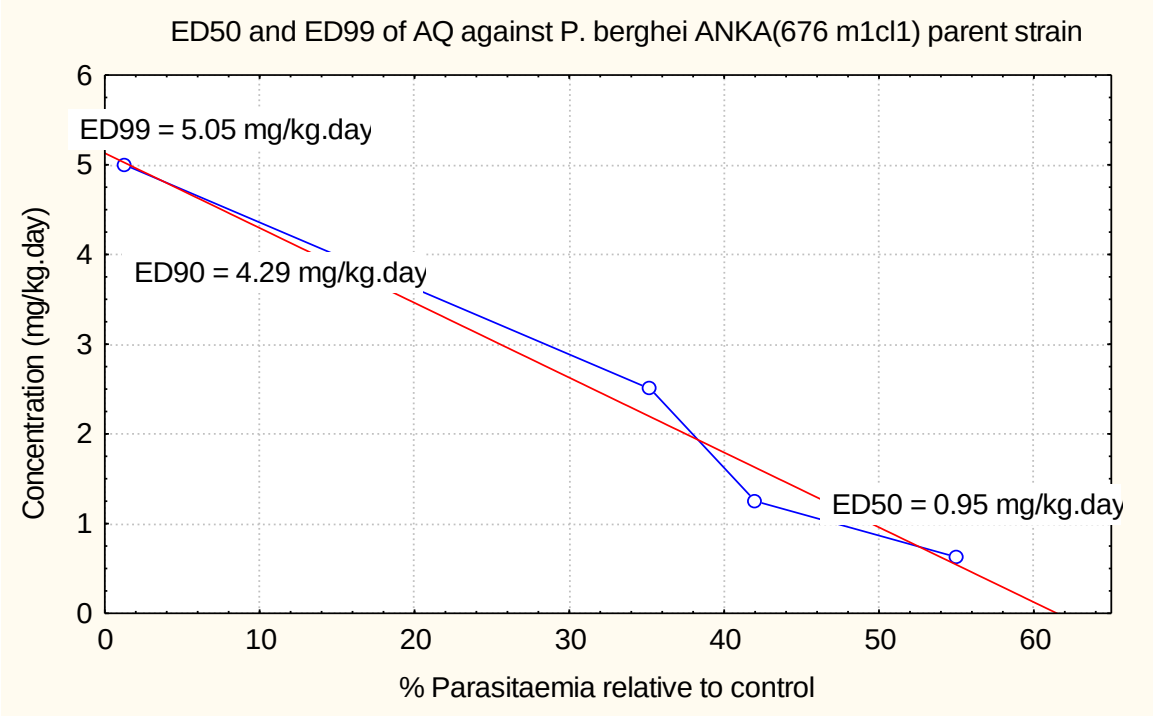
CHAPTER FOUR: RESULTS

4.1 AMODIAQUINE

4.1.1 ED_{50} and ED_{99} of parent strain

The drug concentration that produces a 50% reduction in parasitaemia *in vivo* (ED_{50}), typically in rodent malaria model of AQ against parent *P. berghei* ANKA strain was 0.95mg/kg.day. The ED_{99} of AQ was 5.05mg/kg.day (Fig. 4, Table 7).

Fig. 4: Graph showing activity of AQ against parent strain of *P. berghei* ANKA (reference line 676m1cl1).



4.1.2 Interval between passages

When mice infected with *P. berghei* ANKA reference line 676m1cl1 and treated orally with 5.05 mg/kg of AQ on day 3 (D_3) attained a 2-7 % parasitaemia within 9 days post infection. Collection of parasitized red blood cells for subsequent passage was carried out on day 9 (D_9) for the first 12th passages, however from 13th passage to the 20th

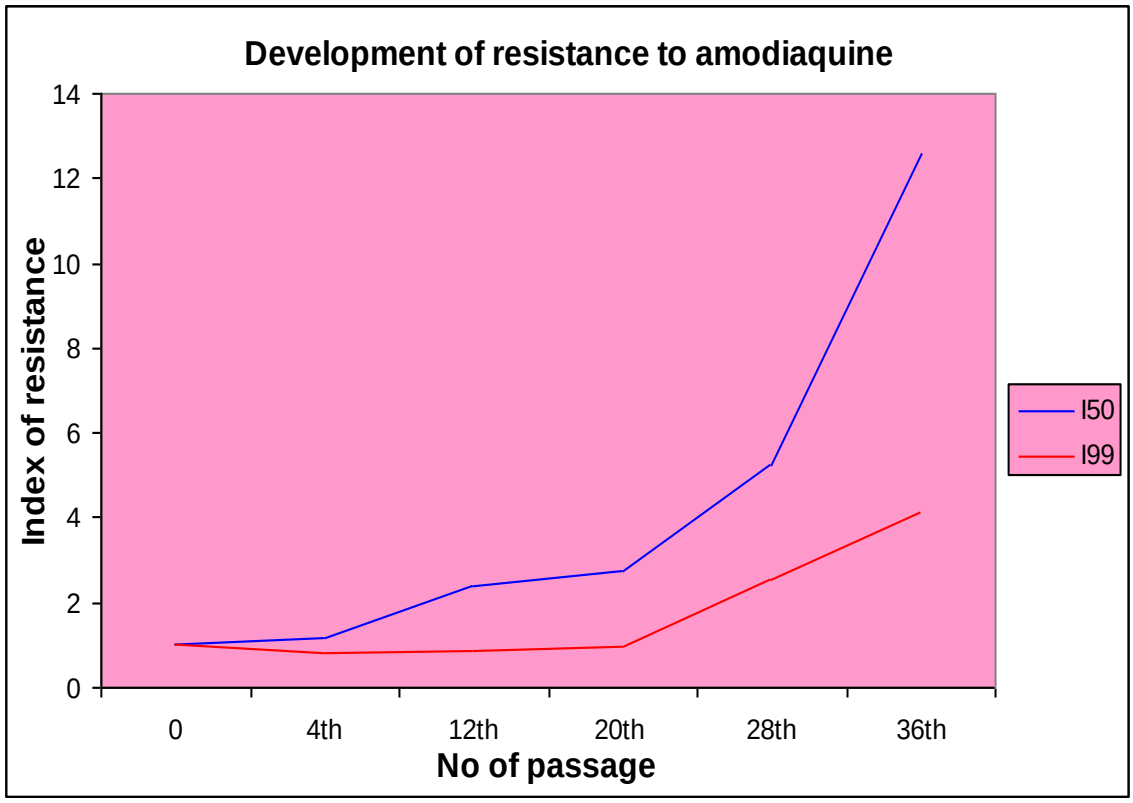
passage parasitaemia reached 2-7 % in within 6 days post infection. The intervals between subsequent passages were therefore shortened to 6 days up to the 20th passage. From the 21st passage and subsequent passages, the drug pressure dose was increased by 5.05 mg/kg depending on the growth of the parasites. After the first increment of the dose to 10.10mg/kg.day, parasitaemia reached 2-7 % in within 9-10 days post infection. The interval between subsequent passages of the *P. berghei* ANKA parasitized red blood cells was every 9 days throughout the experiment. In this experiment *P. berghei* ANKA was passed for a total of 36 passages.

4.1.3 Acquisition of resistance

Table 1: Summary of the ED_{50}, ED_{99} (mg/kg.day) and I_{50} and I_{99} values of AQ measured in the 4-day test (Peter *et al.*, 1975).

Passage No	Amodiaquine			
	ED_{50}	I_{50}	ED_{99}	I_{99}
Parent line	0.95	1.00	5.05	1.00
4th	1.09	1.15	4.04	0.80
12th	2.26	2.38	4.51	0.89
20th	2.63	2.77	5.00	0.99
28th	5.00	5.26	13.00	2.57
36th	12.01	12.64	20.73	4.10

Fig. 5: Graph showing increase in indices of resistance I_{50} and I_{99} in development of resistance to amodiaquine.

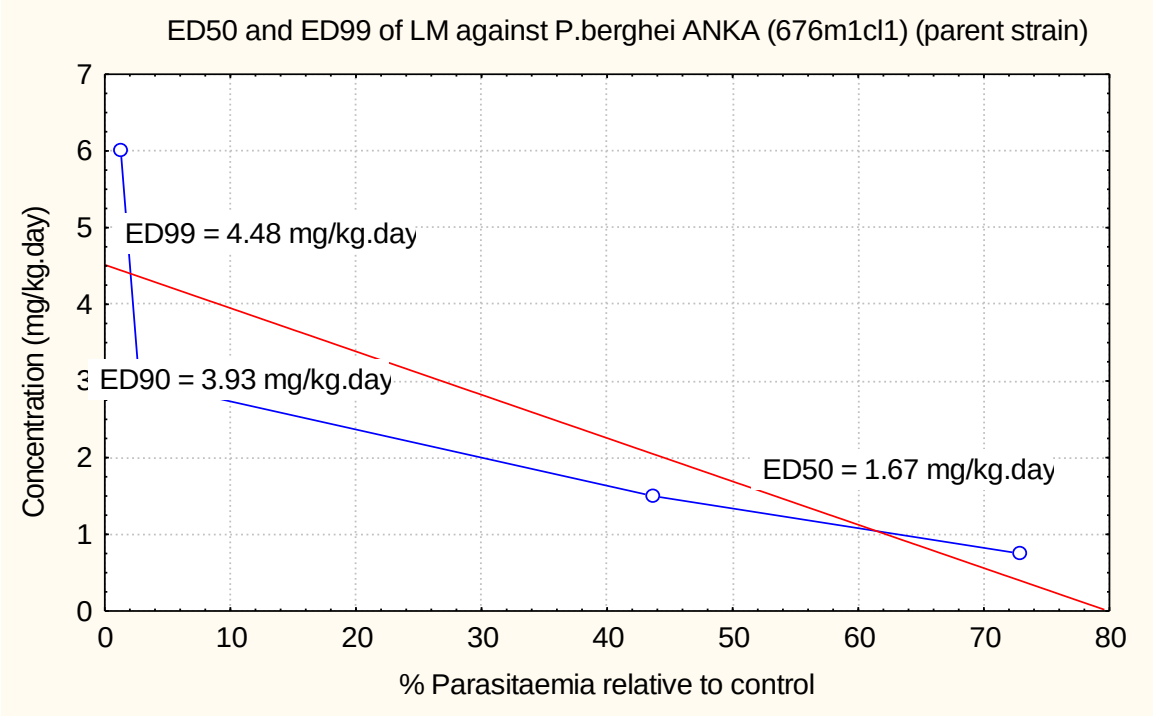


4.2 LUMEFANTRINE

4.2.1 ED_{50} and ED_{99} of parent strain

The ED_{50} and ED_{99} of LM against parent *P. berghei* ANKA strain (reference line: 676m1cl1) was 1.67mg/kg.day and 4.48mg/kg.day respectively (Fig. 6).

Fig. 6: Graph showing activity of LM against parent strain of *P. berghei* ANKA (reference line 676m1cl1).



4.2.2 Interval between passages

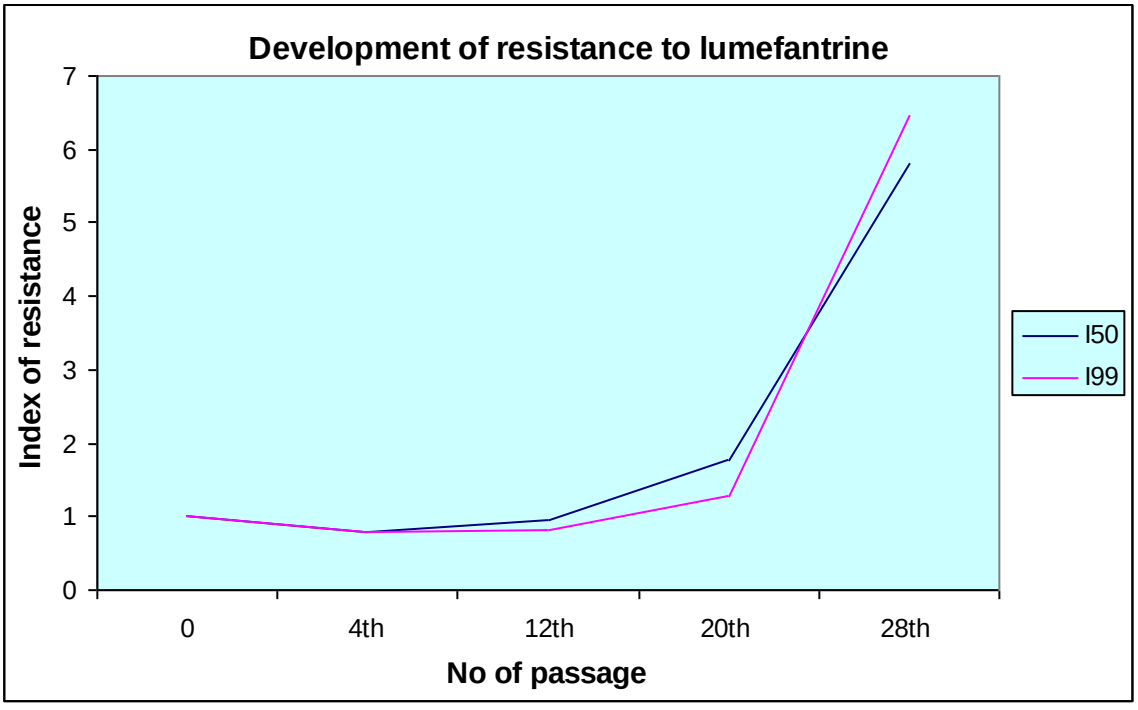
The mice infected with *P. berghei* ANKA reference line 676m1cl1 and treated orally with 4.48mg/kg of LM on day 3 (D_3) attained a 2-7 % parasitaemia within 10 days (D_{10}) post infection. Parasitized red blood cells were therefore passed once every 10 days. From the 12^{th} passage and subsequent passages, the drug pressure dose was increased by 25% of the 4.48mg/kg depending on the growth of the parasites, consequently the PRBC were passed every 10-11 days throughout the experiment. In this experiment, *P. berghei* ANKA was passed for a total of 28 passages.

4.2.3 Acquisition of resistance

Table 2: Summary of the ED_{50}, ED_{99} (mg/kg.day) and I_{50} and I_{99} values of LM measured in the 4-day test (Peter *et al.*, 1975).

Passage No	Lumefantrine			
	ED_{50}	I_{50}	ED_{99}	I_{99}
Parent line	1.67	1.00	4.48	1.00
4^{th}	1.34	0.80	3.49	0.78
12^{th}	1.58	0.95	3.61	0.81
20^{th}	2.96	1.77	5.75	1.28
28^{th}	9.76	5.84	29.02	6.48

Fig. 7: Graph showing increase in indices of resistance I_{50} and I_{99} in development of resistance to lumefantrine.



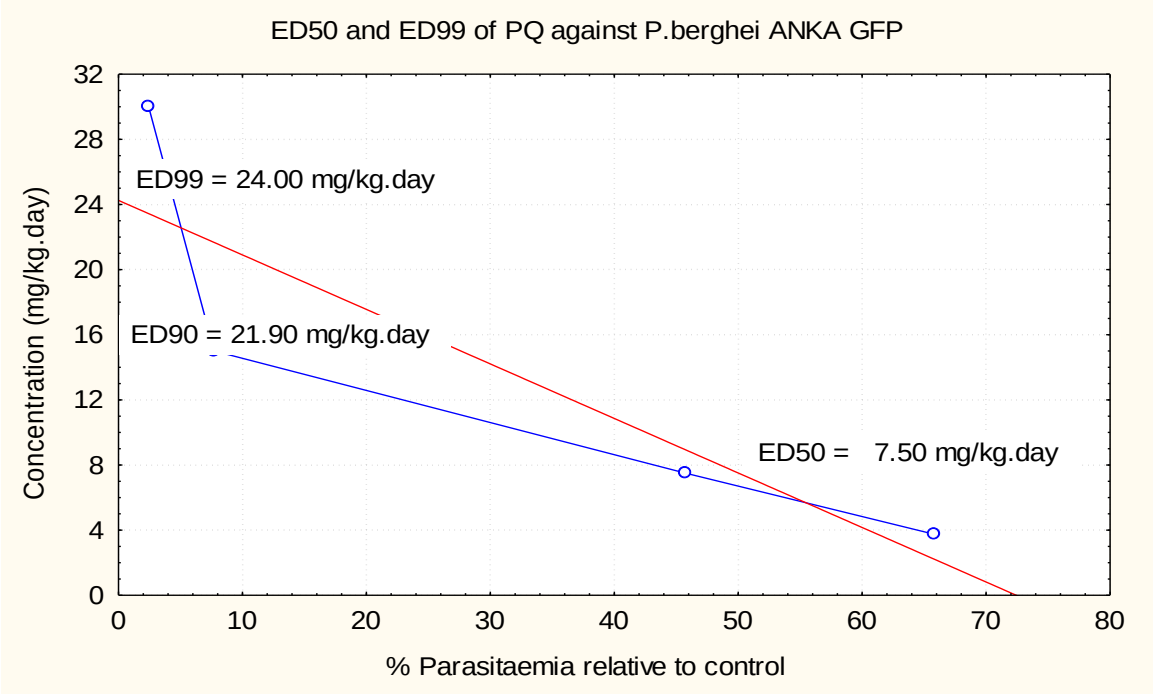
4.3 PIPERAQUINE

4.3.1 ED_{50} and ED_{99} of parent strain

Selection of PQ resistance was a follow up study initially carried out at Swiss Tropical Institute (STI) by Beatrice Irungu. In studies carried out at STI, the ED_{50} and ED_{99} of piperaquine against parent *P. berghei* ANKA GFP was 1.30mg/kg.day and 8.10mg.kg.day respectively. The parasites were exposed to 8.10mg.kg.day and stepwise increase of the dose by 8.10mg/kg.day at each passage. A 123 folds increase in I_{50} (ED_{50} of 160.20 mg/kg.day) was obtained within fifteen passages of drug pressure. The isolate was cryopreserved for 6 month.

The current study revived PQ resistant *P. berghei* ANKA GFP and consequently the ED_{50} and ED_{99} was determined and found to be 7.50mg/kg.day and 24.00mg/kg.day respectively (Fig. 8).

Fig. 8: Graph showing activity of PQ against revived *P. berghei* ANKA GFP before further exposure to selection pressure.



4.3.2 Interval between passages

Mice infected with *P. berghei* ANKA GFP and treated orally with 48.6mg/kg (6× ED_{99}) of PQ and attained a parasitaemia of 2-7 % within 7 days (D_7) post infection. 48.6mg/kg was the highest dose administered at STI before the cryopreservation of the parasites. Parasitized red blood cells were therefore passed once every 7 days. From the 1^{st} passage and subsequent passages, the drug pressure dose was increased by 8.1mg/kg (ED_{99}) depending on the growth of the parasites. The interval between subsequent passages of PRBC was once every 7 days throughout the experiment. In this experiment *P. berghei* ANKA GFP was passed 12 times and in total, 28 passages including the study at STI of drug selection pressure.

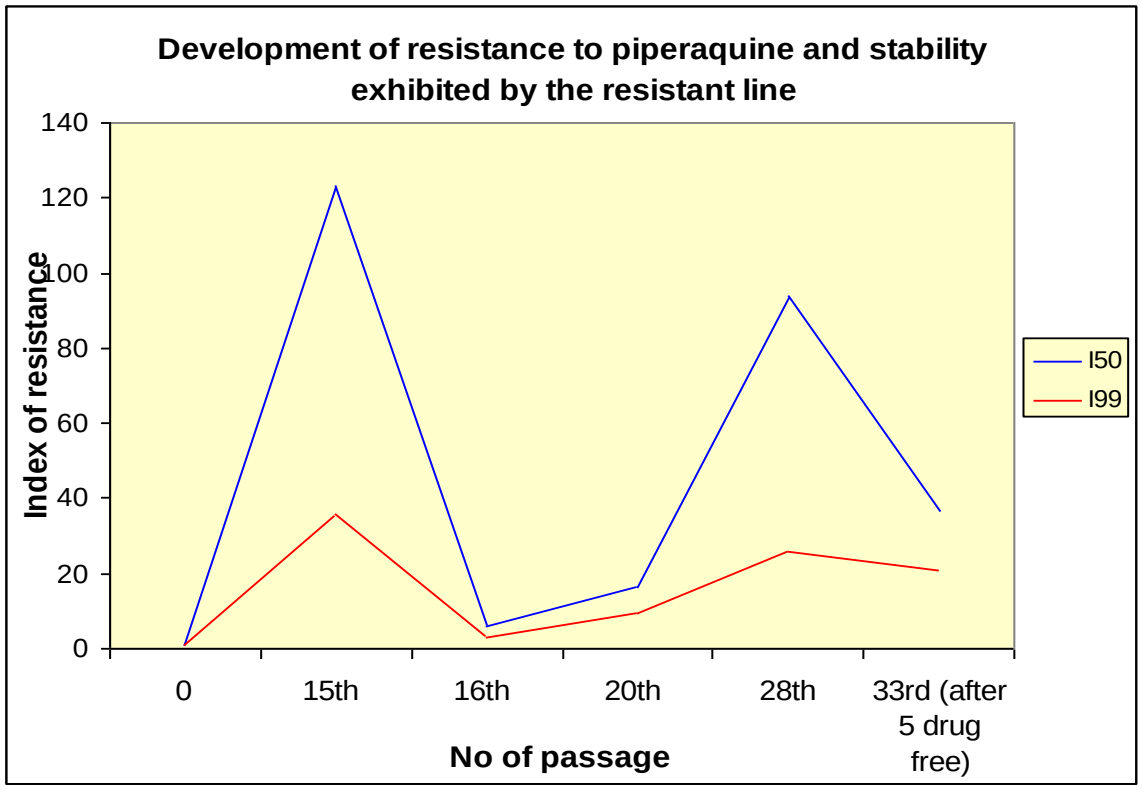
4.3.3 Acquisition of resistance and assessment of stability

After revival from cryopreservation and further exposure of *P. berghei* ANKA GFP to 12 passages of selection pressure, I_{50} and I_{99} of 93.85 and 25.93 respectively were recorded. The PQ resistant line was then passaged five times without the drug pressure and I_{50} and I_{99} of 36.23 and 20.85 respectively were recorded (Table 3, Fig. 9).

Table 3: Summary of the ED_{50}, ED_{99} (mg/kg.day) and I_{50} and I_{99} values of PQ measured in the 4-day test (Peter *et al.*, 1975).

Passage No	Piperaquine			
	ED_{50}	I_{50}	ED_{99}	I_{99}
Parent strain	1.30	1.00	8.10	1.00
15^{th} passage	160.28	123.29	289.64	35.76
PQ resistant line revived after six months of cryopreservation (16^{th} passage)	7.50	5.77	24.00	2.96
20^{th}	21.40	16.46	74.50	9.20
28^{th}	122.00	93.85	210.00	25.93
After removal of drug pressure				
5	47.10	36.23	168.90	20.85

Fig. 9: Graph showing increase in indices of resistance I_{50} and I_{99} in development of resistance to piperaquine and relative stability exhibited by piperaquine resistant line after five drug free passages.



CHAPTER FIVE: DISCUSSION

The complex genome of plasmodium parasites has endowed them with the ability to survive the adverse environments of nearly all antimalarials (Hyde, 2005b). In this phase of increasing antimalarials resistance, old drug such as AQ have been resurrected while new drugs such as LM and PQ are being introduced. LM, PQ and AQ are potent partners in ACTs, which have been proposed to be the novel strategy against drug resistant malaria parasites.

The experimental work presented here focused on selection of AQ, LM and PQ resistant lines. Moderate PQ resistance with an I_{99} of 25.93 was induced while slight resistance against AQ with an I_{99} of 4.10 and LM with an I_{99} of 6.48 were selected (Table1, 2 and 3).

5.1 Development of amodiaquine resistance

Our observations derived from these studies indicate that administration of 5.05 mg/kg at each passage resulted to slow development of resistance with an I_{50} of only 2.77 after 20 passages. This is consistent with other results from selection of AQ resistant *P. berghei* N line in 2% RT method where slow emergence of resistance was also recorded (Peters and Robinson, 1992). It would appear that emergence of AQ resistance is slow regardless of the method or the dose used to induce the resistance. The rate of acquisition of *P. berghei* ANKA resistance to AQ when exposed to 5.05 mg/kg also seemed unstable over the first twelve passages (Table 1, Fig. 5). There was a reduction in the ED_{99} and an increase in ED_{50} after 4^{th} to 12^{th} passages of drug pressure. This is comparable to results from selection of CQ-resistant *P. c. chabaudi* (Coquelin *et al*., 1997).

The gradual increase in the ED_{50} and consequently slight increase in I_{50} recorded at 20th passage probably resulted in test group mice attaining 2-7% parasitaemia in 6 days hence the shortened interval between passages recorded in the 13^{th} to 20^{th} passage. However, quantification of an I_{50} of only 2.77 after 20^{th} passage shows that the parasites were only less susceptible but not resistant to the 5.05 mg/kg (Fig.5). The results conform to other studies that administration of a constant dose at every passage results to slow emergence of resistance as compared with stepwise increase of the dosage at every passage (Xiao *et al*., 2004). Evidence has also been presented that resistance to single compounds may emerge more rapidly when a high dose is employed in the 2% RT than a lower dose (Peters and Robinson, 2000). Though the 2% RT method was not adopted in the study, 5.05 mg/kg of AQ seems too low a dose for inducing resistance. Studies indicate that exposing parasites to low doses might select for parasites with increased expression of genes as opposed to selecting parasites with functional mutations (Carlton *et al*., 2001). Probably administration of 5.05 mg/kg resulted only in increased expression of certain genes but not induction of functional mutation. It may also imply that conversion of AQ into other active metabolites serves to delay emergence of resistance and several genetic events are involved in its resistance.

From the 20^{th} passage the stepwise increase of the drug selection dose was adopted and by 28^{th} passage the parasites tolerated up to 40.40 mg/kg and a significant increase in resistance was observed, an I_{50} of 5.26. A higher index of resistance might have been expected since the parasites tolerated 40.40 mg/kg but studies also indicate that a strain tolerating a higher dose might only show a small index of resistance in 4-DT (Peters and Robinson, 1999). This is reminiscent to other studies where a *P. berghei* line

highly resistant to artemisinin tolerated up to 700 mg/kg after five passages but I_{90} never rose above 10 (Peters and Robinson, 1999). When the parasites develop inside the red blood cells it becomes vulnerable to the drug (Coquelin *et al*., 1997). This is probably the reason why the 4-DT gives a low index of resistance since most parasites are eliminated within the experimental period. Observed results indicate that stepwise increase of drug pressure dose eliminated the drug susceptible parasites and only the resistant parasites were selected between the 20^{th} and 28^{th} passage.

The confirmation of increased AQ selection pressure was clearly shown by the drastic increase in the I_{50} to 12.64 at 36^{th} passage however the I_{99} seems not to increase by a big margin, with only 4.10 recorded at the same passage (Table 1, Fig. 5). This increase confirms the ease of maintaining and increasing AQ resistance once the initial probable physiological adaptations or genetic changes occurs. The observed results emphasize importance of monitoring AQ resistance in areas where the drug is in use.

5.2 Development of lumefantrine resistance

Lumefantrine is one of the newest antimalarial drugs that has not been used extensively in malarias areas (Mutabingwa, 2005). So far no genuine LM resistance has been documented in *P. falciparum*. However, significant differences in levels of *in vitro* and *in vivo* sensitivity have been documented (Basco *et al*., 1998; Sisowath *et al*., 2005; Dokomajilar *et al*., 2006).

From this study, *P. berghei* ANKA resistance to LM proved difficult to induce. Minimal difference between the use of single dose (4.48 mg/kg) and stepwise increase of drug pressure dose was seen in the rate at which the parasites became resistant to LM. In the first 12 passages, the rate of acquisition of *P. berghei* ANKA resistance to LM was

considerably slow, I_{99} of 0.81 (Table 2, Fig. 7), virtually no resistance, the parasites were still in the sensitive level (1.00-1.01). Unexpected trend observed in the first 12 passages was the reduction in both ED_{50} and ED_{99}, the parasite seems to have been more sensitive to the drug. Probably the initial alterations in proteins expression involved in uptake or influx of the drug occurred resulting increased absorption of the drug. Resistance to LM therefore proved very difficult to induce in *P. berghei* ANKA by administration of single dose (4.48 mg/kg) at each passage.

The increase in the I_{99} to 1.28 at 20^{th} passage indicates that stepwise increase of drug pressure dose increased selection pressure (Fig. 7). Although the increased drug pressure dose eliminated more sensitive parasites, the parasites were exposed to a higher drug concentration, a determining factor in selection of resistance resulting in the slight increase in resistance index observed at 20^{th} passage. The observed resistance (I_{99} of 1.28) was however too low to allow any conclusion to be made on possible breakthrough on selection of LM resistant *P. berghei* ANKA. Probably several initial genetic events are involved in LM resistance.

The trend seemed to change as I_{50} and I_{99} of 5.84 and 6.48 respectively was recorded at 28^{th} passage. Probably the increased selection pressure resulted in induction of functional mutation. Contrary to selection of AQ resistance, the I_{99} seemed to increase rapidly than I_{50} (Fig. 5 and Fig. 7). The increment of the LM selection pressure probably resulted to amplification of certain genes or fixation of intrinsic (genetic) mutation faster than the AQ selection pressure. The emergence of resistance is considered in two parts namely the first initial genetic event which produces the resistant mutant and secondly survival advantage that sustains selected resistant mutants in presence of the drug (White

and Pongtavornpinyo, 2003). Initial genetic events leading to LM resistance seems difficult to induce compared AQ and PQ resistance.

Although the selection process seems slow and very promising in the fight against malaria, persistent parasitaemia in malaria endemic areas will increase the risk of LM resistance. The fact that LM has not been used extensively as a monotherapy however gives hope to the recently introduced ACT (LM/ATM). The strong argument for deployment of LM in combination with ATM may only delay the selection for resistance but not avoid emergence of resistance to LM. However, their success is still not clear owing to the documented selection of *pfmdr1*Y86N mutation as a potential marker of LM resistance *in vivo*. The fact that the parasites tolerating residues LM have mutated *pfmdr1* gene (Y86N allele) (Sisowath *et al*., 2005; Dokomajilar *et al*., 2006) suggest that the selected LM resistant phenotypes in this study (an I_{99} of 6.48) may probably have amplified or mutated *pbmdr1*gene. LM is therefore not immune from being selected for resistance. Hence, monitoring LM resistance in areas where the drug is currently being used is therefore essential.

5.3 Development of piperaquine resistance

The study shows that PQ resistance in *P. berghei* ANKA GFP can be selected relatively easily. An I_{50} and I_{99} of 16.46 and 9.20 respectively (Table 3, Fig. 9) recorded within four passages of selection pressure after revival of *P. berghei* ANKA GFP imply rapid selection of resistance compared to AQ and LM. The potential for PQ resistance was shown in China when piperaquine was used widely as a monotherapy and in mass prophylaxis against CQ-resistant *P. falciparum* (Davis *et al*., 2005). Probably resistance developed rapidly since the *P. falciparum* were resistant to CQ and due to the similarity

in the mode of action of PQ to other quinoline antimalarials. However, from this study, resistance to PQ was more pronounced signifying that even drug naive parasites can select PQ resistance rapidly as long as the drug selection pressure is maintained. Other investigators foresaw a similar problem with PQ when they succeeded in selecting a line of *P. berghei* highly resistant to this compound (Li, 1985; Li *et al*., 1985).

Within 12 passages of selection pressure, I_{50} and I_{99} of 93.85 and 25.93 respectively were recorded (Table 3, Fig. 9) confirming the ease with which PQ resistance can be maintained. Since the number of parasites exposed to the selection pressure dosage determines the rate of emergence of resistance (White and Pongtavornpinyo, 2003), higher numbers of recrudescing parasites were exposed to the trailing sub-therapeutic levels of PQ in the blood resulting to rapid acquisition of mutations that decreased the parasite sensitivity to PQ within a shorter period of 90 days. Probably mechanisms allowing frequent genetic recombination exist which resulted in rapid emergence of PQ resistance. It may also imply that only few genetic events are involved in emergence of PQ resistance.

Although it is speculative to extrapolate resistance across species and hosts, increased use of PQ will increase selection pressure of *P. falciparum* resistance to this drug. Studies carried out in Cameroon suggest that PQ is highly and equally active against the CQ-sensitive and the CQ-resistant clinical isolates of *P. falciparum* (Basco and Ringward, 2003). However, PQ-resistant line of *P. berghei* obtained by serial passage under drug pressure in a rodent malaria model, exhibited low level of cross-resistance between PQ and CQ (Li, 1985). The existence of cross-resistance between CQ and PQ coupled with the ease with which PQ resistance was selected in this study

suggests that PQ may be selected for resistance easily especially in East Africa where high CQ resistance exists.

PQ rates well on cost, tolerability and pre-existing drug resistance (Davis *et al*., 2005) however the ease with which the resistant mutant were selected also cast a huge shadow over PQ/DHA. Since long half-life is the most crucial pharmacokinetic feature favouring the development of resistance (Hastings *et al*., 2002), the long terminal elimination half-life of PQ is a predisposing factor for the emergence of resistance even when in a combination.

In relating all these findings to the real-life situation in areas of malaria endemicity, there will be quite a difference between areas with low malaria transmission (low immunity) and those with intensive transmission (high naturally acquired immunity). Focusing on the ACTs, artemisinin derivative reduces the number of asexual parasites biomass exposed to the second drug, hence reducing the chances of a resistant mutant emerging during treatment (Mutabingwa, 2005). This concept might not necessarily apply where rate of infections are high. Rapidly eliminated artemisinin derivatives never present an immediate drug concentration to infecting malaria parasites (Hastings *et al*., 2002). However, the long elimination phase of PQ, LM and DEAQ will provides strong selective filter for resistant infections acquired after the drug treatment. Of great concern is that the naturally acquired immunity will mask a failing drug resulting to total loss of the drug to resistance. In effective antimalarial drug will therefore allow persistent parasite biomass even if the treatment may provide a temporary reduction of parasitaemia and clearance of symptoms. This argument further strengthens the need to identify LM, PQ and AQ resistance makers and the importance of monitoring

the emergence of LM, PQ and AQ resistance in areas where the drugs have been extensively used.

5.4 Stability of PQ resistant line

Stability test for the resistant phenotypes were initiated by withdrawal of the drug selection pressure. On withdrawal of the PQ selection pressure, the growth of PQ resistant strains was slow, this can also be evidenced by 4-DT performed after 12^{th} passage of drug pressure (Table 17, Fig. 19). This may be associated with fitness disadvantages (where the mutant parasites are less fit and multiply less well in absence of the drug as compared to their drug sensitive counterparts) (White, 2004). Mutations that render pathogens resistant to drug treatment are likely to result in a loss of fitness in absence of the drug (Walliker *et al*., 2005).

Selection of unstable PQ resistant *P. berghei* ANKA was first reported by Li *et al*., 1985. However, data obtained in this study indicate relative stability exhibited by the PQ resistant line, a major breakthrough towards understanding mechanism of resistance of PQ. Although a decrease in I_{50} from 93.85 to 36.23 was observed, it is apparent that I_{50} and I_{99} of 20.85 was still high enough to warrant the conclusion of attaining stable PQ resistant line to be used for mechanism of resistance studies (Table 3, Fig. 9).

CHAPTER SIX: CONCLUSION AND RECOMMEDATIONS

6.1 Conclusion

6.1.1 Selection of AQ resistance

The ED_{50}, ED_{90} and ED_{99} of AQ against parent *P. berghei* ANKA strain (reference line: 676m1cl1) was 0.95, 4.29 and 5.05 mg/kg.day respectively.

Increases in ED_{50}, ED_{90} and ED_{99} to 12.01, 19.13 and 20.73 mg/kg.day respectively within 36^{th} passage of drug pressure indicate selection of slight AQ resistant line of *P. berghei* ANKA with I_{50}, I_{90} and I_{99} of 12.64, 4.46 and 4.10 respectively.

6.1.2 Selection of LM resistance

The ED_{50}, ED_{90} and ED_{99} of LM against parent *P. berghei* ANKA strain (reference line: 676m1cl1) was 1.67, 3.93 and 4.48 mg/kg.day respectively

The increases in ED_{50}, ED_{90} and ED_{99} to 9.76, 25.50 and 29.02 mg/kg.day respectively after 28^{th} of drug pressure show selection of slight LM resistant line of *P. berghei* ANKA with I_{50}, I_{90} and I_{99} of 5.84, 6.48 and 6.48 respectively.

6.1.3 Selection of PQ resistance

The ED_{50}, ED_{90} and ED_{99} of revived PQ resistant *P. berghei* ANKA GFP before further exposure to drug pressure were 7.50, 21.90 and 24.00 mg/kg.day respectively.

The ED_{50}, ED_{90} and ED_{99} of PQ against *P. berghei* ANKA GFP after 12^{th} passage of drug selection pressure was 122.00, 193.30 and 210.00 mg/kg.day respectively. The increase in EDs and consequent rise in I_{50}, I_{90} and I_{99} by 93.85, 54.91 and 25.93 respectively clearly signifies selection of moderate PQ resistant line.

Finally, it is apparent that I_{50} of 36.23 and I_{99} of 20.85 observed after withdrawal of selection pressure indicate selection of stable PQ resistant line that could be used for molecular studies.

6.2 Recommendations

To select highly PQ, AQ and LM resistant lines (I_{99} of >100) and to maintain the resistance already obtained, drug selection pressure should be continued.

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

Studies should also be initiated to investigate whether the stability of PQ resistant phenotypes will be maintained once the resistant line is cryopreserved. Stability of the AQ resistant and LM resistant line should be assessed when moderate resistance (an I_{50} and I_{99} of above 10) is attained.

Finally, following selection of relatively stable PQ resistant line the study recommends investigations into PQ resistance markers mainly targeting the two implicated genes (*crt* and *mdr1*) associated with reduction in drug efficacy of most quinoline based drugs.

REFERENCES

Afonso, A., Hunt, P., Cheesman, S., Alves, A. C., Cunha, C. V., do Rosario, V. and Cravo, P. (2006) Malaria Parasites Can Develop Stable Resistance to Artemisinin but Lack Mutations in Candidate Genes *atp6* (Encoding the Sarcoplasmic and Endoplasmic Reticulum Ca_2ATPase), *tctp*, *mdr1*, and *cg10*. *Antimicrobial Agents and Chemotherapy*, 50: 480–489

Alin, M. H., Bjorkman, A. and Wernsdorfer, W. H. (1999) Synergism of benflumetol and artemether in *Plasmodium falciparum*. *The American Journal of Tropical Medicine and Hygiene*, 61: 439–445

Alonso, P. L., Sacarlal, J., Aponte, J. J., Leach, A., Macete, E., Milman, J., Mandomando, I., Spiessens, B., Guinovart, C., Espasa, M., Bassat, Q., Aide, P., Ofori- Anyinam, O., Navia, M. M., Corachan, S., Ceuppens, M., Dubois, M., Demoitié, M., Dubovsky, F., Menéndez, C., Tornieporth, N., Ballou, W. R., Thompson, R and Cohen, J. (2004) Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. *Lancet*, 364:1411-1420

Anderson, T. J. C. and Roper, C. (2005) The origins and spread of antimalarial drug resistance: Lessons for policy makers. *Acta Tropica*, 94: 269–280

Angulo, I. and Fresno, M. (2002) Cytokines in the Pathogenesis of and Protection against Malaria. *Clinical and Diagnostic Laboratory Immunology*, 9:1145-1152

Baird, J.K. (2005) Effectiveness of Antimalarial Drugs. *The New England Journal of Medicine*, 352:1565-1577

Barry, A. E. (2005) Malaria epidemiology: Insights from the genome of the malaria parasite. *Journal of Molecular and Genetic Medicine*, 1: 76-86

Basco, L. K. and Ringwald, P. (2003) *In vitro* activities of piperaquine and other 4-Aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrobial Agents and Chemotherapy*, 47: 1391-1394

Basco, L. K., Bickii, J. and Ringwald, P. (1998) *In Vitro* Activity of Lumefantrine (Benflumetol) against Clinical Isolates of *Plasmodium falciparum* in Yaounde, Cameroon. *Antimicrobial Agents and Chemotherapy*, 42:2347–2351

Bertino, J. R., Goker, E., Gorlick, R., Li, W. W. and Banerjee, D. (1996) Resistance mechanisms to methotrexate in tumors. *Oncologist*, 1: 223–226

Biagini, G. A., O'Neill, P. M., Bray, P.G. and Ward, S. A. (2005) Current drug development portfolio for antimalarial therapies. *Current Opinion in Pharmacology*, 5:473–478

Bjorkman, A. and Bhattarai, A. (2005) Public health impact of drug resistant *Plasmodium falciparum* malaria. *Acta Tropica*, 94: 163–169

Breman, J. G., Mills, A., Snow, R. W., Mulligan, J., Lengeler, C., Mendis, K., Sharp, B., Morel, C., Marchesini, P., White, N. J., Steketee, R. W. and Doumbo, O. K. (2006) "Conquering Malaria." *Disease Control Priorities in Developing Countries (2nd Edition), ed.* 413-432. New York: Oxford University Press [http://www.dcp2.org/pubs/DCP/21/Section/2811] Assessed on 09 May 2007

Carlton, J. M-R., Hayton, K., Cravo, P. V. L. and Walliker, D. (2001) Of mice and malaria mutants: unraveling the genetics of drug resistance using rodent malaria models. *Trends in Parasitology*, 17: 236- 241

Carlton, J., Mackinnon, M. and Walliker, D. (1998b) A chloroquine resistance locus in the rodent malaria parasite *Plasmodium chabaudi*. *Molecular and Biochemical Parasitology*, 93: 57–72

Carlton, J., Vinkenoog, R., Waters, A. P., Walliker, D. (1998a) Gene synteny in species of *Plasmodium*. *Molecular and Biochemical Parasitology*, 93: 285–294

CDC, 2007 Laboratory Identification of Parasites of Public Health Concern. Parasites and Health, Malaria [http://www.dpd.cdc.gov/DPDx/HTML/Malaria.htm] Assessed on 09 May 2007

Chiang, P. K., Bujnicki, J. M., Xinzhuan, S. and Lanar, D. E. (2006) Malaria: Therapy, Genes and Vaccines. *Current Molecular Medicine*, 6: 309-326

Coquelin, F., Biarnais, T., Deharo, E., Peters, W., Chabaud, A. and Landau, I. (1997) Modifications in the rhythm of schizogony in *Plasmodium chabaudi chabaudi* associated with the selection of chloroquine resistance. *Parasitology Research*, 83: 504-509

Cravo, P. V. L., Carlton, J. M. R., Hunt, P., Bisoni, L., Ann Padua, R and Walliker, D (2003) Genetics of Mefloquine Resistance in the Rodent Malaria Parasite *Plasmodium chabaudi*. *Antimicrobial Agents and Chemotherapy*, 47: 709–718

D'Alessandro, U. (2001). Insecticide treated bed nets to prevent malaria**.** *British Medical Journal*, 322: 249-250

D'Alessandro, U. and Buttiens, H (2001) History and importance of antimalarial drug resistance. *Tropical Medicine and International Health*, 6: 845-848

Davis, T. M. E., Hung, T., Sim, I., Karenajeewa, H. A. and Ilett, K. F. (2005) Piperaquine: A Resurgent Antimalarial Drug. *Drugs*, 65: 75-87

de Koning-Ward, T. F., Janse, C. J and Waters, A. P. (2000) The Development of Genetic Tools for Dissecting the Biology of Malaria Parasites. *Annual Review of Microbiology*, 54: 157-185

de Koning-Ward, T. F., Thomas, A. W., Waters, A. P and Janse, C. J. (1998) Stable expression of green fluorescent protein in blood and mosquito stages of *Plasmodium berghei*. *Molecular and Biochemical Parasitology*, 97: 247-252

Deitsch, K. W. and Hviid, L. (2004) Variant surface antigens, virulence genes and the pathogenesis of malaria. *Trends in parasitology*, 20: 562-566

Dokomajilar, C., Samuel L. Nsobya, S. L., Greenhouse, B., Rosenthal, P. J. and Dorsey, G. (2006) Selection of *Plasmodium falciparum pfmdr1* Alleles following Therapy with Artemether-Lumefantrine in an Area of Uganda where Malaria Is Highly Endemic. *Antimicrobial Agents and Chemotherapy*, 50: 1893–1895

Duraisingh, M. T. and Cowman, A. F. (2005) Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Tropica*, 94: 181–190

EANMAT (2003) The efficacy of antimalarial monotherapies, sulphadoxine–pyrimethamine and amodiaquine in East Africa: implications for sub-regional policy. *Tropical Medicine and International Health*, 8: 860–867

Eckstein-Ludwig, U., Webb, R. J., Van Goethem, I. D., East, J. M., Lee, A. G., Kimura, M., O'Neill, P. M., Bray, P. G., Ward, S. A. and Krishna, S. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature*, 424: 957–961.

Ezzet, F., van Vugt, M., Nosten, F., Looareesuwan, S. and White, N. J. (2000) Pharmacokinetics and Pharmacodynamics of Lumefantrine (Benflumetol) in Acute Falciparum Malaria. *Antimicrobial Agents and Chemotherapy*, 44: 697–704

Faye, F. B. K., Spiegel, A., Tall, A., Sokhna, C., Fontenille, D., Rogier, C. and Trape, J. F. (2002) Diagnostic Criteria and Risk Factors for *Plasmodium ovale* Malaria. *The Journal of Infectious Diseases*, 186:690–695

Fidock, D. A., Rosenthal, P. J., Croft, S. L., Brun, R. and Nwaka, S. (2004) Antimalarial drug discovery: Efficacy model for compound screening. *Nature Reviews*, 3: 509-517

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B., Tabb, D. L., Witney, A. A., Wolters, D., Wu, Y., Gardner, M. J., Holder, A. A., Sinden, R. E., Yates, J. R. and. Carucci, D. J. (2002) A proteomic view of the *Plasmodium falciparum* life cycle. *Nature*, 419: 520-526

Franke-Fayard, B., Waters, A. P. and Janse, C. J. (2006) Real-time *in vivo* imaging of transgenic bioluminescent blood stages of rodent malaria parasites in mice. *Nature Protocols*, 1: 476-485

Gamble, C., Ekwaru, P. J., Garner, P. and ter Kuile, F. O. (2007) Insecticide-Treated Nets for the Prevention of Malaria in Pregnancy: A Systematic Review of Randomised Controlled Trials. *PLoS Medicine*, 4: 0506-0515

Gervais, G. W., Trujillo, K., Robinson, B. L., Peters, W. and Serrano, A. E. (1999) *Plasmodium berghei*: Identification of an *mdr*-like Gene Associated with Drug Resistance. *Experimental Parasitology*, 91: 86–92

Giao, P. T., de Vries, P. J., Le Q. Hung, L. Q., Binh, T. Q., Nam, N. V., and Kager, P. A. (2004) CV8, a new combination of dihydroartemisinin, piperaquine, trimethoprim and primaquine, compared with atovaquone–proguanil against

falciparum malaria in Vietnam. *Tropical Medicine and International Health*, 9: 209 216

Goodman, C. A. and Mills, A. J. (1999) The evidence base on the cost-effectiveness of malaria control measures in Africa. *Health Policy and Planning*, 14: 301–312

Gorissen, E., Ashruf, G., Lamboo, M., Bennebroek, J., Gikunda, S., Mbaruku, G. and Kager, P. A. (2000) *In vivo* efficacy study of amodiaquine and sulfadoxine/pyrimethamine in Kibwezi, Kenya and Kigoma, Tanzania. *Tropical Medicine and International Health*, 5: 459-463

Greenwood, B. (2005) Malaria vaccines Evaluation and implementation. *Acta Tropica*, 95: 298–304

Gregson, A. and Plowe, C. V. (2005) Mechanisms of Resistance of Malaria Parasites to Antifolates. *Pharmacological Review*, 57:117–145

Guerra, C. A., Hay, S. I., Lucioparedes, L. S., Gikandi, P. W., Tatem, A. J., Noor, A. M. and Snow, R. W. (2007) Assembling a global database of malaria parasite prevalence for the Malaria Atlas Project. *Malaria Journal*, 6:1-13

Hastings, I. M. (2004) The origins of antimalarial drug resistance. *Trends in Parasitology*, 20:512-518

Hastings, I. M., Watkins, W. M. and White, N. J. (2002) The evolution of drug-resistant malaria: the role of drug elimination half-life. *Philosophical Transactions of Royal Society of London Biology*, 357: 505–519

Hayton, K., Ranford-Cartwright, L. C. and Walliker, D. (2002) Sulfadoxine-Pyrimethamine Resistance in the Rodent Malaria Parasite *Plasmodium chabaudi*. *Antimicrobial Agents and Chemotherapy*, 46: 2482–2489

Hearn, J., Rayment, N., Landon, D. N., Katz, D.R. and de Souza, J. B (2000) Immunopathology of Cerebral Malaria: Morphological Evidence of Parasite Sequestration in Murine Brain Microvasculature. *Infection and Immunity*, 68: 5364-5376

Hien, T. T., Dolecek, C., Mai, P. P., Dung, N. T., Truong, N. T., Thai, L. H., Hoai An D. T., Thanh, T. T., Stepniewska, K., White, N. J and Farrar, J. (2004) Dihydroartemisinin-piperaquine against multidrug-resistant *Plasmodium falciparum* malaria in Vietnam: randomised clinical trial. *Lancet*, 363:18–22

Hoffman, S. L., Subramanian, G. M., Collins, F. H. and Venter, J. C. (2002) Plasmodium, human and Anopheles genomics and malaria. *Nature*, 415: 702-709

Holmgren, G., Gil, J. P., Ferreira, P. M., Veiga, M. I., Obonyo, C. O and Bjorkman A. (2005) Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of pfcrt 76T and pfmdr1 86Y. *Infection, Genetics and Evolution*, [in press], doi: 10.1016/j.meegid.2005.09.001

Hung, T. Y., Davis, T. M. E., Ilett, K. F., Karunajeewa, H., Hewitt, S., Denis, M. B., Lim, C. and Socheat, D (2004). Population pharmacokinetics of piperaquine in adults and children with uncomplicated *falciparum* or *vivax* malaria. *British Journal of Clinical Pharmacology*, 57: 253-262

Hviid, L. (2005) Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. *Acta Tropica*, 95: 270–275

Hyde, J. E. (2005a) Exploring the folate pathway in *Plasmodium falciparum*. *Acta Tropica*, 94: 191–206

Hyde, J. E. (2005b) Drug resistant malaria. *Trends in Parasitology*, 21:494-498

Janse, C. and Waters, A. (1995) *Plasmodium berghei*: The application of cultivation and purification techniques for molecular studies of malaria parasites. *Parasitology Today*, 11: 138-143

Janse, C. and Waters, A. (2006) Leiden University Medical Center, Parasitology, Malaria, The *Plasmodium berghei* research model of malaria. [http://www.lumc.nl/1040/research/malaria/model.html] Assessed on 12 July 2007

Janse, C. J., Franke-Fayard, B., Mair, G. R., Ramesar, J., Thiel, C., Engelmann, S., Matuschweski, K.,Jan van Gemert, G., Sauerwein, R. W and Waters, A. P. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Molecular and Biochemical Parasitology*, 145: 60-70

Labbé, A., Patel, S., Crandall, I. and Kain, K. C. (2003) Molecular Surveillance System for Global Patterns of Drug Resistance in Imported Malaria. *Emerging Infectious Diseases*, 9: 33-36

Lengeler, C. (2004) Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database of Systematic Reviews*. (http://www.mrw.interscience.wiley.com/cochrane/clsysrev/articles/CD000363/frame.html) Assessed on 12 July 2007

Lengeler, C., Armstrong-Schellenberg, J., D'Alessandro, U., Binka, F and Cattani, J. (1998) Relative versus absolute risk of dying reduction after using insecticide treated nets for malaria in Africa. *Tropical Medicine and International Health*, 3: 286-290

Li, G. D. (1985) Development of piperaquine-resistant line of *Plasmodium berghei* K173 strain. *Acta Pharmaceutica Sinica*, 20:412–417.

Li, G. D., F. Y. Qu, and L. Chen (1985) Development of piperaquine-resistant line of *Plasmodium berghei* ANKA strain. *Chinese Journal of Parasitology and Parasitic. Diseases*, 3:189–192.

Li, X., Bjorkman, A., Andersson, T. B., Ridderstrom, M and Masimirembwa, C. M. (2002) Amodiaquine Clearance and Its Metabolism to *N*-Desethylamodiaquine Is Mediated by CYP2C8: A New High Affinity and Turnover Enzyme-Specific Probe Substrate. *The Journal of Pharmacology and Experimental Therapeutics*, 300: 399-407

Lou, J., Lucas, R. and Grau, G. E. (2001) Pathogenesis of Cerebral Malaria: Recent Experimental Data and Possible Applications for Humans. *Clinical Microbiology Reviews*, 14: 810-820

Mariga, S. T., Gil, J. P., Sisowath, C., Wernsdorfer, W. H. and Bjorkman, A. (2004) Synergism between Amodiaquine and Its Major Metabolite, Desethylamodiaquine, against *Plasmodium falciparum In Vitro*. *Antimicrobial Agents and Chemotherapy*, 48: 4089–4096

Mendis, K., Sina, B. J., Marchesini, P. and Carter, R. (2001) The neglected burden of *Plasmodium vivax* malaria. *The American Journal of Tropical Medicine and Hygiene*, 64: 97-106

Merkli, B. and Richle, R. W. (1980) Studies on the resistance to single and combined antimalarials in the *Plasmodium berghei* mouse model. *Acta Tropica*, 37, 228–231

Moorthy, V. S., Good, M. F. and Hill, A. V. S. (2004) Malaria vaccine developments. *Lancet*, 363: 150–156

Mutabingwa, T. K. (2005) Artemisinin-based combination therapies (ACTs): Best hope for malaria treatment but inaccessible to the needy! *Acta Tropica*, 95: 305–315

Nduati, E., Hunt, S., Kamau, E. M., Nzila, A. (2005) 2, 4-Diaminopteridine-Based Compounds as Precursors for De Novo Synthesis of antifolates: a Novel Class of Antimalarials. *Antimicrobial Agents and Chemotherapy*, 49:3652-3657

Nzila, A. (2006) The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *Journal of Antimicrobial Chemotherapy*, 57:1043–1054

Ochong, E.O., van den Broek, I.V., Keus, K. and Nzila, A. (2003) Short report: association between chloroquine and amodiaquine resistance and allelic variation in the *pfmdr1* gene and the *crt* gene in isolates from the Upper Nile in Southern Sudan. *The American Journal of Tropical Medicine and Hygiene*, 69:184-187

O'Neill, P. M. and Posner, G. H. (2004) A Medicinal Chemistry Perspective on Artemisinin and Related Endoperoxides. *Journal of Medicinal Chemistry*, 47: 2945-2964

Peters, W. (1999) The chemotherapy of rodent malaria. LVII. Drug combinations to impede the selection of drug resistance, part 1: which model is appropriate? *Annals of Tropical Medicine and Parasitology*, 93: 569-587

Peters, W. and Robinson, B. L. (1992) The chemotherapy of rodent malaria. XLVII. Studies on pyronaridine and other Mannich base antimalarials. *Annals of Tropical Medicine and Parasitology*, 86:455-465

Peters, W. and Robinson, B. L. (1999) The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. *Annals of Tropical Medicine and Parasitology*, 93: 325-339

Peters, W. and Robinson, B. L. (2000) The chemotherapy of rodent malaria. LVIII. Drug combinations to impede the selection of drug resistance, part 2: the new generation—artemisinin or artesunate with long-acting blood schizontocides. *Annals of Tropical Medicine and Parasitology*, 94: 23-35

Peters, W., Portus, J.H. and Robinson, B.L. (1975) The chemotherapy of rodent malaria. XXII. The value of drug-resistant strains of *Plasmodium berghei* in screening for schizontocidal activity. *Annals of Tropical Medicine and Parasitology*, 69: 155-171

Phillips-Howard, P. A., Ter Kuile, F., Nahlen, B. L., Alaii, J. A., Gimnig, J. E., Kolczak, M. S., Terlouw, D. J., Kariuki, S. K., Shi, Y., Kachur, S. P., Hightower, A. W., Vulule, J. M and Hawley, W. A. (2003) The efficacy of permethrin-treated bed nets on child mortality and morbidity in Western Kenya II study design and methods. *The American Journal of Tropical Medicine and Hygiene*, 68; 10-15

Plowe, C. V. (2003) Monitoring antimalarial drug resistance: making the most of the tools at hand. *The Journal of Experimental Biology*, 206: 3745-3752

Rathod, P. K., McErlean, T. and Lee, P.C. (1997) Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America*, 94: 9389–9393

Ridley, R.G. (2002) Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature*, 415: 686-693

Robert, A and Meunier, B. (1998) Is alkylation the main mechanism of action of the antimalarial drug artemisinin? *Chemical Society Reviews*, 27: 273-279

Robert, A., Benoit-Vical, F., Dechy-Cabaret, O. and Meunier, B. (2001) From classical antimalarial drugs to new compounds based on the mechanism of action artemisinin. *Pure and Applied Chemistry*, 73: 1173-1188

Sidhu, A. B. S., Uhlemann, A. C., Valderramos, S. G., Valderramos, J. C., Krishna, S. and Fidock, D. A. (2006) Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *The Journal of Infectious Diseases*, 194: 528–535

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A. and Gil1, J. P. (2005) *In vivo* Selection of *Plasmodium falciparum pfmdr1* 86N Coding Alleles by Artemether-Lumefantrine (Coartem). *The Journal of Infectious Diseases*, 191:1014–1017

Smith, D. F. and Parsons, M. (1996) Molecular Biology of Parasitic Protozoa. Oxford University Press New York USA, p 81

Statsoft, Inc.2000. STATISTICA for Windows. Computer Program Manual. Oklahoma. Tulsa

Stewart, L. B., Peters, W. and Robinson, B. L. (2004) The chemotherapy of rodent malaria. LXII. Drug combinations to impede the selection of drug resistance, part 5: rates of development of resistance to some inhibitors of folate metabolism and to artesunate. *Annals of Tropical Medicine and Parasitology*, 98: 763–783

Sullivan, Jr D. J., Gluzman, I. Y., Russell, D.G. and Goldberg, D. E. (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proceedings of the National Academy of Sciences of the United States*, 93: 11865-11870

Tarning, J., Lindegardh, N., Annerberg, A., Singtoroj, T., Day, N. P. J., Ashton, M and White, N. J. (2005) Pitfalls in Estimating Piperaquine Elimination. *Antimicrobial Agents and Chemotherapy*, 49: 5127–5128

Tona, L., Mesia, K., Ngimbi, N.P., Chrimwami, B., Okondahoka Cimanga, K., De Bruyne, T., Apers, S., Hermans, S., Totte, J., Peters, L. and Vlientinck, A.J (2001) *In vivo* antimalarial activity of *Cassia occidentalis, Morinda morindoides* and *Phyllanthus niruri*. *Annals of Tropical Medicine and Parasitology*, 95: 45-57

Toovey, S (2006) Summaries: Act of resistance. *Travel Medicine and Infectious Disease*, 4: 99–105

Toovey, S. and Jamieson, A. (2004) Audiometric changes associated with the treatment of uncomplicated falciparum malaria with co-artemether. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 98: 261-267

Trape, J. F. (2001) The public health impact of chloroquine resistance in Africa. *The American Journal of Tropical Medicine and Hygiene*, 64: 12 -17

Valderramos, S. G. and Fidock D. A. (2006) Transporters involved in resistance to antimalarial drugs. *Trends in Pharmacological Sciences*, 27:594-601

Vangapandu, S., Jain, M., Kaur, K., Patil, P., Patel, S. R. and Jain, R. (2006) Recent Advances in Antimalarial Drug Development. *Medicinal Research Review,* 1: 65-107

Walliker, D. (2005) The hitchhiker's guide to malaria parasite genes. *Trends in Parasitology*, 21: 489-493

Walliker, D., Hunt, P. and Babiker, H. (2005) Fitness of drug-resistant malaria parasites. *Acta Tropica*, 94: 251-259

Warhurst, D. C., Steele, J. C. P., Adagu, I. S., Craig, J. C. and Cullander, C. (2003) Hydroxychloroquine is much less active than chloroquine against chloroquine-resistant *Plasmodium falciparum*, in agreement with its physicochemical properties. *Journal of Antimicrobial Chemotherapy*, 52:188–193

Watkins, W.M., Sibley, C.H and Hastings, I.M (2005) The search for effective and sustainable treatments for *Plasmodium falciparum* malaria in Africa: a model of the selection of resistance by antifolate drugs and their combinations. *The American Journal of Tropical Medicine and Hygiene,* 72: 163-173

Webster, D and Hill, A.V.S. (2003) Progress with new malaria vaccines. Bulletin of the World Health Organization, 81: 902–909

Wellems, T. E. and Plowe, C. V. (2001) Chloroquine Resistant Malaria. *The Journal of Infectious Diseases*, 184:770–776

White, N. J. (2002) The assessment of antimalarial drug efficacy. *Trends in Parasitology*, 18: 458- 464

White, N. J. (2004) Antimalarial drug resistance. *The Journal of Clinical Investigation*, 113:1084–1092

White, N. J. and Pongtavornpinyo, W. (2003) The *de novo* selection of drug-resistant malaria parasites. *Proceedings of Royal Society of London Biology*, 270: 545–554

Win, T., Jalloh, A., Tantular, I. S., Tsuboi, T., Ferreira, M. U., Kimura, M and Kawamoto, F. (2004) Molecular analysis of *Plasmodium ovale* variants. *Emerging Infectious Diseases*, 10: 1235-1240

Winstanley, P., Ward, S., Snow, R. and Breckenridge, A. (2004) Therapy of Falciparum Malaria in Sub-Saharan Africa: from Molecule to Policy. *Clinical Microbiology Reviews*, 17: 612–637

Wiseman, V., Kim, M., Mutabingwa, T. K., Whitty, C. J. M. (2006) Cost-Effectiveness Study of Three Antimalarial Drug Combinations in Tanzania. *PLoS Medicine*, 3:e373

Wongsrichanalai, C., Pickard, A. L., Wernsdorfer, W. H. and Meshnick, S. R. (2002) Epidemiology of drug-resistant malaria. *Lancet Infectious Diseases*, 2: 209–218

World Health Organization: Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria. Geneva; WHO; 2003

World Health Organization: Drug resistance in Malaria: Malaria Epidemiology Branch Centers for Disease Control and Prevention Chamblee, GA, United States of America; WHO; 2001. WHO/CDS/CSR/DRS/2001.4

World Health Organization: Guidelines for the treatment of malaria. Geneva; WHO; 2006

World Health Organization: TDR strategic direction for Research: Malaria. Geneva; WHO; 2002

Xiao, S.H., Yao, J.M., Utzinger, Y.C., Chollet, J. and Marcel, T. (2004) Selection and reversal of *Plasmodium berghei* resistance in the mouse model following repeated high doses of artemether. *Parasitology Research*, 92: 215-219

Yeung, S., Pongtavornpinyo, W., Hastings, I. M., Mills, A. J. and White, N. J. (2004) Antimalarial drug resistance, Artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. *The American Journal of Tropical Medicine and Hygiene*, Supplement: 71: 179-186

APPENDICES

APPENDIX 1: PREPARATION OF BUFFERS AND SOLUTIONS

Phosphate saline glucose buffer (PSG)

5.392g	di-sodium hydrogen phosphate (Na_2HPO4)
0.312g	Sodium di-hydrogen phosphate ($NaH_2PO4.2H2O$)
1.7g	Sodium chloride (NaCl)
10.0	D-glucose

Dissolve the mixture in 1 litre of distilled water, sterilize by autoclaving at 121ºC for 15 minutes and store in dark at 4ºC

20% glycerol in Phosphate saline glucose buffer

Two parts of 100% Glycerol solution is mixed with eight parts of PSG to obtain 20% glycerol solution.

Sorensen staining buffer

1.2705g	Potassium di-hydrogen phosphate (KH_2PO4)
4.275g	di-sodium hydrogen phosphate ($Na_2HPO4.\ 2H2O$)

Dissolve the mixture in 2.5 litres of distilled water, pH 7.2. Store at room temperature

Giemsa stock solution

3g	Giemsa powder
300ml	Methanol
200ml	100% glycerol

Mix the solution thoroughly for 45-60 minutes. Store at room temperature

70% Tween-80 and 30% ethanol (stock solution)

7 parts of Tween-80 is mixed with 3 parts of analytical ethanol

APPENDIX 2: *IN VIVO* ACTIVITY PROFILES OF AMODIAQUINE

Table 4: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) parent strain.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage (mg/kg.day)	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	5	0.19	1.26	98.74
2	AQ	2.5	5.27	35.28	64.72
3	AQ	1.25	6.28	41.99	58.01
4	AQ	0.625	8.23	55.02	44.98
Control	7% Tween-80 in 3% ethanol solution		14.95		

Fig. 10: Graph showing activity of AQ against parent strain of *P. berghei* ANKA (reference line 676m1cl1).

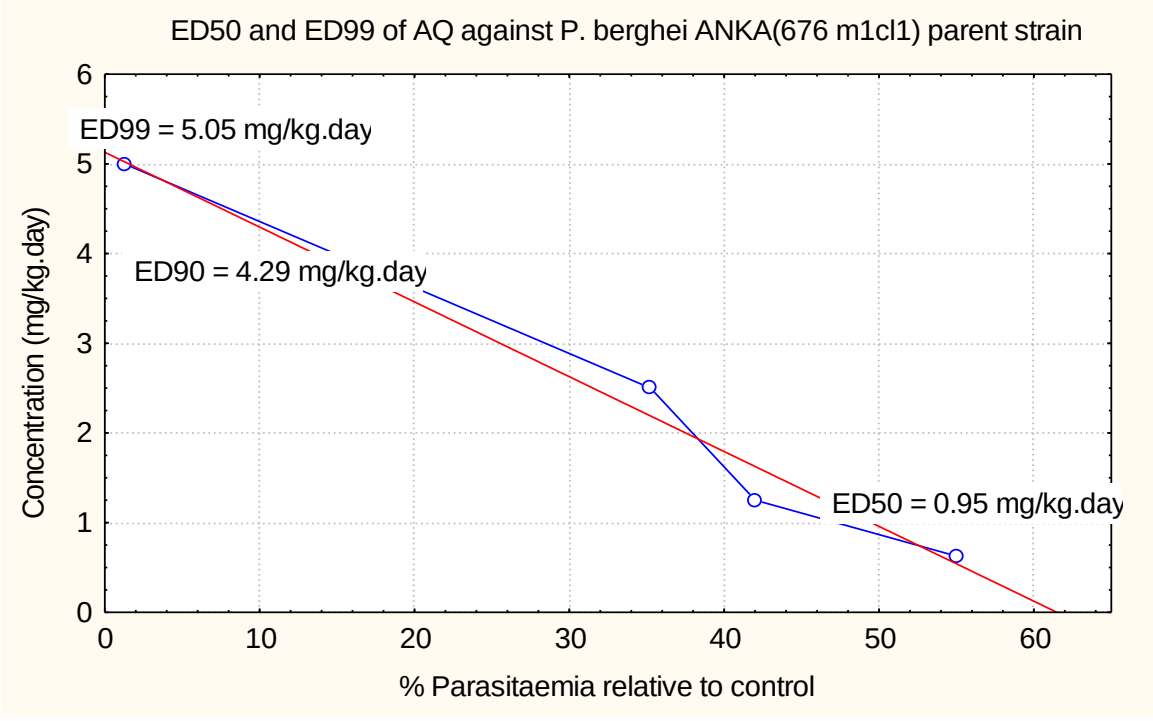


Table 5: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) after 4th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage (mg/kg.day)	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	5	0.15	0.66	99.34
2	AQ	2.5	2.30	10.32	89.68
3	AQ	1.25	9.00	40.34	59.66
4	AQ	0.625	14.25	63.91	36.09
Control	7% Tween-80 in 3% ethanol solution		22.30		

Fig.11: Graph showing of activity of AQ against *P. berghei* ANKA (reference line 676m1cl1) after 4th passage of drug pressure

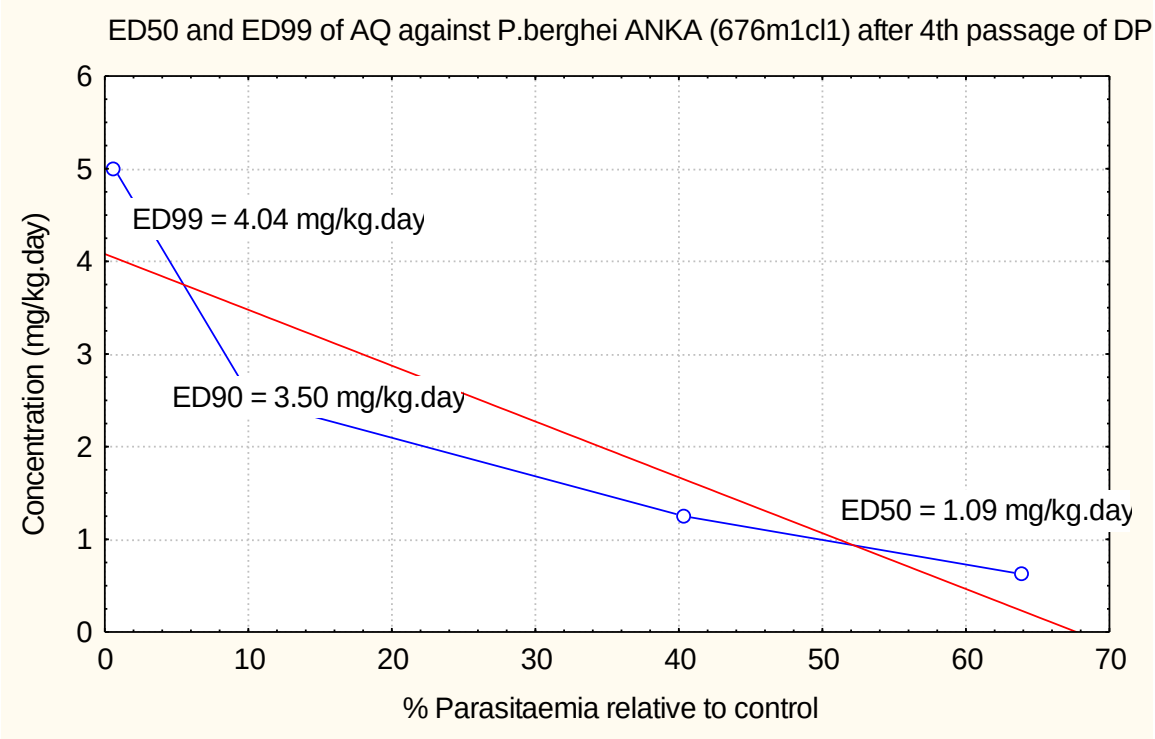


Table 6: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) after 12th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	5	0.09	0.33	99.67
2	AQ	2.5	8.18	29.76	70.24
3	AQ	1.25	23.04	83.87	16.13
4	AQ	0.625	21.86	79.59	20.41
Control	7% Tween-80 in 3% ethanol solution		27.47		

Fig. 12: Graph showing of activity of AQ against *P. berghei* ANKA (reference line 676m1cl1) after 12th passage of drug pressure

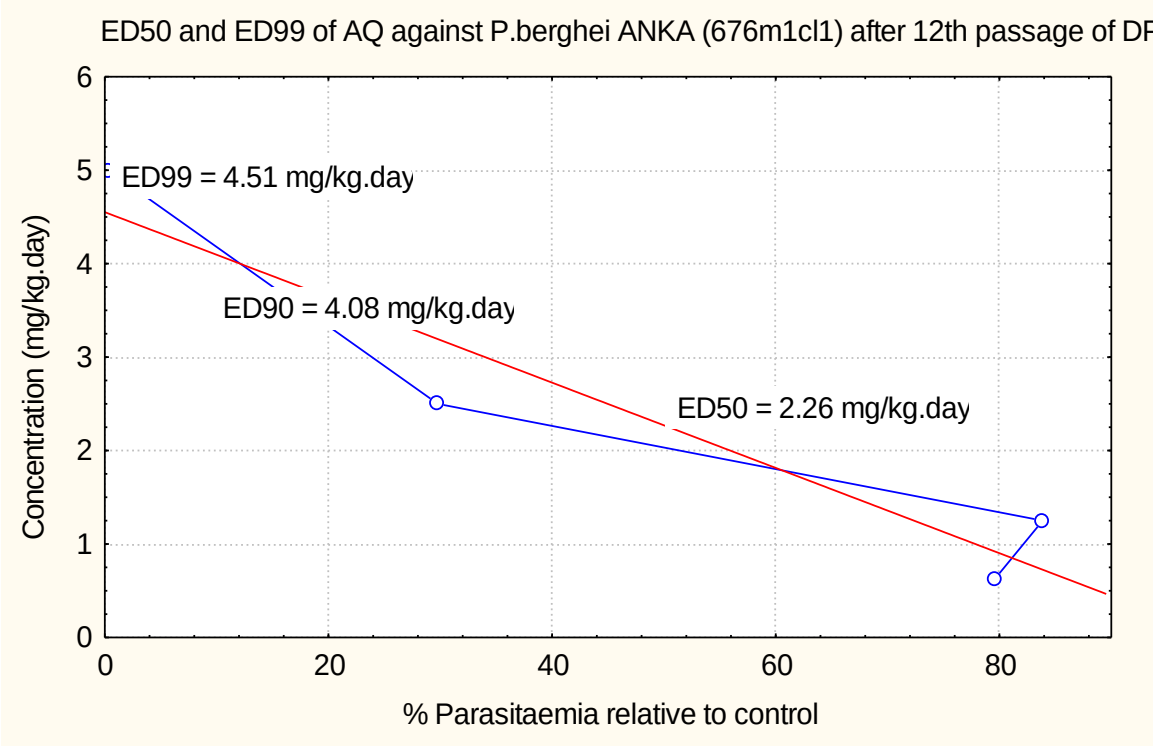


Table 7: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) after 20th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	5	0.11	0.52	99.48
2	AQ	2.5	10.67	52.29	47.71
3	AQ	1.25	16.85	82.52	17.48
4	AQ	0.625	17.96	87.96	12.04
Control	7% Tween-80 in 3% ethanol solution		20.41		

Fig. 13: Graph showing of activity of AQ against *P. berghei* ANKA (reference line 676m1cl1) after 20th passage of drug pressure

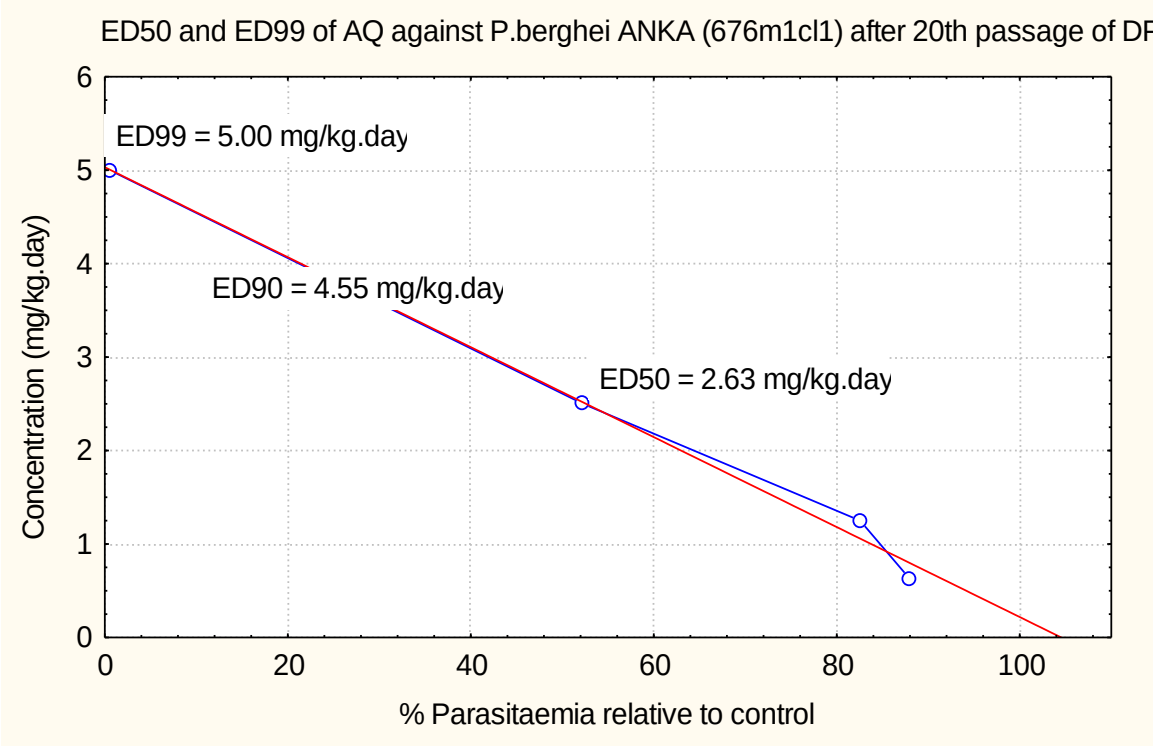


Table 8: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) after 28th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	20	0.12	1.32	98.68
2	AQ	10	0.32	3.56	96.44
3	AQ	5	1.28	14.11	85.89
4	AQ	2.5	6.55	71.92	28.08
Control	7% Tween-80 in 3% ethanol solution		9.10		

Fig. 14: Graph showing of activity of AQ against *P. berghei* ANKA (reference line 676m1cl1) after 28th passage of drug pressure.

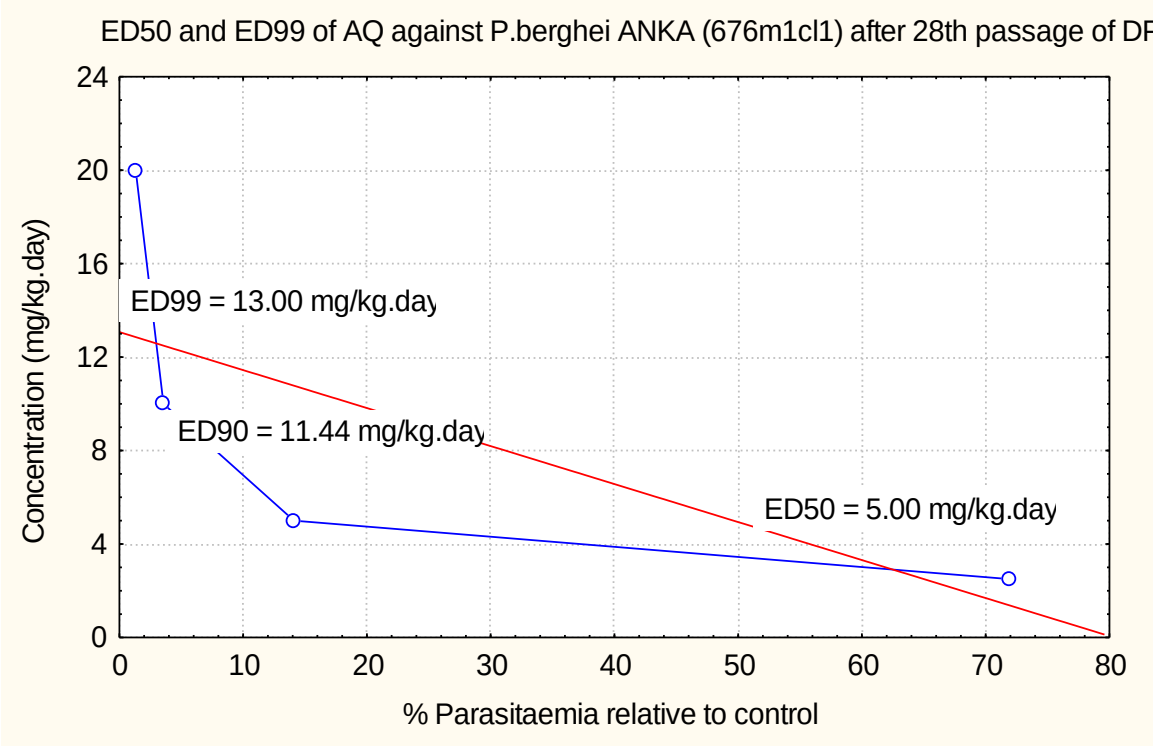
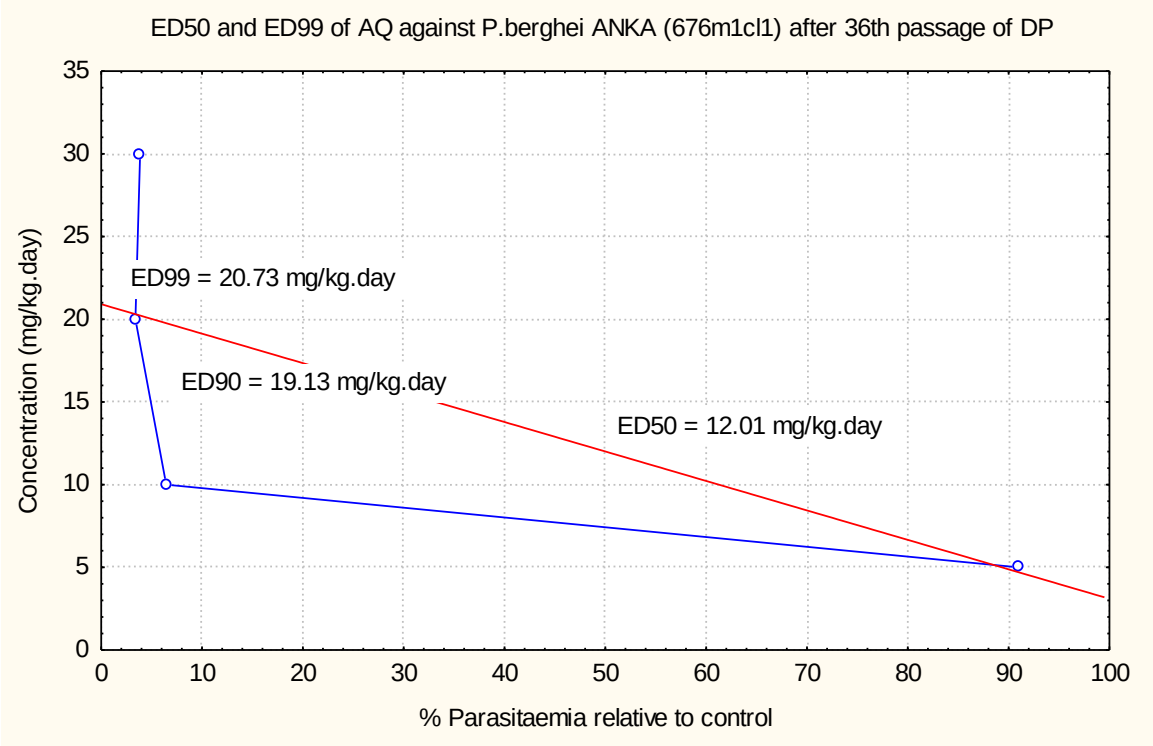


Table 9: *In vivo* activity of AQ against *P. berghei* ANKA (reference line: 676 m1cl1) after 36th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	AQ	30	0.31	3.87	96.13
2	AQ	20	0.28	3.40	96.60
3	AQ	10	0.52	6.45	93.55
4	AQ	5	7.39	90.94	9.06
Control	7% Tween-80 in 3% ethanol solution		8.12		

Fig. 15: Graph showing of activity of AQ against *P. berghei* ANKA (reference line 676m1cl1) after 36th passage of drug pressure



APPENDIX 3: *IN VIVO* ACTIVITY PROFILES OF LUMEFANTRINE

Table 10: *In vivo* activity of LM against *P. berghei* ANKA (reference line: 676 m1cl1) parent strain.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	LM	6	0.18	1.27	98.73
2	LM	3	0.39	2.73	97.27
3	LM	1.5	6.17	43.65	56.35
4	LM	0.75	10.30	72.87	27.13
Control	7% Tween-80 in 3% ethanol solution		14.13		

Fig. 16: Graph showing activity of LM against parent strain of *P. berghei* ANKA (reference line 676m1cl1).

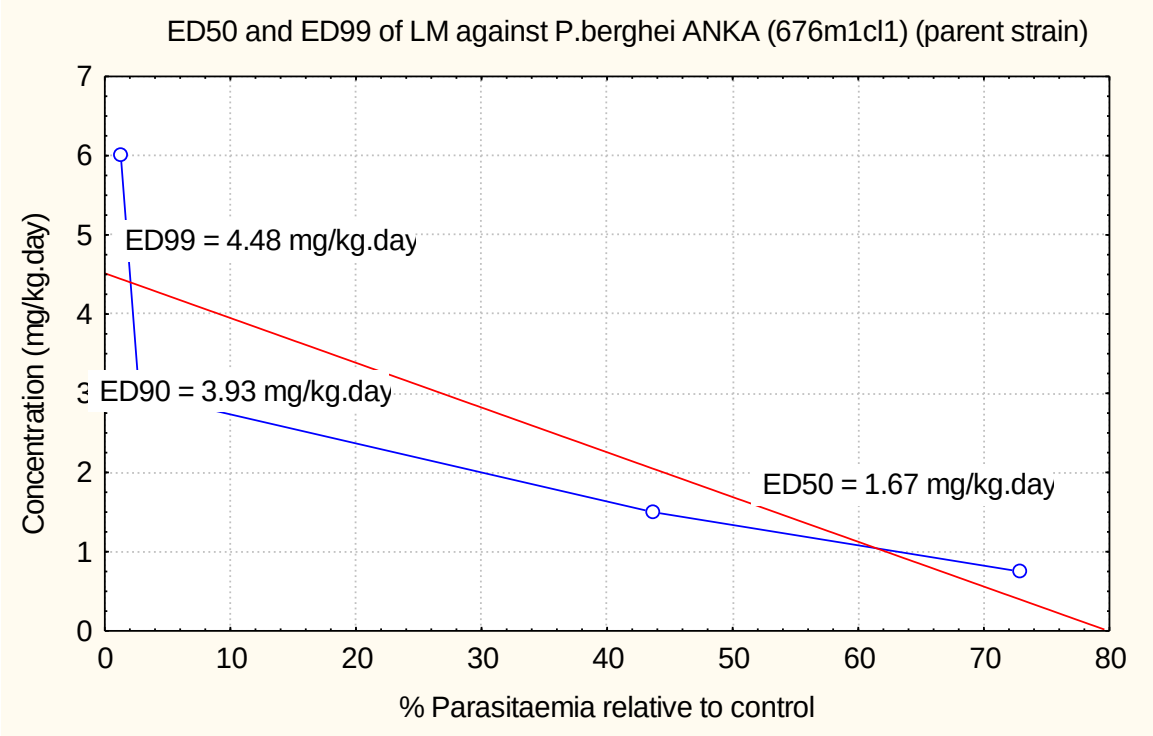


Table 11: *In vivo* activity of LM against *P. berghei* ANKA (reference line: 676 m1cl1) after 4th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	LM	6	0.08	0.87	99.13
2	LM	3	0.18	1.94	98.06
3	LM	1.5	1.83	19.32	80.68
4	LM	0.75	6.51	68.69	31.31
Control	7% Tween-80 in 3% ethanol solution		9.47		

Fig. 17: Graph showing of activity of LM against *P. berghei* ANKA (reference line 676m1cl1) after 4th passage of drug pressure.

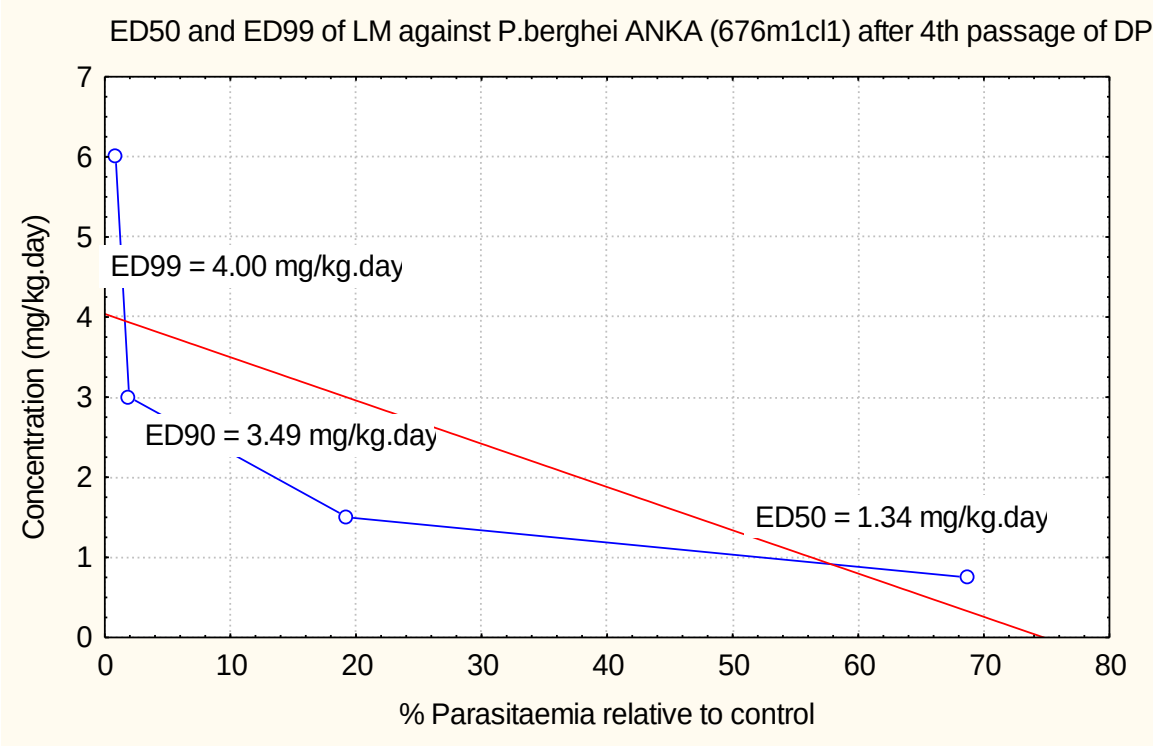


Table 12: *In vivo* activity of LM against *P. berghei* ANKA (reference line: 676 m1cl1) after 12th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	LM	6	0.10	0.45	99.55
2	LM	3	0.16	0.72	99.28
3	LM	1.5	1.39	6.20	93.80
4	LM	0.75	16.39	73.35	26.65
Control	7% Tween-80 in 3% ethanol solution		22.34		

Fig. 18: Graph showing of activity of LM against *P. berghei* ANKA (reference line 676m1cl1) after 12th passage of drug pressure

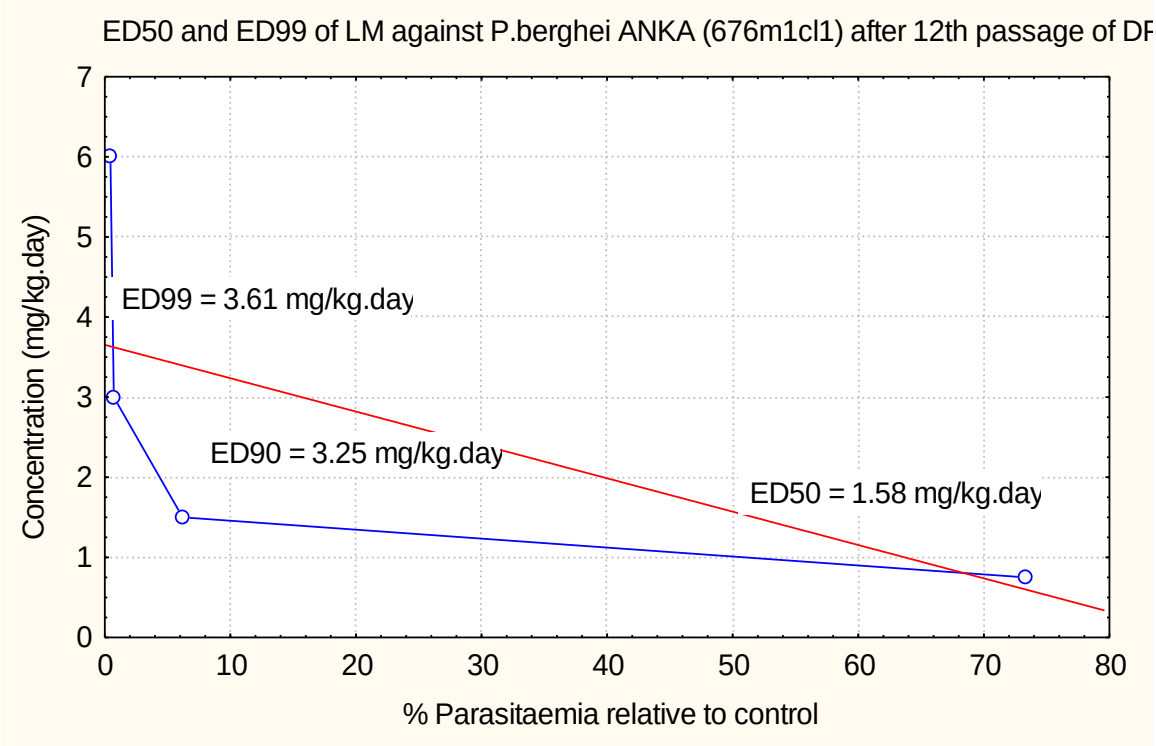


Table 13: *In vivo* activity of LM against *P. berghei* ANKA (reference line: 676 m1cl1) after 20th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	LM	6	1.90	15.69	84.31
2	LM	3	3.97	32.77	67.23
3	LM	1.5	7.51	61.98	38.02
4	LM	0.75	12.06	99.45	0.55
Control	7% Tween-80 in 3% ethanol solution		12.12		

Fig. 19: Graph showing of activity of LM against *P. berghei* ANKA (reference line 676m1cl1) after 20th passage of drug pressure

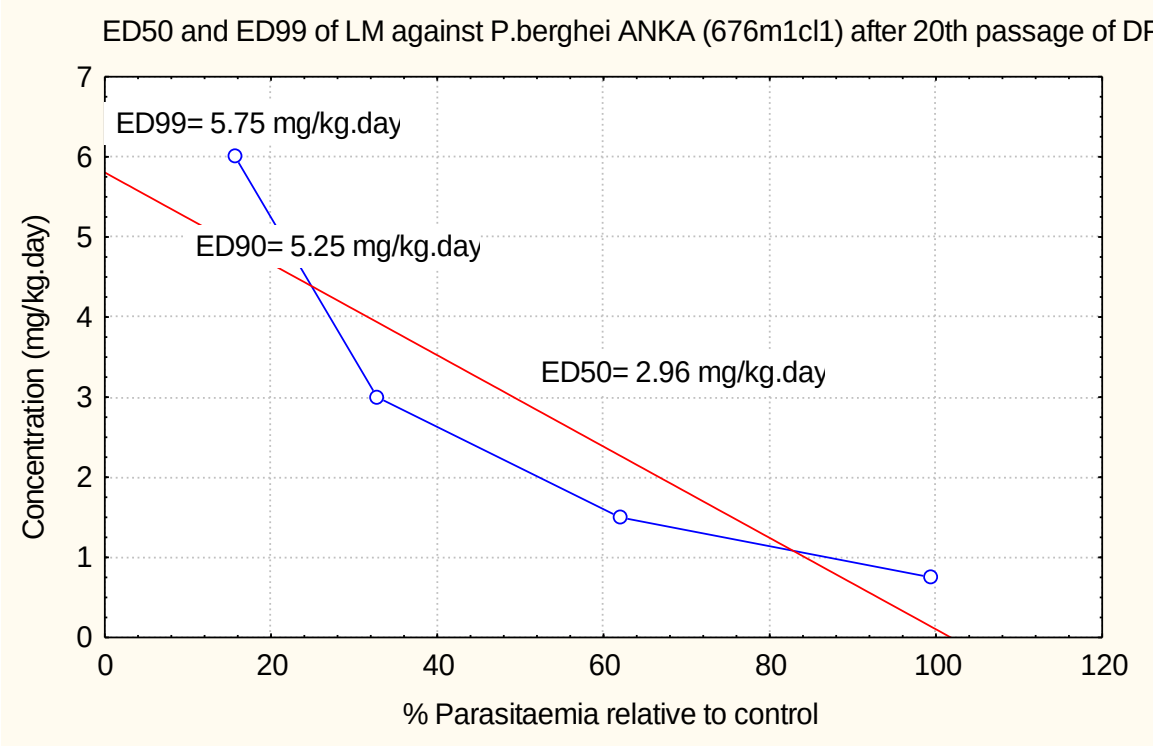
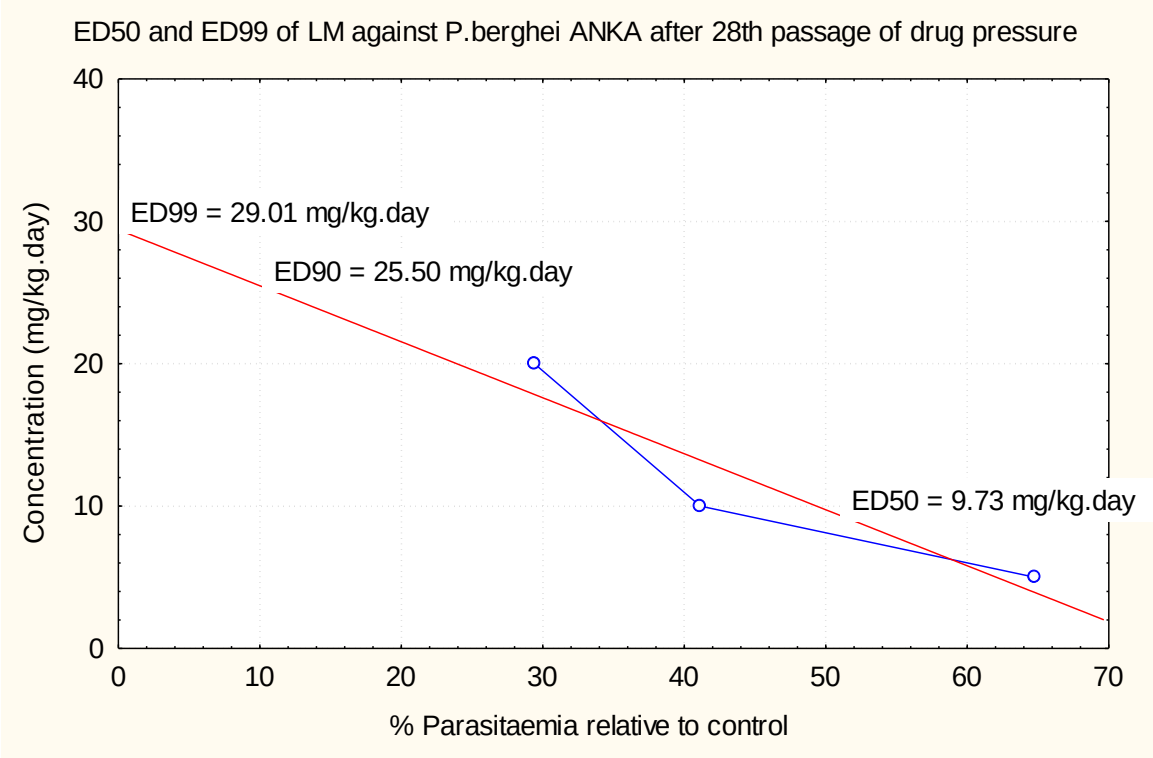


Table 14: *In vivo* activity of LM against *P. berghei* ANKA (reference line: 676 m1cl1) after 28th passage of drug pressure.

P. berghei ANKA (676 m1cl1)		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	LM	20	2.93	29.41	70.59
2	LM	10	4.10	41.13	58.87
3	LM	5	6.45	64.77	35.23
4	LM	2.5	3.97	39.79	60.21
Control	7% Tween-80 in 3% ethanol solution		9.96		

Fig 20: Graph showing of activity of LM against *P. berghei* ANKA (reference line 676m1cl1) after 28th passage of drug pressure



APPENDIX 4: *IN VIVO* ACTIVITY PROFILES OF PIPERAQUINE

Table15: *In vivo* activity of PQ against *P. berghei* ANKA GFP before further exposure to selection pressure.

P. berghei ANKA GFP		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	PQP	30	0.22	2.43	97.57
2	PQP	15	0.69	7.72	92.28
3	PQP	7.5	4.06	45.76	54.24
4	PQP	3.75	5.85	65.82	34.18
Control	7% Tween-80 in 3% ethanol solution		8.88		

Fig. 21: Graph showing activity of PQ against *P. berghei* ANKA GFP before further exposure to selection pressure.

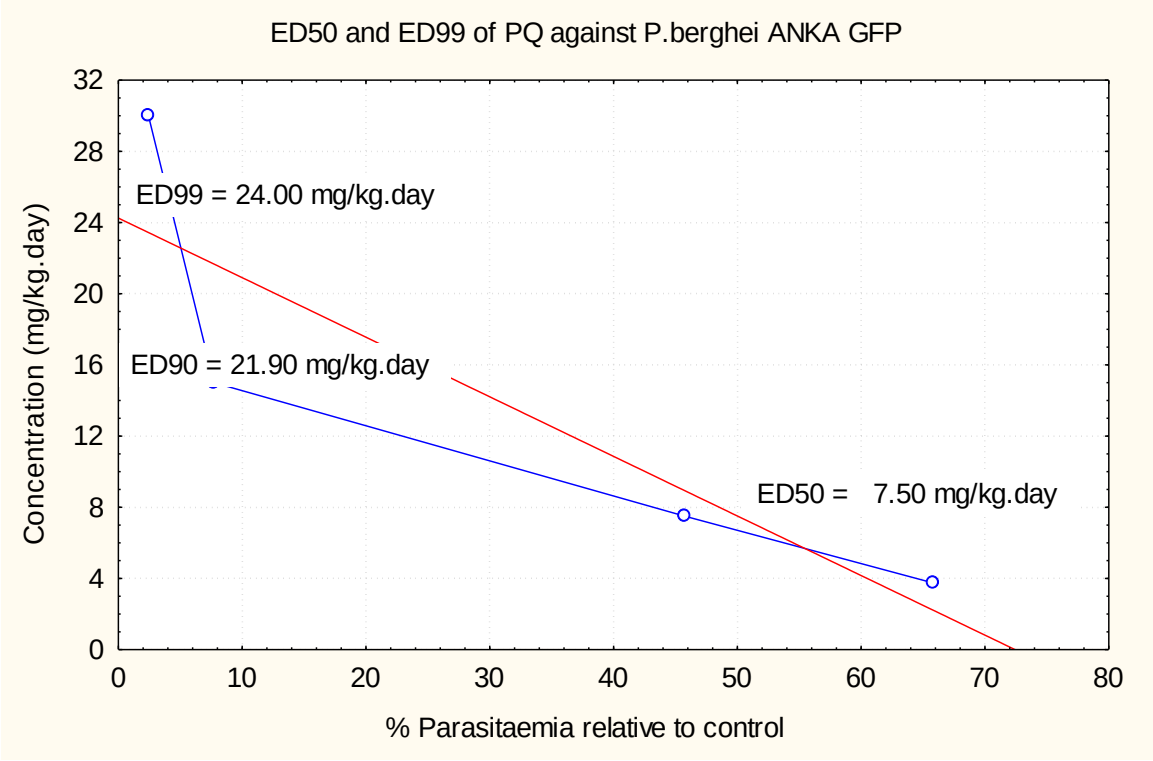


Table 16: *In vivo* activity of PQ against *P. berghei* ANKA GFP after 4th passage of drug pressure

P. berghei ANKA GFP		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	PQP	60	0.92	17.61	82.39
2	PQP	30	2.02	38.45	61.55
3	PQP	15	2.69	51.30	48.70
4	PQP	7.5	3.52	67.04	32.96
Control	7% Tween-80 in 3% ethanol solution		5.25		

Fig. 22: Graph showing of activity of PQ against *P. berghei* ANKA GFP after 4th passage of drug pressure.

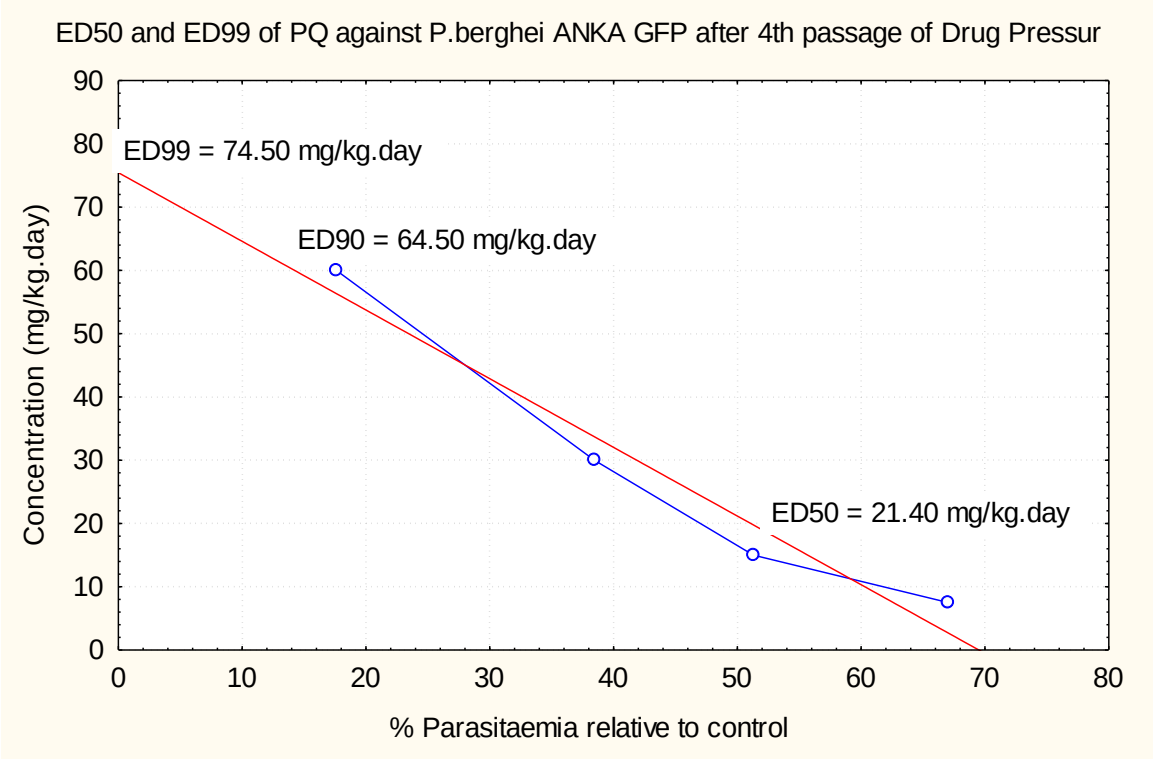


Table 17: *In vivo* activity of PQ against *P. berghei* ANKA GFP after 12th passage of drug pressure

P. berghei ANKA GFP		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	PQP	150	2.31	36.48	63.52
2	PQP	100	4.08	64.37	35.63
3	PQP	50	5.21	82.14	17.86
4	PQP	25	6.91	108.96	-8.96
Control	7% Tween-80 in 3% ethanol solution		6.34		

Fig. 23: Graph showing of activity of PQ against *P. berghei* ANKA GFP after 12th passage of drug pressure.

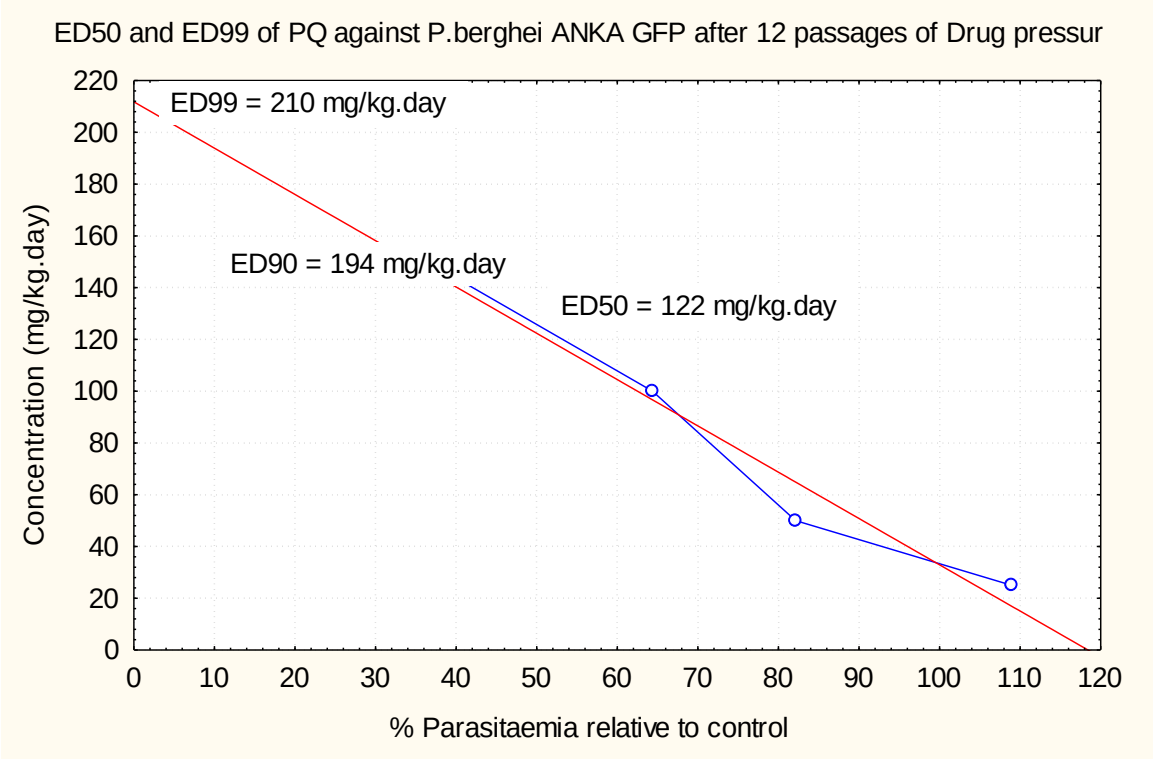


Table 18: *In vivo* activity of PQ against PQ resistant *P. berghei* ANKA GFP after five drug free passages

P. berghei ANKA GFP		TEST N°:			
N°/Cages	Substances	Dosage mg/kg.day	Average parasitaemia	% parasitaemia relative to control	% activity
1	PQP	150	1.63	15.25	84.75
2	PQP	100	3.14	29.38	70.62
3	PQP	50	3.89	36.33	63.67
4	PQP	25	6.86	64.10	35.90
Control	7% Tween-80 in 3% ethanol solution		10.70		

Fig. 24: Graph showing of activity of PQ against PQ resistant *P. berghei* ANKA GFP after five drug free passages.